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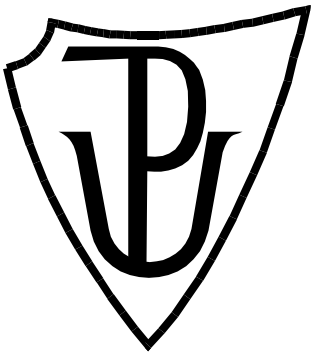

Study of endogenous neuroactive substances in biological material

Ph.D. thesis

Author:	**Mgr. Michal Kaleta**
Study programme:	P0511D030004 – Experimental Biology
Field of Study:	Experimental Biology
Form of study:	Full-time
Supervisor:	Prof. Mgr. Ondřej Novák, Ph.D.
Consultant:	Mgr. Jana Oklešťková, Ph.D.

Bibliographical identification

Author's first name and surname	Mgr. Michal Kaleta
Title of thesis	Study of endogenous neuroactive substances in biological material
Type of thesis	Ph.D.
Department	Laboratory of Growth Regulators
Supervisor	Prof. Mgr. Ondřej Novák, Ph.D.
The year of presentation	2023
Abstract	One of the current hot topics is the increasing proportion of neurodegenerative diseases. The key challenge is to design reliable diagnostic tools capable of detecting these diseases before they progress to an advanced stage. Neuroactive steroids, which have the potential to modulate the development of the nervous system, represent a highly promising avenue of research. Therefore, the availability of dependable methods for monitoring changes in the concentration of these substances is of paramount importance. This study presents the development and validation of a comprehensive method based on ultra-high-performance liquid chromatography–tandem mass spectrometry for the quantification of a selected group of steroids with neuroactive effects in human serum. Additionally, this research involved profiling a range of tryptophan-related compounds in the serum and cerebrospinal fluid of various neuropathological cohorts to map their metabolic alterations. Furthermore, selected candidate protein biomarkers were studied.
Keywords	Neuroactive steroids, neurotransmitters, neurodegenerative disease, UHPLC–MS/MS.
Number of pages	62
Number of appendices	5
Language	English

Declaration

I hereby declare that the thesis summarizes the original results obtained during my Ph.D. study under the great supervision of Prof. Mgr. Ondřej Novák, Ph.D., and Mgr. Jana Oklešťková, Ph.D., using the literary sources listed below.

In Olomouc, ……

Acknowledgement

This work was performed in the Laboratory of Growth Regulators at Palacký University in Olomouc, in collaboration with the colleagues from the Department of Neurology at University Hospital Olomouc. I would like to express my gratitude to Prof. Mgr. Ondřej Novák, Ph.D., and Mgr. Jana Oklešťková, Ph.D., for their expert guidance, valuable insights, and willingness to assist me throughout my studies. I am also appreciative of all my colleagues for their helpful advice and for fostering an excellent working environment. Furthermore, I extend my thanks to my family and friends for their unwavering support.

The work was made possible thanks to the financial support of the student project IGA_PrF_2023_031 of Palacký University in Olomouc and the European Regional Development Fund - ENOCH project (No. CZ.02.1.01/0.0/0.0/16_019/0000868).

Content

List of papers

The presented Ph.D. thesis comprehensively summarizes and links the individual papers presented in the Supplementary section. References to these publications are listed in the text of the thesis as *Supplement I–V*.

I **Kaleta, M.**, Oklestkova, J., Novák, O. and Strnad, M. (2021) Analytical Methods for the Determination of Neuroactive Steroids. *Biomolecules* 11, 553.

II Hényková, E.[1], **Kaleta, M.**[1], Klíčová, K., Gonzalez, G., Novák, O., Strnad, M. and Kaňovský, P. (2022) Quantitative Determination of Endogenous Tetrahydroisoquinolines, Potential Parkinson's Disease Biomarkers, in Mammals. *ACS Chemical Neuroscience* 13, 3230-3246.

III **Kaleta, M.**, Oklestkova, J., Strnad, M., Novák O. Simultaneous Determination of Selected Steroids with Neuroactive Effects in Human Serum by Ultra-High Performance Liquid Chromatography–Tandem Mass Spectrometry (In preparation).

IV **Kaleta, M.**, Hényková, E., Menšíková, K., Friedecký, D., Kvasnička, A., Klíčová, K., Koníčková, D., Strnad, M., Kaňovský, P., Novák O. Patients with Neurodegenerative Proteinopathies Exhibit Altered Tryptophan Metabolism in the Serum and Cerebrospinal Fluid. *ACS Chemical Neuroscience* (Submitted).

V Koníčková, D., Menšíková, K., Klíčová, K., Chudáčková, M., Kaiserová, M., Přikrylová, H., Otruba, P., Nevrlý, M., Hluštík, P., Hényková, E., **Kaleta, M.**, Friedecký, D., Matěj, R., Strnad, M., Novák, O., Plíhalová, L., Rosales, R., Colosimo, C., Kaňovský, P. (2023) Cerebrospinal Fluid and Blood Serum Biomarkers in Neurodegenerative Proteinopathies: A Prospective, Open, Cross-Correlation Study. *Journal of Neurochemistry* 167, 168-182.

[1] E.H. and M.K. contributed equally to the presented paper.

Contribution report

I As the first author, MK contributed to the preparation of a literature review of classical and modern methods for the analysis of neuroactive steroids.

II As the first and corresponding author, MK contributed to the original draft of a literature review on methods for the analysis of endogenous tetrahydroisoquinolines in various types of mammalian samples.

III As the first author, MK developed and validated an extraction technique and a quantification method for the determination of selected steroids with neuroactive effects in human blood serum. MK also wrote the original draft.

IV As the first and corresponding author, MK performed the measurements, processed the experimental data, and wrote the original draft.

V As a co-author, MK contributed to the review and editing of the manuscript.

Abbreviations

3/4R	Three/Four-repeat
3-OH-KYN	3-Hydroxykynurenine (2-Amino-4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid)
4R-Tau	Four-repeat tauopathy
5-OH-TRP	5-Hydroxy-L-tryptophan
ACN	Acetonitrile
AD	Alzheimer's disease
AFMK	N^1-Acetyl-N^2-formyl-5-methoxykynuramine (*N*-[3-(2-Formamido-5-methoxyphenyl)-3-oxopropyl]acetamide)
ALLO	Allopregnanolone ((3α,5α)-3-Hydroxypregnan-20-one)
AMK	N^1-Acetyl-5-methoxykynuramine (*N*-[3-(2-Amino-5-methoxyphenyl)-3-oxopropyl]acetamid)
ANDRO	Androstenedione (Androst-4-ene-3,17-dione)
BSA	Bovine serum albumin
CBS	Corticobasal syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DHEA	Dehydroepiandrosterone ((3β)-3-Hydroxyandrost-5-en-17-one)
DHP	5α-Dihydroprogesterone ((5α)-Pregnane-3,20-dione)
DHT	5α-Dihydrotestosterone ((5α,17β)-17-Hydroxyandrostan-3-one)
DLB	Dementia with Lewy bodies
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMA	European Medicines Agency
EPIA	Epiandrosterone (17β-hydroxy-4-androsten-3-one)
ESI	Electrospray ionization
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
HC	Healthy controls
HQ	High-quality control
IMS	Ion mobility spectrometry
KYN	L-Kynurenine ((2*S*)-2-Amino-4-(2-aminophenyl)-4-oxobutanoic acid)
LBD	Lewy body disease

LC	Liquid chromatography
LLE	Liquid-liquid extraction
LQ	Low-quality control
MeOH	Methanol
MQ	Medium-quality control
MRM	Multiple reaction monitoring
MS	Mass spectrometer/mass spectrometry
MS/MS	Tandem mass spectrometry
MSA	Multiple system atrophy
NAS	Neuroactive steroids
NMDA	*N*-Methyl-D-aspartate
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PREG	Pregnenolone ((3β)-3-Hydroxypregn-5-en-20-one)
PROG	Progesterone (Pregn-4-en-3,20-dione)
PSP	Progressive supranuclear palsy
RIA	Radioimmunoassay
SFC	Supercritical fluid chromatography
SPE	Solid-phase extraction
T	Testosterone ((17β)-17-Hydroxyandrost-4-en-3-one)
TRP	L-Tryptophan
UHPLC–MS/MS	Ultra-high performance liquid chromatography–tandem mass spectrometry
UHQ	Ultra-high quality control

1 Introduction

The increasing human lifespan is, unfortunately, associated with a growing proportion of age-related neurodegenerative diseases (among others). Perhaps unsurprisingly, the most common neurodegenerations are Parkinson's and Alzheimer's diseases. Worldwide, several million people suffer from these diseases. It should not be forgotten that this growing medical and socio-economic problem of an ageing population is also a major burden on the healthcare systems. An early detection of these conditions is very important, preferably before the full onset of the disease.

The nervous system is the target but also the source of a wide variety of neuroactive substances of different chemical natures. The vast majority of available predictive, prognostic, or diagnostic biomarkers of neurodegenerative diseases are proteins, i.e. high molecular weight markers (alpha-synuclein, tau protein, etc.). However, low molecular weight substances are also an interesting, albeit insufficiently explored area. One of the groups of these substances that have attracted interest in recent years is certainly the neuroactive steroids. These substances, capable of modulating the function and development of the nervous system, are being intensively studied, including their use as potential drugs for various diseases of the nervous system. Several synthetic analogues have undergone clinical trials. Some compounds may also be directly involved in the pathogenetic processes of several neuropathological entities or may be potentially useful in their pharmacotherapy. In this respect, tryptophan metabolites are a very interesting and not so thoroughly studied group. They include neuroprotective and neurotoxic substances, which makes them particularly interesting in the context of the neurodegenerative process.

This Ph.D. thesis describes the development and validation of an analytical method based on ultra-high performance liquid chromatography combined with tandem mass spectrometry (UHPLC–MS/MS) for the determination of selected neuroactive steroids in human serum. In addition, metabolic profiling of tryptophan metabolites and analysis of candidate protein biomarkers in several neurodegenerative pathologies are also presented.

2 Aims and scope

The presented Ph.D. thesis focuses mainly on the study of two groups of neuroactive substances, namely neuroactive steroids (NASs) and tryptophan (TRP)-related substances, in human body fluids. Sensitive methods based on UHPLC–MS/MS were used to study these analytes. The last part of this thesis is devoted to selected candidate protein biomarkers of neurodegeneration.

The main aims of the work described in this thesis were as follows:

- preparation of an overview of studied neurodegenerative diseases, neuroactive substances, and classical and modern methods of their analysis,
- development and validation of a purification protocol and a detection UHPLC–MS/MS method for profiling a selected group of steroid analytes with neuroactive properties in human blood serum,
- metabolic profiling of TRP and TRP-related analytes by UHPLC–MS/MS and analysis of protein candidate biomarkers in human cerebrospinal fluid (CSF) and blood serum samples from a healthy control group and several neuropathological cohorts; mapping changes in TRP metabolism and interpretation of results.

3 Literature review

3.1 Neurodegenerative diseases

The hallmark of this heterogeneous group of diseases is neurodegeneration (*Figure 1*), a term referring to a process in which a progressive loss of neurons and damage to their structure and/or function occur (Lamptey *et al.* 2022; Mathur *et al.* 2023; Koníčková *et al.* 2022). This leads to a selective impairment of a subpopulation of cells in the nervous system that eventually manifests itself in the ability to perform certain bodily functions such as speech, motor skills, stability, organ function, cognitive abilities, etc. Neurodegeneration is therefore a significant contributor to mortality and disability on a global scale (Ayeni *et al.* 2022).

Neurodegenerative diseases are usually characterized by intracellular or extracellular accumulation of clumps of various aberrant proteins with altered physicochemical properties (Koníčková *et al.* 2022; Kovacs 2018). These pathological deposits may be related to neuronal death. Neurodegenerative diseases can be categorized based on their molecular pathology into distinct groups, including alpha-synucleinopathies, tauopathies, etc. (Menšíková *et al.* 2022).

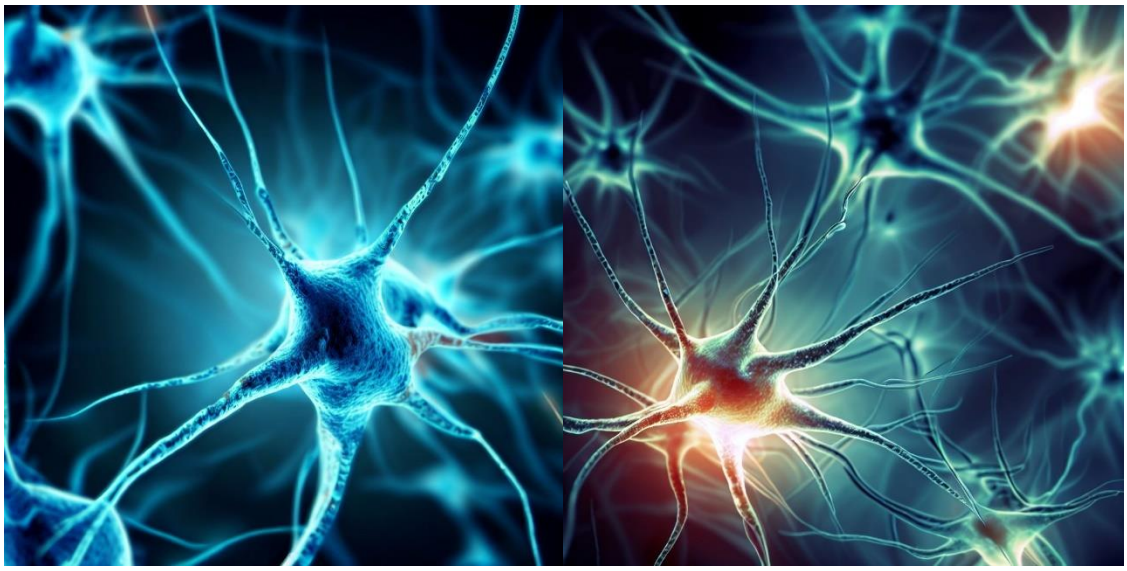

Figure 1. Visualization of neurons affected by neurodegeneration (generated by DALL-E).

Disorders affecting the nervous system represent the second most common cause of death in the contemporary world (Mathur *et al.* 2023), with Alzheimer's disease (AD) and

Parkinson's disease (PD) being the predominant cases (Klatt *et al.* 2021). AD and various forms of dementia currently affect tens of millions of people worldwide (Li *et al.* 2022b). It is well known that the frequency and incidence of these diseases are closely related to ageing (Mathur *et al.* 2023). Nonetheless, the interaction between an individual's genetic composition and environmental influences also plays a role in elevating the risk of neurodegenerative disorders (Lamptey *et al.* 2022). Although the pathogenesis of neurodegenerative diseases is not yet fully understood, aetiological factors include genetic factors, brain injury, stress, and environmental factors (environment, diet, lifestyle, etc.) (Ayeni *et al.* 2022). As life expectancy increases, resulting in a greater likelihood of disease development, the proportion of age-related neurodegenerative diseases also rises (Sorgdrager *et al.* 2019; Van Schependom & D'haeseleer 2023). This “expansion of the ageing population” unfortunately places an increasingly heavy burden on the healthcare system. It is equally crucial to bear in mind the socioeconomic and health repercussions for individuals responsible for the care of these patients (Ayeni *et al.* 2022). The increasing proportion of patients with neurodegeneration is also due to the improving efficiency of early diagnosis (Shusharina *et al.* 2023).

Most medications presently employed for neurodegenerative disorders primarily aim to decelerate disease progression and alleviate associated symptoms, ultimately enhancing the patient's quality of life (Lamptey *et al.* 2022; Shusharina *et al.* 2023). Therapeutic approaches beyond pharmacological treatment, such as complex rehabilitation (e.g. physical exercises, cognitive training) also hold significant importance.

An early, preferably preclinical, identification of these conditions is crucial for the implementation of effective therapeutic interventions (e.g. pharmacotherapy and cognitive training) focused on retarding degeneration and the onset of severe clinical manifestations (Shusharina *et al.* 2023). The clinical diagnosis of neurodegenerative entities is currently based on a wide range of routinely used clinical diagnostic criteria (Armstrong *et al.* 2013; Gilman *et al.* 2008; Höglinger *et al.* 2017; Litvan *et al.* 1996; McKeith *et al.* 2005; McKeith *et al.* 2017; McKhann *et al.* 2011; Postuma *et al.* 2015). These criteria integrate clinical, neuroimaging, laboratory, and other findings. Unfortunately, there is not a solitary characteristic clinical symptom or test for neurodegenerative diseases that could facilitate an accurate diagnosis. Moreover, the determination of the exact and correct diagnosis is complicated by the fact that neurodegenerative diseases often overlap and

combine (Kovacs 2018). Thus, a reliable diagnosis is still possible only by post-mortem histopathological examination of brain tissue. The discovery of new biomarkers in the CSF, as well as in the blood, which would allow reliable and unambiguous diagnosis of neurodegenerative diseases is still a very actual area of research (Koníčková *et al.* 2022). Although CSF is a very valuable biofluid reflecting changes in neural tissue, much effort is also devoted to the possibility of obtaining blood biomarkers, simply because of the lower invasiveness for patients. There are several established and candidate biomarkers for neurodegenerative diseases, but in most cases, these are protein markers (Koníčková *et al.* 2022; Koníčková *et al.* 2023). However, low molecular weight substances are also a very promising and less explored area, some of which will be discussed in sections *3.2 Neuroactive steroids* and *3.3 Other neuroactive substances: tryptophan-related metabolites*.

3.1.1 Selected types of neurodegenerative diseases

This section provides a fundamental overview of the neuropathologies encompassed in the study described in *Supplement IV* and *V*.

3.1.1.1 Alpha-synucleinopathies

A common feature shared by all alpha-synucleinopathies is the accumulation of pathologically altered alpha-synuclein protein, which forms neurotoxic oligomers and consequently fibrillar aggregates in the cytoplasm of nervous system cells (Koníčková *et al.* 2022). These aggregates can either occur intraneuronally as Lewy bodies and Lewy neurites, in the case of Lewy body diseases (LBD), or mainly in oligodendrocytes, in the case of multiple system atrophy (MSA).

MSA is an atypical Parkinsonian syndrome characterised clinically by combination of varying degrees of Parkinsonism, autonomic failure, cerebellar ataxia, and pyramidal signs (Koga *et al.* 2020; Watanabe *et al.* 2023). Apart from the accumulation of alpha-synuclein aggregates in oligodendrocytes, these inclusions can also be observed within a specific subset of neurons. It is important to note that the clinical manifestations of LBD and MSA may overlap, which may complicate the correct diagnosis.

LBD can be further categorised into three subtypes: PD, Parkinson's disease dementia (PDD), and dementia with Lewy bodies (DLB), although these are overlapping conditions at

the clinical, pathological, and biochemical levels (Walker *et al.* 2019). Typical symptoms of PD include bradykinesia, hypokinesia, muscle rigidity, and rest tremor (Lamptey *et al.* 2022; Koníčková *et al.* 2022). These motor symptoms are mainly due to a dysfunction of the nigrostriatal pathway and the associated gradual reduced concentration of dopamine in the *striatum* resulting from the death of dopaminergic neurons in the *substantia nigra pars compacta*. However, other areas of the brain and other parts of the nervous system are also affected, which is the cause of the non-motor symptoms. An early identification of the disease is challenging because typical motor symptoms appear only after 70–80% of dopaminergic neurons have been compromised (Emamzadeh & Surguchov 2018). As previously noted, the prevalence of this disease tends to rise with age, with additional risk factors like smoking and exposure to environmental toxins also contributing to its development (Ayeni *et al.* 2022; Lamptey *et al.* 2022). PD can be sporadic (or idiopathic) or hereditary with various manifestations of the Parkinsonian phenotype (in some cases, the hereditary forms resemble atypical Parkinsonism) (Menšíková *et al.* 2022).

As some forms of PD can lead to cognitive impairment and associated dementia, a PDD group was created (Koga *et al.* 2020; Menšíková *et al.* 2022). PDD is characterized by typical Lewy bodies but also by the presence of amyloid beta inclusions in the limbic system. In contrast, diffuse alpha-synucleinopathy is observed in DLB, which is usually accompanied by Alzheimer's disease-like changes - the appearance of senile plaques (Menšíková *et al.* 2022). The impact on cognitive function is generally greater in this case than in PDD (Walker *et al.* 2019). PDD subtype is diagnosed if the development of cognitive symptoms occurs at least one year after the onset of PD; in the case of DLB, the development of cognitive symptoms precedes or coincides with the onset of Parkinsonian motor symptoms (the so-called one-year rule) (Jellinger & Korczyn 2018). The clinical symptoms of these LBD subtypes overlap and may include cognitive decline and fluctuations, Parkinsonism (which may not be expressed in DLB), visual hallucinations, and others (Walker *et al.* 2019).

Unfortunately, the treatment of PD is not causal, but only alleviates the course of the disease. Dopamine deficiency in the *substantia nigra* can be compensated by administration of its precursor levodopa, most commonly in combination with the peripheral dopamine carboxylase inhibitor carbidopa (Lamptey *et al.* 2022). PD treatment with levodopa is still the gold therapeutic standard (Murakami *et al.* 2023). The bioavailability of levodopa to the nervous system can also be increased by using catechol-O-methyltransferase inhibitors

(e.g. entacapone). In addition, dopamine agonists (such as apomorphine hydrochloride) that stimulate dopamine neuronal receptors can be administered (Emamzadeh & Surguchov 2018). The target may also be to limit dopamine metabolism, reduce the production of reactive oxidative metabolites, and protect neurons from oxidative damage and cell death through the inhibition of monoamine oxidase B (e.g. rasagiline) (Emamzadeh & Surguchov 2018; Murakami *et al.* 2023). Currently, several disease-modifying drug candidates (neuroprotective, immunosuppressive, anti-inflammatory, anti-oxidative stress, and alpha-synuclein-targeting agents) are also part of clinical trials (Murakami *et al.* 2023). Non-motor symptoms such as depression, sleep disorders, and constipation problems are treated with symptomatically appropriate medications (Lamptey *et al.* 2022).

3.1.1.2 Tauopathies

As the name implies, this heterogeneous group of neurodegenerative diseases is characterized by neuronal and/or glial accumulation of pathologically conformed tau protein with different clinical manifestations depending on the localization of pathological deposits (Ganguly & Jog 2020). The clinical picture is characterised by a combination of levodopa non-responsive Parkinsonism and cognitive impairment, which are accompanied by other symptoms typical for individual clinical entities (oculomotor disorder, apraxia, etc.).

Based on the predominant isoform of tau protein in the aggregates, tauopathies can be divided into three-repeat (3R), four-repeat (4R), and mixed subtypes (3R + 4R) (Zhang *et al.* 2022; Sexton *et al.* 2021). Pathologically, this group can be classified into primary [tau protein a prominent component of the pathology; e.g. progressive supranuclear palsy (PSP), corticobasal syndrome (CBS), or Pick´s disease] and secondary (tau protein accumulation in response to the presence of beta-amyloid, or other insults such as trauma, neuroinflammation, or autoimmune insult; e.g. AD and chronic traumatic encephalopathy) tauopathies (Ganguly & Jog 2020). However, other geographically isolated forms also exist (such as Guadeloupean Parkinsonism).

PSP and CBS can be classified within the subgroup of four-repeat tauopathies (4R-Tau) (Ganguly & Jog 2020). PSP (4R isoform of tau protein predominates) can present itself with a wide range of phenotypic manifestations. Characteristic features of classic PSP include axial rigidity, early postural instability with frequent backward falls, vertical gaze palsy, and cognitive dysfunction. Based on the combination of these cardinal symptoms and their

severity, eight clinical phenotypes of PSP are currently described (Höglinger *et al.* 2017). Another type of primary tauopathy is CBS, which is clinically characterized by progressive asymmetric rigidity and apraxia together with symptoms suggestive of cortical involvement (e.g. alien limb phenomena, cortical sensory loss, myoclonus, or mirror movements) and basal ganglia dysfunction, e.g. bradykinesis, dystonia, or tremour (Zhang *et al.* 2022).

A representative of the mixed 3R and 4R secondary tauopathies and the most prevalent form of tauopathy overall is AD (Silva & Haggarty 2020). Typical morphological findings in AD are beta-amyloid deposits forming characteristic plaques located extracellularly and neurofibrillary tangles formed by hyperphosphorylated tau proteins (Drummond *et al.* 2018; Ayeni *et al.* 2022). The main clinical feature of AD is disorders of short-term memory and other cognitive domains, which gradually progress to the clinical picture of severe dementia (Lamptey *et al.* 2022).

Even in the case of tauopathies, there is currently no treatment that addresses the underlying cause (Silva & Haggarty 2020). Treatment of AD (especially limiting its progression) is based on several approaches: cholinesterase inhibitors (drugs of first choice - reduction of cognitive deficits due to increased acetylcholine supply, e.g. donepezil), antibodies targeting amyloid beta plaques (aducanumab approved by FDA in 2021), or glutamate regulators [e.g. memantine - an uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, prevention of excessive NMDA receptor activation by glutamate and neuronal damage] (Lamptey *et al.* 2022). However, therapeutic strategies aimed at reducing the accumulation and aggregation of tau protein or promoting its increased clearance are also being investigated in the context of tauopathies (Silva & Haggarty 2020).

3.2 Neuroactive steroids

The development and functioning of the human nervous system are modulated by a variety of endogenous and exogenous stimuli; substances of steroid origin also play an important role (Melcangi *et al.* 2016). The name NASs has been established for this wide group, including hormonal steroids of the peripheral glands, steroids produced by nervous tissue, and synthetic representatives (Giatti *et al.* 2019). Their endogenous representatives can be found among several classical steroid classes (see *Figure 2*).

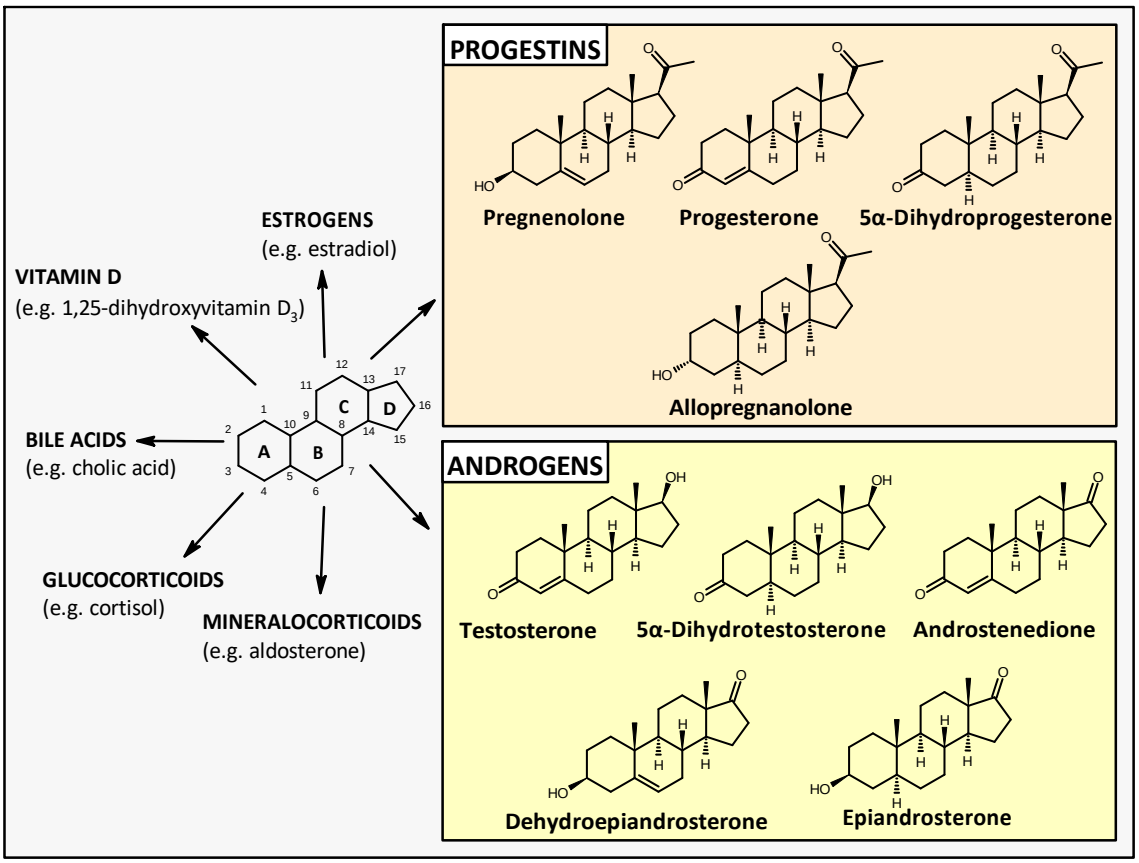

Figure 2. Main steroid classes and representatives of progestins and androgens. The analysis of the shown progestins and androgens is presented in the practical part of the thesis.

Typical representatives of NASs include, for example, pregnenolone (PREG), progesterone (PROG), allopregnanolone (ALLO), dehydroepiandrosterone (DHEA), testosterone (T), estradiol, some of their sulfate esters, and many others (Zheng 2009). Synthetic analogues of NASs are presented in section *3.2.3 Cellular and supracellular responses.*

3.2.1 Endogenous sources of neuroactive steroids

The endogenous sources of steroid hormones in the body are usually the adrenal glands (specifically the adrenal cortex) and gonads, the testes in men and the ovaries in women (placenta and *corpus luteum*) (Holst *et al.* 2004). However, the enzymatic machinery enabling steroidogenesis has been found in other tissues and organs (see *Figure 3*).

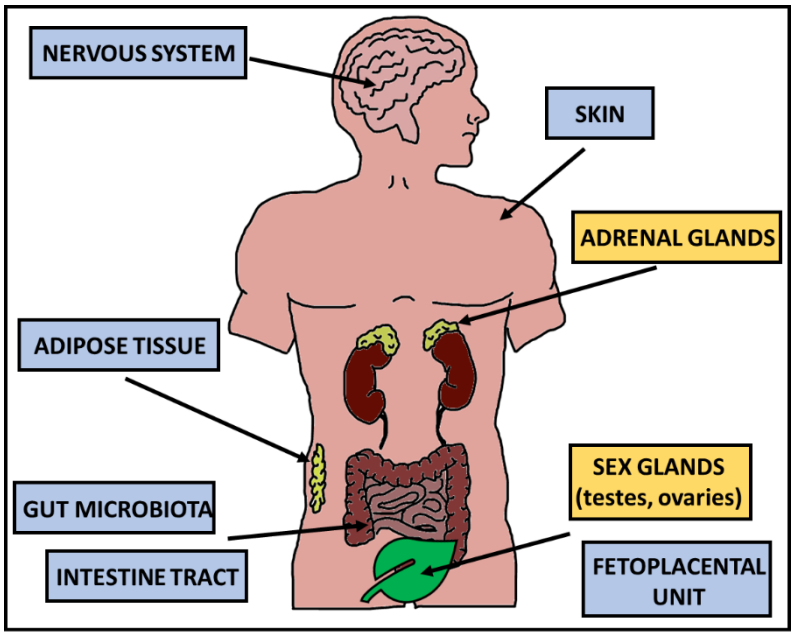

Figure 3. Major endogenous sources of steroids.

The peripheral and central nervous system (CNS) itself (specifically glial cells and neurons) is capable of biosynthesising steroid substances (Baulieu 1998; Le Goascogne *et al.* 1989). The key steps of steroid biosynthesis are subcellularly localised in the mitochondria and smooth endoplasmic reticulum (Giatti *et al.* 2019). As early as the 1980s, it was suggested that the biosynthesis of some steroids may occur directly in the brain tissue (i.e., independent of the peripheral endocrine system) (Corpéchot *et al.* 1981; Corpéchot *et al.* 1983). Corpéchot and colleagues (1981, 1983) determined dehydroepiandrosterone sulphate, PREG, and its sulphate metabolite in the brains of male rodents in which the gonads and adrenal glands had been surgically removed. Based on the localisation of their synthesis, this group was named neurosteroids (the term was first used in the Czech literature in 1980 by Schreiber and one year later by other authors in France) (Schreiber 1980; Corpéchot *et al.* 1981). Neurosteroids are produced, for example, in the hippocampus and neocortex (Reddy & Estes 2016). The concept of neurosteroidogenesis is also supported by the presence of biologically active enzymes or their mRNA in nervous tissue (studied and

demonstrated by immunohistochemical or *in situ* hybridisation techniques) (Mensah-Nyagan *et al.* 1996; Mensah-Nyagan *et al.* 1999; Mukai *et al.* 2006; Do Rego *et al.* 2009; Le Goascogne *et al.* 1989; Mensah-Nyagan *et al.* 1994; Le Goascogne *et al.* 1987; Guennoun *et al.* 1995). The biosynthesis and metabolism of steroids in the nervous system are described in detail in publications (Mensah-Nyagan *et al.* 1999; Do Rego *et al.* 2009).

In addition, steroid compounds are also formed in other tissues and organs: skin (Slominski *et al.* 1996; Slominski *et al.* 2013), adipose tissue (Li *et al.* 2015), intestinal tissue (Bouguen *et al.* 2015; Cima *et al.* 2004), and others. Currently, the interaction between the biosynthesis of steroids and the gut microbiota is also quite intensively studied (Diviccaro *et al.* 2021; Li *et al.* 2022a). Surprisingly, the gut microbiota can influence the levels of available steroids, even with functional effects on organisms. It is suggested that microbiota expressing the enzyme 3β-hydroxysteroid dehydrogenase (in this case *Mycobacterium neoaurum*) may be responsible for the decline in T levels and associated depressive symptoms (Li *et al.* 2022a).

3.2.2 Mechanism of neuroactive steroid action

NASs may exert their effects through two mechanisms called genomic and non-genomic (McEwen 1991). A visualisation of these mechanisms at the receptor level is available in *Figure 4*. These effects of NASs can be observed in different cell types of the nervous tissue, such as neurons, oligodendrocytes, astrocytes, microglia, or endothelial cells (Melcangi *et al.* 2016).

The essence of the first is the interaction between steroid molecules and "classical" steroid receptors (Reddy 2010). These are localised intracellularly, either in the cytosol or in the cell nucleus (Holst *et al.* 2004). After the receptor activation, they function as ligand-activated transcription factors capable of regulating gene expression (Zheng 2009; Wang 2011). However, the response is slow (minutes to hours) as it depends on the rate of protein biosynthesis. Progesterone, androgen, estrogen, and corticosteroid receptors have also been identified directly in some regions of the mammalian brain (Taylor & Al-Azzawi 2000; Reul & de Kloet 1985; Brinton *et al.* 2008; Sarkey *et al.* 2008). No specific nuclear receptors have been identified yet for other steroids such as DHEA and dehydroepiandrosterone sulfate (Stárka *et al.* 2015). The ability of some NASs to interact with intracellular steroid receptors and regulate gene expression is dependent on their metabolic transformation

(Reddy 2010; Rupprecht *et al.* 1993). The genomic effect of DHEA may be mediated only after its conversion to T, 5α-dihydrotestosterone (DHT), or estradiol (Stárka *et al.* 2015). These compounds are already capable of activating androgen and estrogen receptors, respectively. Similarly, in the case of ALLO, its intracellular oxidation to the 5α-pregnane metabolite 5α-dihydroprogesterone (DHP), which has an affinity for the progesterone receptor, is required (Rupprecht *et al.* 1993).

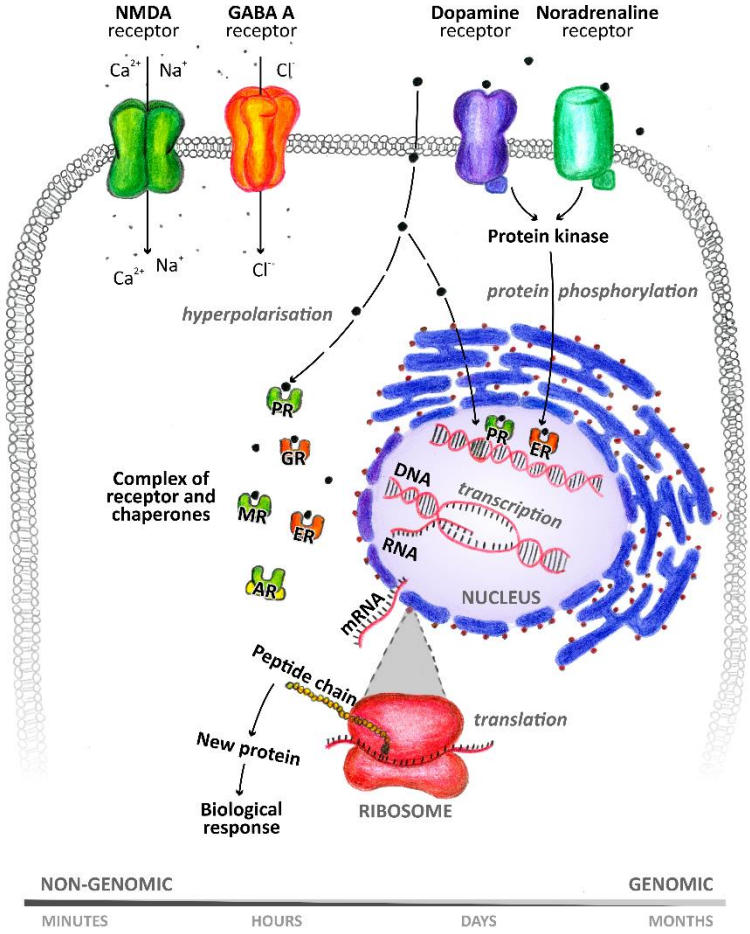

Figure 4. Genomic and nongenomic mechanism of neuroactive steroid action (NMDA: *N*-methyl-D-aspartate, GABA: gamma-aminobutyric acid, PR: progesterone receptor, GR: glucocorticoid receptor, MR: mineralocorticoid receptor, ER: estrogen receptor, AR: androgen receptor) (*Supplement I*).

However, NASs can also interact with a variety of membrane receptors and ion channels, resulting in rapid changes in neuronal membrane excitability (Reddy 2010; Wang 2011; Paul & Purdy 1992). This mechanism of action is referred to as non-genomic and occurs within milliseconds to seconds. Typical representatives of this group are gamma-aminobutyric acid (GABA) or NMDA receptors (Tuem & Atey 2017).

The basic knowledge of the interaction between NASs and GABA receptors dates back to the 1980s (Harrison & Simmonds 1984; Majewska *et al.* 1986). The $GABA_A$ subtype, widely distributed in the CNS, is one of the main targets of NAS action - depending on their structure at these receptors, they act as positive or negative modulators of inhibitory GABAergic neurotransmission (Carta *et al.* 2012). NASs with a sulfate group in the C_3 position (see *Figure 2*) function as negative modulators (such as pregnenolone sulfate and dehydroepiandrosterone sulfate) (Park-Chung *et al.* 1999). This inhibitory effect is probably mediated by the negative charge of the sulfate group. In contrast, the structural formula patterns for strong positive modulators involve a 3α-hydroxyl group on the A ring and a single bond between the C_5 and C_6 of the steroid molecule (e.g. ALLO) (*Figure 2*). After these ligand-gated chloride channels are activated by the inhibitory neurotransmitter GABA, the ion channels open and the influx of chloride ions occurs (Reddy & Estes 2016; Reddy 2010). This results in membrane hyperpolarisation and a reduction in action potential conduction. In the case of NAS-induced positive modulation of the $GABA_A$ receptor (by ALLO, androstanediol, tetrahydrodeoxykortikosteron, etc.) an increase occurs in the time or frequency of chloride channel opening - enhancement of inhibitory neurotransmission (Carta *et al.* 2012; Reddy 2010). Conversely, when the receptor is negatively modulated, the frequency of ion channel opening is reduced (Mienville & Vicini 1989). Interestingly, at low concentrations, NASs act as allosteric modulators, while at higher concentrations (above physiological values) they can activate $GABA_A$ receptors themselves (Reddy & Estes 2016; Puia *et al.* 1990).

Another very widespread CNS receptor with which NASs can interact are NMDA receptors (Wang *et al.* 2007; Reddy 2010; Rambousek *et al.* 2011). These ligand-gated ion channels are interesting in several aspects. First, their excessive activation by the excitatory amino acid L-glutamate can lead to excitotoxic neuronal cell death, which is associated with many neurodegenerative and other diseases (Sattler & Tymianski 2001; Wang & Qin 2010). The application of NMDA receptor antagonists may have a neuroprotective effect on the nervous system (Rambousek *et al.* 2011). However, the choice of an appropriate glutamate receptor antagonist is complicated as their use is often associated with serious side effects. Another area of interest is the involvement of NMDA receptors in neuronal plasticity and related processes such as memory formation and learning (Korinek *et al.* 2011). Similar to GABA receptors, NASs can function as positive (e.g. pregnenolone sulfate) or negative

regulators (epipregnanolone sulphate, etc.) of NMDA receptors (Park-Chung *et al.* 1997). The modulatory effect again depends on the structural characteristics of the steroid molecules.

There is evidence for interactions of NASs with several other receptor types, voltage-gated calcium channels, voltage-dependent anion channels, serotonin, alpha-adrenoceptors, microtubule-associated protein 2, and others (Tuem & Atey 2017).

3.2.3 Cellular and supracellular responses

The anaesthetic and anticonvulsant effects of deoxycorticosterone and PROG were observed by Hans Selye more than 80 years ago (Reddy a Estes, 2016). In addition to the above-mentioned neuroprotection of some NASs (Pike *et al.* 2009; Djebaili *et al.* 2005), anxiolytic (Bitran *et al.* 1999; Wieland *et al.* 1997), anticonvulsant (Wieland *et al.* 1997; Belelli *et al.* 1989), antidepressant (Khisti *et al.* 2000), anesthetic (Larsson-Backström *et al.* 1988), or analgesic (Kavaliers & Wiebe 1987) effects have been observed. They are probably mainly based in the interaction of NASs with $GABA_A$ receptors (Porcu *et al.* 2016). Moreover, the antitumour activity of PROG was also observed in experimental models of glioblastoma multiforme (an aggressive malignant brain tumour in adults) (Atif *et al.* 2015).

Changes in NAS levels occur physiologically, however, they can also be disturbed by various neuropathological processes: traumatic brain injury (Lopez-Rodriguez *et al.* 2015; Lopez-Rodriguez *et al.* 2016), multiple sclerosis (Caruso *et al.* 2014; Kanceva *et al.* 2015), AD or non-Alzheimer's dementia (Akwa 2020; Smith *et al.* 2006), PD (di Michele *et al.* 2003), Huntington's disease (Markianos *et al.* 2005), etc. Therefore, the study of the role of NASs in these and other pathologies is essential, also regarding their early diagnosis or possible therapy.

However, the direct therapeutic use of natural NAS substances is limited (in the case of ALLO - short biological half-life, low bioavailability, poor aqueous solubility, development of tolerance, sedation, memory impairment, addiction) (Porcu *et al.* 2016). One possibility is therefore to promote neurosteroidogenesis at different levels (translocator protein - TSPO, enzymes, etc.). Another thoroughly studied area is the preparation of synthetic analogues. Synthetic analogues of endogenous NASs are being studied for their therapeutic potential in diseases of the nervous system (epilepsy, status epilepticus, traumatic brain injury, AD, etc.) (Blanco *et al.* 2018; Reddy & Estes 2016). Compared to natural steroids, they

may be inactivated more slowly by glucuronidation and sulphation, resulting in longer bioavailability and half-life. Several potential NAS-based drugs (alphaxolone, sepranolone, ganaxolone, etc.) have been tested in clinical trials with varying degrees of success (Blanco *et al.* 2018). In 2022, ganaxalone was approved by the FDA for the treatment of seizures associated with cyclin-dependent kinase-like 5 deficiency disorder in patients two years of age and older (Lamb 2022; Vossler 2022). The use of this antiepileptic drug for other indications is being evaluated in further clinical trials.

3.3 Other neuroactive substances: tryptophan metabolites

L-tryptophan (TRP) is an essential neutral amino acid that cannot be biosynthesized by the human body and must be obtained through diet (legumes, meats, etc.) (Poeggeler *et al.* 2022; Perez-Castro *et al.* 2023). It is a pivotal constituent of proteins (Ostapiuk & Urbanska 2022). However, most of the ingested TRP is metabolised into numerous metabolic intermediates and only 1–2% is incorporated into peptides and proteins. TRP is an important precursor and can be metabolized through several metabolic pathways, including kynurenine, methoxyindole, kynuramine, and the intestinal bacterial indole pathway, resulting in the formation of a diverse and extensive range of bioactive compounds (Hényková *et al.* 2016; Anesi *et al.* 2019; Ostapiuk & Urbanska 2022; Bender 1983; Hardeland *et al.* 2009; Keszthelyi *et al.* 2009). These compounds include various neurotransmitters, neuromodulators, and neurohormones, among others, which can have an impact on health and the quality of life (Poeggeler *et al.* 2022). Some of these substances also exhibit pharmacological activity (Hényková *et al.* 2016).

The kynurenine pathway is dominant in the human body and catabolises approximately 95% of ingested TRP (e.g. kynurenic acid, quinolinic acid), 1–2% is converted by the enzymatic cascade known as methoxyindole pathway (e.g. serotonin, melatonin), 4–6% is bacterially degraded in the gut lumen (e.g. indole, indican), and approximately 0.5% is excreted in urine without any change (Ostapiuk & Urbanska 2022; Anesi *et al.* 2019; Keszthelyi *et al.* 2009). Selected representatives (target molecules analysed in the practical part of the thesis, *Supplement IV*) of these metabolic routes are shown in *Figure 5*. The products of these pathways not only target the nervous system but also play a crucial role in various processes throughout the body, including protein synthesis, biomass production, growth, immune response, etc. (Perez-Castro *et al.* 2023). TRP metabolites play a significant

role in processes related to neurophysiology, depression, and obesity, as well as the regulation of immune response and inflammation (Anesi *et al.* 2019).

There is evidence that certain pathological processes can disrupt TRP metabolism, leading to alterations in the levels of its intermediates. Changes in the human serum and CSF levels of some metabolites covering kynurenine, methoxyindole, kynuramine, and intestinal bacterial indole pathways in association with selected neurodegenerative proteinopathies are described in detail in *Supplement IV*.

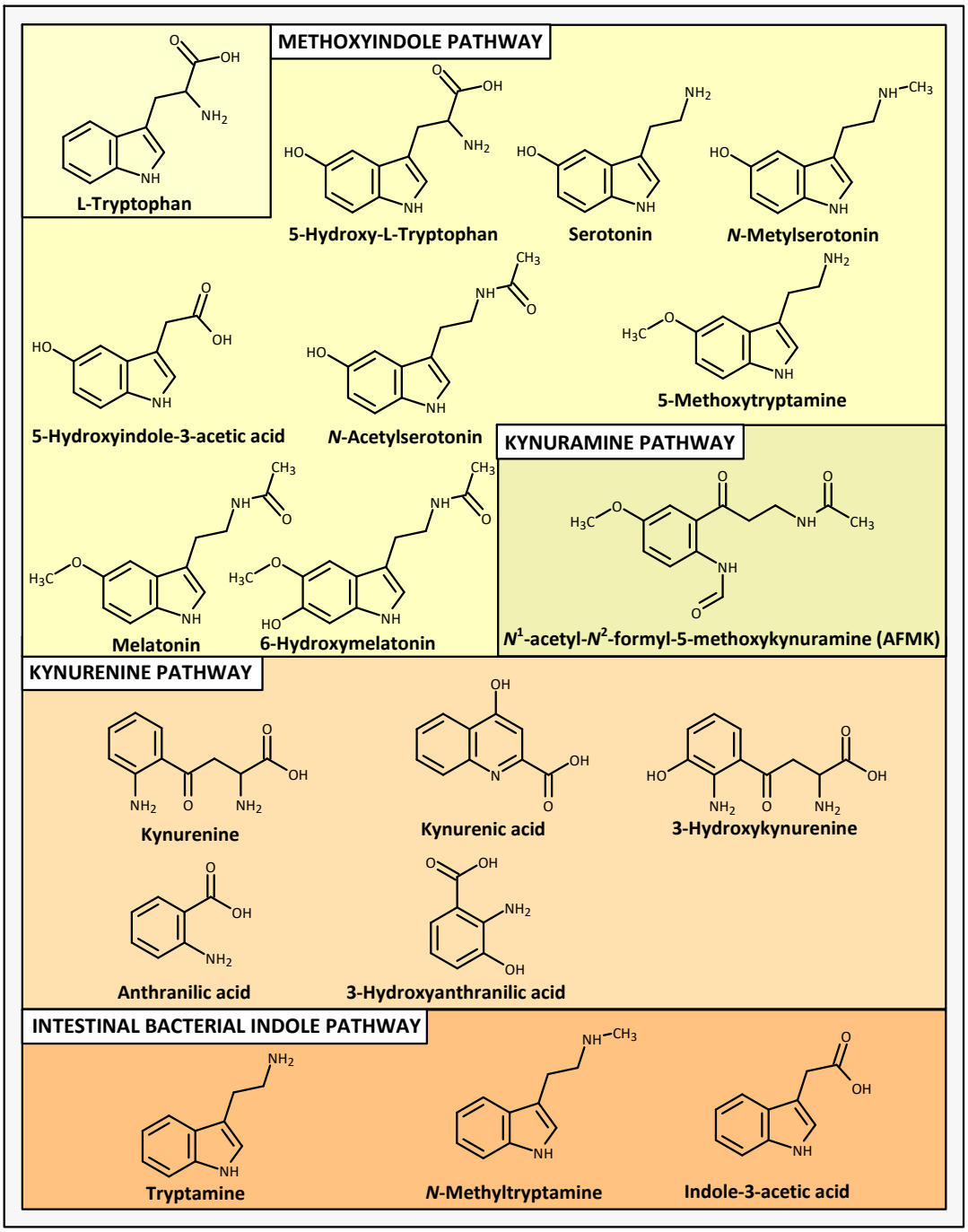

Figure 5. Structures of selected representatives of tryptophan metabolites.

3.3.1 Kynurenine pathway

The products of the kynurenine pathway (called kynurenines) are a variety of neuroprotective (e.g. kynurenic and picolinic acid) and neurotoxic (e.g. 3-hydroxykynurenine, 3-OH-KYN; quinolinic acid) substances (Guillemin 2012; Heilman *et al.* 2020). The dominant product of this route (and overall degradation of ingested TRP) in mammals is L-kynurenine (KYN) (Hényková *et al.* 2016). The balance between the production of neurotoxic and neuroprotective substances is essential for the proper functioning and survival of neurons (Ostapiuk & Urbanska 2022). The neurotoxicity of quinolinic acid leading to neuronal dysfunction or death is due to several mechanisms: its ability to induce microglial activation and neuroinflammation, increase glutamate release from neurons, inhibit glutamate uptake by astrocytes, lipid peroxidation, etc. (Heilman *et al.* 2020; Guillemin 2012). In addition, in high concentrations, it over-activates glutamate receptors with excitotoxic consequences (excessive influx of calcium ions; mentioned in the previous section) (Heilman *et al.* 2020). Another very important metabolite of TRP that is probably related to the pathogenesis of PD is 3-OH-KYN, which can induce mitochondrial dysfunction, neuronal damage, and cell death due to free radical formation and increased oxidative activity (Heilman *et al.* 2020; Klatt *et al.* 2021). This pathway also generates several metabolites that play roles in immune response, inflammation, excitatory neurotransmission, and communication between the nervous and immune systems (Anesi *et al.* 2019; Hényková *et al.* 2016).

There is much evidence for changes in the metabolism of the kynurenine pathway in PD and AD (Chang *et al.* 2018; Heilman *et al.* 2020; Klatt *et al.* 2021; Oxenkrug *et al.* 2017; Sorgdrager *et al.* 2019). Its disruption is probably one of the important factors contributing to the development of these neurodegenerative diseases (Oxenkrug *et al.* 2017). Moreover, the initial enzymes of the kynurenine pathway are probably preferentially induced by pro-inflammatory cytokines and glucocorticoids, i.e. during infection, inflammation, and chronic stress (Höglund *et al.* 2019). For instance, the previously mentioned neurotoxin 3-OH-KYN might function as a peripheral biomarker of PD progression and/or its severity (Heilman *et al.* 2020)

3.3.2 Methoxyindole pathway

Although other metabolic pathways are in the minority, they also serve as a source of bioactive intermediates. Several other TRP-related substances may have potentially beneficial effects in some neuropathologies, e.g. immunomodulatory, antioxidant, anti-inflammatory, analgesic, anticarcinogenic, antiapoptotic, and neuroprotective properties have been described for melatonin (Escribano *et al.* 2014; Escribano *et al.* 2022; Muñoz-Jurado *et al.* 2022; Bahamonde *et al.* 2014; Esposito & Cuzzocrea 2010). Melatonin is primarily secreted by the pineal gland in a circadian rhythm, but other sources may be cells of the skin, bone marrow, gastrointestinal tract, retina, brain as well as cells of the immune system (Muñoz-Jurado *et al.* 2022; Escribano *et al.* 2014). On the level of the whole organism, this neurohormone is involved in the regulation of circadian rhythms, reproductive development, seasonal adaptation, memory formation, or behaviour (Kema *et al.* 2000; Muñoz-Jurado *et al.* 2022).

In addition to melatonin, other metabolites show antioxidant activity. It has been observed that the metabolites *N*-acetylserotonin (immediate precursor of melatonin) and 6-hydroxymelatonin enhance the overall antioxidant protection against oxidative stress that melatonin exerts, mainly due to its higher free radical scavenging activity compared to other melatonin-related compounds (Álvarez-Diduk *et al.* 2015). *N*-acetylserotonin has also been attributed to the ability to participate in maintaining optimal fluidity of biological membranes, improving cognition, protecting against beta-amyloid-induced neurotoxicity, and possessing anti-ageing properties.

Serotonin, also known as 5-hydroxytryptamine, is physiologically involved in the regulation of sexual activity, appetite control, sleep, blood pressure regulation, neurotransmission, smooth muscle contraction, and it also functions as a vasoconstrictor and platelet proaggregator (Kema *et al.* 2000). Additionally, there are suggestions that conditions such as hypertension, depression, anxiety, bipolar disorder, migraine, anorexia, and others are linked to dysregulation and abnormalities of the serotonergic system (Hényková *et al.* 2016; Yubero-Lahoz *et al.* 2014).

In addition, some metabolites also exhibit pharmacologically attractive properties. Preliminary observations suggest a positive effect of 5-hydroxy-L-tryptophan (5-OH-TRP) on depressive symptoms (Meloni *et al.* 2020a), overall sleep quality (Meloni *et al.* 2022), or levodopa-induced motor complications (dyskinesia) (Meloni *et al.* 2020b) in PD patients.

3.3.3 Kynuramine and bacterial intestine pathways

Another interesting group of TRP metabolites is the biogenic amines, kynuramines, e.g. N_1-acetyl-N_2-formyl-5-methoxykynuramine (AFMK) and N_1-acetyl-5-methoxykynuramine (AMK) (Hardeland *et al.* 2009; Hényková *et al.* 2016). These two major brain metabolites of melatonin can scavenge reactive nitrogen and oxygen species and protect tissues from damage by reactive intermediates. Possible anti-inflammatory and immunomodulatory effects have also been reported (Mayo *et al.* 2005; Silva *et al.* 2005).

The biogenic monoamine tryptamine as a representative of bacterial intestinal degradation products can stimulate intestinal motility, release of serotonin by enteric neurons, accelerate whole gut transit, and increase colonic secretion of anions and fluids (Benech *et al.* 2021; Bhattarai *et al.* 2018). It can also undergo metabolic conversion into *N*-acetyltryptamine (Backlund *et al.* 2017). This metabolite, exhibiting a plasma diurnal rhythm, is considered an evolutionary precursor of melatonin. It acts as a mixed agonist-antagonist of melatonin receptors, however, its exact role in the organism has still not been sufficiently investigated (perhaps this is a chronobiological signal).

3.4 Determination of steroids and tryptophan metabolites

The rapid development of omics methods (genomics, transcriptomics, proteomics, and metabolomics) and their introduction into biomedical and clinical research is a great support in the study of human health and disease (Gonzalez-Covarrubias *et al.* 2022). Although there has been a massive development of molecular genetic methods in recent years, other approaches are useful to fill in the missing pieces of the puzzle (Wudy *et al.* 2018). Indeed, not all processes occurring in the human organism can be assessed at the level of genome analysis (monitoring the course of the disease or treatment, etc.). In this respect, the study of the metabolome (a complex, dynamic, sensitive, and precise measure of phenotype) can be a huge source of valuable information (Gonzalez-Covarrubias *et al.* 2022; Smoleńska & Zdrojewski 2015).

Metabolomics is a modern scientific discipline that deals with the comprehensive study (targeted or non-targeted approach) of metabolites, i.e. intermediates of metabolism in living organisms with a typical molecular weight ≤ 1.5 kDa (Resurreccion & Fong 2022; Smoleńska & Zdrojewski 2015). This analytical profiling approach is currently used in many

fields such as environmental epidemiology, food science, or medicine (e.g. oncology) (Resurreccion & Fong 2022). Since the actual metabolic profile (or the levels of specific metabolites) is influenced by several factors, including physiological processes, environmental stimuli, but also ongoing pathologies, metabolomics is a suitable laboratory tool to reveal potential biomarkers providing valuable information on these conditions (Gonzalez-Covarrubias *et al.* 2022; Smoleńska & Zdrojewski 2015). Their monitoring may be useful for prevention, more reliable diagnosis, monitoring of progression, or, for example, efficacy of therapy for certain diseases. The findings may also contribute to the wider application of personalised pharmacotherapy. Metabolomic studies are based on highly reliable and sensitive analytical methods, often nuclear magnetic resonance (NMR) and/or mass spectrometry (MS) in combination with various separation techniques (electrophoresis, chromatography, etc.). Their clear advantage is the minimal sample consumption (below 1 ml or mg) (Smoleńska & Zdrojewski 2015). The choice of appropriate techniques reflects the physicochemical properties of the analytes and the nature of the matrix (different types of biofluids or tissues) (Gonzalez-Covarrubias *et al.* 2022). The aim of metabolomic studies may involve the discovery, validation, or introduction of specific biomarkers (molecular patterns) that have the potential to drive advancements in medicine.

Different approaches to the determination of selected neuroactive substances (steroids with neuroactive effects and potential PD biomarkers - tetrahydroisoquinolines) are discussed in detail in the publications listed in *Supplements I* and *II*. The following sections discuss both the classical and especially modern approaches to the analysis of NASs and TRP-related substances in different types of human matrices.

3.4.1 Detection and quantification techniques

There are several points of convergence in the development and application of analytical methods for the determination of steroids and compounds related to TRP.

In the early days of steroid and TRP analysis, various insufficiently sensitive, laborious, and complicated bioassays (e.g. whole animal *in vivo* steroid bioassays) and/or colorimetric tests were used (Cohen & Bates 1947; Pincus *et al.* 1936; Fischl 1960; Friedman & Finley 1971; Handelsman 2017; Lewy & Markey 1978). A breakthrough was the development of the so-called immunoassay, specifically radioimmunoassay (RIA for the determination of insulin in human plasma - Yalow & Berson 1959), whose application potential in many

modifications (enzyme-, fluorescence-, or chemiluminescence immunoassay; faster and simpler automated platforms) was extended over time to a whole portfolio of analytes (hormones including steroids, vitamins, tumour antigens, viruses, serum proteins, etc.) (Yalow 1978; Glick 2011; Zendjabil *et al.* 2016). Different variants of immunoassays (RIAs, Enzyme-Linked ImmunoSorbent Assay known as ELISA, automated multianalyte analysers, etc.) have also been applied in the analysis of steroid hormones (Abraham 1969; Zendjabil *et al.* 2016; Taieb *et al.* 2003) and TRP metabolites (Engbaek & Voldby 1982; Li & Cassone 2015; Geffard *et al.* 1982) in various type of samples. Unfortunately, there are several limitations associated with the use of these methods - one type of immunoassay allows the determination of only one analyte, interference with matrix components, cross-reactivity with structurally related substances, limited dynamic range, etc. (Yuan *et al.* 2020; Magliocco *et al.* 2021).

Techniques using liquid chromatographic (LC) separation in combination with UV spectrometry, fluorometric, or electrochemical detection are also available for the determination of TRP and some of its major metabolites (Eugster *et al.* 2022; Sadok *et al.* 2017). However, a reliable application of these approaches is usually complicated by their interference with endogenous matrix artefacts. Continuous advances have enabled a wider use of MS detection, which allows simultaneous analysis of multiple analytes with excellent specificity, sensitivity, accuracy, precision, and reliability (Nilsson *et al.* 2015; Eugster *et al.* 2022). Moreover, the clear advantage is the need for a relatively small amount of sample (injection volume in microlitres) (Sadok *et al.* 2017). However, MS is still not a commonly used instrument in laboratories because of its high acquisition and operating costs, as well as the requirement for qualified personnel. Despite these challenges, MS detection is now generally regarded as the gold standard for therapeutic monitoring of hormone levels (the method of choice for steroid measurement) (Conklin & Knezevic 2020; Zendjabil *et al.* 2016). However, other MS methods and the extension of their applicability are continuously being improved and developed (e.g. miniaturization and on-site and real-time monitoring) (Mielczarek *et al.* 2020).

Analytes in unknown samples can be quantified using the isotope dilution method, where a defined concentration of internal standards is added to the sample (Ciccimaro & Blair 2010; Stokvis *et al.* 2005). Structural analogues or, in the best case, stable isotopically labelled analogues of analytes (several atoms are replaced by their isotopes, such as ^{13}C,

^{15}N, ^{17}O, or ^{2}H) are used as internal standards. The addition of a defined concentration of internal standards before sample processing can compensate not only for the loss of analytes during preparation (due to variability in dilution, recovery, evaporation, degradation, derivatisation, etc.) but also for instrumentation variability (such as injection volume and ionisation efficiency). Moreover, this step enables the normalisation of the matrix effects, thereby enhancing the overall accuracy and reproducibility of the analysis (Sadok *et al.* 2017).

MS detection with chromatographic separation, specifically gas chromatography (GC) were combined as early as the 1950s (Picó 2020). A few years later, this hyphenated technique was first used for the identification and quantification of steroid analytes (neutral steroids in human faeces) (Eneroth *et al.* 1964). The development of GC–MS-based methods for the determination of metabolite of the TRP pathway has also not been left behind (Degen *et al.* 1972; Lewy & Markey 1978; Peura *et al.* 1988). However, GC–MS is not the most suitable method for routine use in clinical practice due to its typically more time-consuming and complex sample preparation, often requiring derivatisation of polar, thermolabile, and/or non-volatile analytes (Taylor *et al.* 2015; Storbeck *et al.* 2018; Beale *et al.* 2018). This limitation also hinders the analysis of larger sample sets, making high-throughput analysis impractical. In contrast, liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a more suitable choice in this regard. Nevertheless, it is important not to overlook the importance of the GC–MS technique (more suitable for non-targeted analysis, characterisation of steroid metabolomes), which, complementary to LC–MS/MS, plays a key role in research (Taylor *et al.* 2015; Wudy *et al.* 2018).

The combination of MS with LC became feasible only a few decades later (1980s) due to its higher technical complexity, while its wider dissemination did not occur until the turn of the millennium (Picó 2020). Currently, a full spectrum of LC–MS(/MS) methods are available for the analysis of TRP metabolites (Hényková *et al.* 2016; Tömösi *et al.* 2020; Eugster *et al.* 2022; Ohki *et al.* 2022; Sadok *et al.* 2017) and steroids (reviewed in *Supplement I*). The chromatographic separation of these two groups of compounds in LC is typically achieved through reversed-phase separation, with hydrophobic and van der Waals interactions mainly responsible for analyte retention (Tömösi *et al.* 2020). Stationary phases used in reverse phase chromatography often include hydrocarbon chains, such as octadecyl C_{18} (Hényková *et al.* 2016; Eugster *et al.* 2022; Magliocco *et al.* 2021; van der Veen *et al.*

2019; Yesildal *et al.* 2019; Naldi *et al.* 2016), as well as other options, such as diphenyl (van der Veen *et al.* 2020; van Faassen *et al.* 2021), phenyl-hexyl (Márta *et al.* 2018), pentafluorophenyl (Tömösi *et al.* 2020; Yuan *et al.* 2020), and various other modifications. Currently, one of the most popular MS/MS instruments used for the quantitative analysis of these endogenous substances is the triple quadrupole (van der Veen *et al.* 2019; Yuan *et al.* 2020; Hényková *et al.* 2016; Naldi *et al.* 2016; Eugster *et al.* 2022; van Faassen *et al.* 2021; van der Veen *et al.* 2020; Kaleta *et al.* 2021). This tool enables the quantification of low-abundance target compounds using the multiple reaction monitoring (MRM) mode, which has excellent reliability, selectivity, and sensitivity among other MS acquisition modes (Stachniuk & Fornal 2016).

Although the technique of supercritical fluid chromatography (SCF) has been around for several decades (since the 1960s), it has only become more widespread in recent years due to technological advances and the introduction of commercial instruments (hybrid SFC/UHPLC system; ultra-high performance supercritical fluid chromatography system) (Pilařová *et al.* 2019; Picó 2020). The primary mobile phase currently used in SFC is supercritical carbon dioxide with the addition of various organic modifiers (Teubel *et al.* 2018). The SFC has been applied to various fields, including steroid analysis (Storbeck *et al.* 2018; de Kock *et al.* 2018). This approach enables the development of highly rapid, selective, reproducible, and robust SFC–MS applications while also reducing the use of toxic organic solvents, promoting green chemistry (Pilařová *et al.* 2019; Dhoru *et al.* 2020). In addition, it can be useful in the analysis of chiral, non-volatile, thermolabile, and otherwise unstable compounds (e.g. presence of water). This technique can also be employed for the analysis of polar analytes, as demonstrated by Wolrab and colleagues (2016, 2017), who introduced SFC–MS/MS methods enabling the analysis of selected amino acids and metabolites of the TRP pathways. However, developing methods for the analysis of polar, highly polar, and ionisable analytes with this technique is challenging, typically requiring gradient elution with a mobile phase containing polar additives and organic modifiers (more than 40%) (Pilařová *et al.* 2019).

Development continues to progress rapidly, with ion mobility spectrometry (IMS) emerging as another technique in the field of steroid analysis (Rister & Dodds 2020b). In addition to low physiological concentration and reduced ionisation efficiency, another challenge in steroid analysis is the frequent presence of isomers and isobaric compounds

(Rister & Dodds 2020a). Moreover, they exhibit vastly different biological effects. Several complications may arise in the analysis of steroids by LC–MS/MS such as (i) limited resolution of structurally similar substances and the need to prolong the separation, (ii) the occurrence of similar fragmentation patterns in some isomers (especially stereoisomers) (Chouinard *et al.* 2017). A solution may be to incorporate an additional separation dimension using IMS. The general principle of IMS involves the separation of ions in the gas phase using an electric field and a drift gas, depending on their size, shape, and charge (Rister & Dodds 2020b). In the analysis of steroids, the prevailing methods typically employ temporal separators, such as drift tube ion mobility spectrometry and travelling wave ion mobility spectrometry, as well as spatial separators, such as differential ion mobility spectrometry (also known as high-field asymmetric ion mobility spectrometry). Currently, commercial instruments containing these types of IMS are available. This technique can be integrated with existing GC–MS and LC–MS (potential for higher signal-to-noise ratio and identification reliability) methods or used with IMS–MS alone (with the potential to significantly reduce sample preparation and acquisition time) (Chouinard *et al.* 2017; Rister & Dodds 2020b).

Another emerging area is the development of various biosensors using so-called molecularly imprinted polymers (e.g. for selective determination of TRP or T) (Liu *et al.* 2020; Prabakaran *et al.* 2021). These possess unique cavities that precisely correspond to the size, shape, and functional groups of template molecules. Due to this uniqueness, higher levels of selectivity and/or sensitivity can be achieved when using them.

3.4.2 Sample preparation

Despite the constant advances associated with the availability of highly sensitive and robust analytical techniques, sample preparation should certainly not be overlooked. It is still a key part of the analytical process. It must be adapted to the nature of the sample and the chosen analytical endpoint. The main objectives of sample processing may be extraction, purification (removal of interfering contaminants), and pre-concentration of analytes.

A major challenge and source of analytical problems discussed in connection with the determination of analytes using LC–MS is the presence of what is commonly referred to as the matrix effect (Antignac *et al.* 2005; Wudy *et al.* 2018; Keevil 2013). These reflect the complexity of biological matrices - the presence of phospholipids, carbohydrates, salts,

urea, pigments, analyte analogues, etc. Interferents can co-elute with analytes and thus affect the ionisation efficiency (ion suppression or enhancement) in MS detection. However, the negative influence of matrix effects can be minimised or at least compensated by several procedures: the use of internal standards (the use of deuterium labelling should be considered - a small change in lipophilicity and possible shift in retention time), methods of standard addition, matrix-matched calibration curves, or optimisation of LC–MS parameters (injection volume, flow rate, gradient program, post-column split, choice of ion source, ionisation polarity, etc.) (Zhou *et al.* 2017). The influence of the biological matrix can also be significantly reduced by a properly chosen sample preparation and a clean-up procedure. The possible photosensitivity of some analytes (e.g. melatonin) should also be taken into account and protected from light during all sample handling (Andrisano *et al.* 2000; Hényková *et al.* 2016). In addition, to minimise the degradation of the analytes, it is advisable to work quickly and maintain the samples at a low temperature (Sadok *et al.* 2017).

Some endogenous NASs and various TRP metabolites have been measured by LC–MS/MS in a wide range of body fluids and tissues over the years, e.g. in blood serum (Hényková *et al.* 2016; Dury *et al.* 2015), plasma (Sosvorova *et al.* 2015; Eugster *et al.* 2022), CSF (Hényková *et al.* 2016; Sosvorova *et al.* 2015), urine (Naldi *et al.* 2016; Magliocco *et al.* 2021), saliva (Jurgens *et al.* 2019), brain tissue (Wang *et al.* 2016), hair (Gomez-Gomez & Pozo 2020), or nails (Voegel *et al.* 2018). The most frequently used biofluids for both groups of analytes are blood serum and plasma (reviewed in *Supplement I* and *IV*).

The typical preparation procedure for LC–MS(/MS) analysis of low-abundance analytes such as NASs often includes a series of steps, such as protein precipitation, liquid-liquid extraction (LLE), or solid-phase extraction (SPE), which can be modified and combined in different ways (Keevil 2013). Some of these procedures are also used in the processing of samples for TRP metabolite analysis (Hényková *et al.* 2016; Eugster *et al.* 2022; Magliocco *et al.* 2021). Nevertheless, it is important to realise that each additional step in the sample preparation not only prolongs the total time required to obtain the results but may also raise the financial burden, and, more importantly, increase the likelihood of procedural errors.

The purpose of precipitation is to remove proteins that could compromise analytical instruments (clogging of instrument capillaries, chromatographic columns, etc.) (Keevil

2013). Additionally, the addition of precipitating agents such as acetonitrile (ACN) and methanol (MeOH) allows the release of analytes from their carrier proteins (Wooding & Auchus 2013). This is especially important in the case of steroid hormones, where their bound form represents the majority in the blood (due to their chemical properties; such as albumin, sex hormone-binding globulin, and corticosteroid-binding globulin) (Hammond 2016; Rao 1981). The non-protein-bound fraction of circulating steroid hormones is considered biologically active. The following reagents (in some cases ice-cold) are usually used for protein precipitation, e.g. MeOH (Hényková *et al.* 2016; Márta *et al.* 2018), ACN (Zhao *et al.* 2016; Tömösi *et al.* 2020; Eugster *et al.* 2022; Backlund *et al.* 2017; Yuan *et al.* 2020), acetone/MeOH (Tömösi *et al.* 2020), or zinc sulfate solution/MeOH (Yesildal *et al.* 2019; Gao *et al.* 2015). The precipitate obtained can be removed by centrifugation, in some cases it is also used in combination with sub-micron filtration (e.g. microtubes with an insert including a filtration membrane with porosity 0.20 μm) (Hényková *et al.* 2016). However, the choice of precipitating reagent is not universal and needs to be optimised based on the selected group of analytes (Sadok *et al.* 2017). For example, in the case of kynurenines, the use of acids (since indole derivatives are sensitive to acidic pH) does not seem to be appropriate. A better choice in this case might be precipitation with MeOH, ethanol, or ammonium acetate in either MeOH or water.

Unfortunately, despite the simplicity and speed of precipitation and the possibility of its automation (e.g. using a plate format), the extract obtained after the extraction process is usually not pure enough (Keevil 2013). This is caused by, in addition to proteins, the sample containing other undesirable substances, mainly salts and phospholipids, which can interfere with the analysis (there are also commercial platforms combining protein precipitation and targeted phospholipid removal) (Keevil 2013; Hényková *et al.* 2016). Compared to protein precipitation, extracts obtained by SPE or LLE are cleaner (Keevil 2013). In the case of LLE, organic solvents such as methyl *tert*-butyl ether, diethyl ether, dichloromethane, ethyl acetate, and hexane, can be used (Sosvorova *et al.* 2015; Yuan *et al.* 2020; Wang *et al.* 2016). However, the implementation of this classical and simple approach is associated with significant consumption of organic reagents (Wozniak *et al.* 2012).

A frequently employed purification method nowadays is SPE. Unlike LLE, it has a broader range of applications as it also enables the extraction of ionic compounds (Keevil 2013). The fundamental principle of SPE involves extracting analytes based on their

reversible interactions (whether polar, non-polar, ion exchange, or mixed) with a sorbent (silica or polymer-based), followed by their elution using an appropriate solvent (Sadok *et al.* 2017). There are various options for SPE available for the clean-up of steroids: conventional cartridges, SPE columns or miniaturised approaches employing 96-well plates, offline and online configurations, and various types of sorbents, such as C_8, C_{18}, HLB (universal Hydrophilic-Lipophilic-Balanced reversed-phase polymeric sorbent), mixed-mode type (reversed-phase interaction and anion exchange), and weak anion exchange materials, among others (Caruso *et al.* 2014; van der Veen *et al.* 2019; Gao *et al.* 2015; Hobo *et al.* 2020; Li *et al.* 2018; Márta *et al.* 2018; Wang *et al.* 2020; Dury *et al.* 2015). This variably modified procedure is also commonly used to purify and concentrate samples for LC–MS profiling of TRP metabolites (Sadok *et al.* 2017; Magliocco *et al.* 2021; van der Veen *et al.* 2020; van Faassen *et al.* 2021; Eugster *et al.* 2022).

Sample processing for the determination of conjugated and unconjugated forms of these analytes in urine may also involve enzymatic hydrolysis (e.g. by β-glucuronidase/arylsulfatase from *Helix pomatia*) (Magliocco *et al.* 2021; Wozniak *et al.* 2012). This procedure, on the one hand, allows for the determination of the total concentration of both conjugated (sulphated and glucuronidated) and unconjugated forms. Additionally, it may also improve the detection of the unconjugated form for certain analytes.

Ultimately, incorporating chemical derivatisation before LC–MS analysis may prove advantageous in some cases, such as enhancing chromatographic separation or detection properties (Tömösi *et al.* 2020; Sosvorova *et al.* 2015). There is, for example, the derivatisation technique of 3-OH-KYN, picolinic acid, and quinolinic acid with a mixture of *n*-butanol and acetyl chloride to form their respective butyl ester derivatives (Tömösi *et al.* 2020) or the derivatisation of oestrogens with dansyl chloride (Zhang *et al.* 2019). Other derivatising agents include, for example, isonicotinoyl chloride, 2-hydrazinopyridine, and hydroxylamine hydrochloride (Sharp *et al.* 2018; Sosvorova *et al.* 2015; Yuan *et al.* 2020).

4 Materials and methods

This part of the Ph.D. thesis is devoted to an overview of individual research projects. Detailed information on each method (chemicals used, instrument parameters, etc.) is provided in the research publications in the Supplements section (*Supplement III*, *IV*, and *V*).

4.1 Chemicals

- Unlabelled standards and stable isotopically labelled internal standards (mostly deuterated) were obtained from Sigma–Aldrich (Germany), Fluka (Netherlands), National Measurement Institute (Australia), Cambridge Isotope Laboratories, Inc. (USA), C/D/N Isotopes (Canada), Olchemim Ltd. (Czech Republic), Toronto Research Chemicals (Canada). Some of them were synthesized in the Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany ASCR, Olomouc (*Supplement III–V*).
- All chemicals and solvents for sample preparation and analysis were purchased from Merck Millipore (Germany), Fluka (USA), Sigma-Aldrich (USA), Lach-ner (Czech Republic), Linde Industrial Gases (Czech Republic), and Tocris Bioscience (UK). Ultra-pure water was produced using the Direct-Q® 3 UV Water Purification System (Merck Millipore, Germany) (*Supplement III–V*).

4.2 Biological material

Human serum and CSF samples were provided by the Department of Neurology, University Hospital Olomouc, Czech Republic. Peripheral blood and CSF were collected, processed, transported, and stored according to the standardised protocol of the Department of Neurology. The detailed pre-treatment procedure of these biofluids is listed in *Supplement III–V*. All samples (i.e. CSF and serum obtained) were stored in the dark at -80 °C until analysis.

This biological material was used following an approval of the ethics committee of the Faculty of Medicine and Dentistry, Palacký University Olomouc and University Hospital Olomouc. Ethical approval was granted according to the standard of the University Hospital Olomouc SM-L031 and the reference numbers of the ethics committee: 139/10 and 76/15.

All volunteers were informed about the purpose of the study and signed an informed consent.

4.3 Instrumentation

- ACQUITY™ UPLC™ H-Class PLUS System (Waters, USA) connected to a triple quadrupole mass spectrometer (MS) Xevo® TQ-S micro (Waters, UK) (*Supplement III*)
- ACQUITY® UPLC® (Waters, USA) system connected to a triple MS Xevo® TQ (Waters, UK) (*Supplement IV*)
- Kinetex® Biphenyl column (100 × 2.1 mm, 1.7 µm, 100 Å; Phenonemex, USA), ACQUITY Column In-Line Filter kit (Waters, UK) (*Supplement III*)
- ACQUITY UPLC® HSS T3 column (2.1 × 100 mm, 1.8 µm, 100 Å; Waters, UK), ACQUITY UPLC® HSS T3 VanGuard™ pre-column (2.1 mm × 5 mm, 1.8 µm, 100Å; Waters, UK) (*Supplement IV*)
- Atellica® CH analyser (Siemens, USA; SEKK certified) (*Supplement V*)
- ELISA kits (CE-IVD; BioVendor, Euroimmun, EPITOPE Diagnostic) (specified in *Supplement V*)

4.4 Methods

4.4.1 Extraction and purification methods (*Supplement III, IV*)

- Serum samples (150 µL) for steroid analysis were subjected to a protocol involving precipitation of serum proteins and extraction of steroid analytes using ice-cold ACN (595 µL, -20 °C) containing 0.05% butylated hydroxytoluene (prevention of oxidation). The protocol included incubation of samples on a rotator (1 h, -20 °C) with the addition of ACN, MeOH (45 µL; instead of standard solution in the calibration), and internal standards (5 µL; the defined addition shown in *Supplement III*), centrifugation, filtration (centrifuge filter microtubes, nylon, 0.2 µm), and evaporation under a stream of nitrogen. Before analysis, the samples were reconstituted in 100% MeOH (50 µL) and filtered again (centrifuge filter microtubes, nylon, 0.2 µm). The quantification of the analytes was performed using matrix-matched calibration curves prepared from artificial serum: 4% bovine serum albumin (BSA) in 10 mmol/L phosphate-buffered

saline (PBS), pH 7.4. The same purification and extraction protocol was used for calibration samples and serum samples (*Supplement III*).

- TRP-related analytes were analysed in blood serum and CSF samples (100 µL). The samples were protected from light during processing and placed in a CoolBox™ (Biocision). The corresponding isotopically labelled internal standards were added to each sample at the beginning of the extraction (listed in Hényková *et al.* 2016). The purification protocol included protein precipitation with 100% MeOH for 1 h at -20 °C, centrifugation, filtration (centrifuge filter tubes, nylon, 0.2 µm), and evaporation (under nitrogen). Samples were reconstituted in 2% aqueous MeOH (30 µL) before LC–MS/MS analysis. The quantification was performed using serum (4% BSA in 10 mmol/L PBS) and CSF (CSF calibrator; Tocris Bioscience, UK) matrix-matched calibration curves (*Supplement IV;* Hényková *et al.* 2016*)*.

4.4.2 UHPLC–MS/MS analysis (*Supplement III, IV*)

- Selected steroid analytes were determined by ACQUITY™ UPLC™ H-Class PLUS System (Waters, USA) connected to a triple quadrupole MS Xevo® TQ-S micro (Waters, UK) with electrospray ionisation (ESI). The samples were injected (2 µL) onto a reversed-phase chromatography column (Kinetex® Biphenyl column; 100 × 2.1 mm, 1.7 µm, 100 Å; Phenonemex, USA) maintained at 40 °C. Analytes were eluted with 100% MeOH (A) and 7.5 mmol/L aqueous formic acid (B) as mobile phases at a flow rate of 0.5 mL/min using the following gradient: 0–10 min, 60–75% A; 10–12 min, 75–85% A; 12–12.25 min, 85–99% A; 12.25–12.75 min, 99% A; 12.75–13 min, 99%–60% A; 13–15 min, 60% A. The tandem MS with positive ESI was operated in MRM mode using quantification and confirmation transitions. Based on the expected retention times of the analytes, data acquisition was divided into five separate MRM scan segments. The defined addition of isotopically labelled (deuterated) internal standards enabled the quantification of analytes by the isotopic dilution method (*Supplement III*).
- UHPLC–MS/MS analysis of targeted TRP and TRP-related analytes was performed by ACQUITY® UPLC® (Waters, USA) system connected to a triple MS Xevo® TQ (Waters, UK). The analytes (sample injection 10 µL) were separated using an ACQUITY UPLC® HSS T3 column (2.1 × 100 mm, 1.8 µm, 100 Å, Waters, UK; temperature 30 °C) equipped with a pre-column ACQUITY UPLC® HSS T3 VanGuard™ (2.1 mm × 5 mm, 1.8 µm, 100Å;

Waters, UK) and were eluted in a gradient (0–2 min, 98% A; 2–10 min 40% A) of aqueous 0.1% formic acid (A) and 100% MeOH (B) at a flow rate of 0.3 mL/min. A wash and equilibration steps were performed at the end of the gradient. The MS instrument with ESI source was operated in MRM mode (*Supplement IV*).

4.4.3 Biochemical determination of protein analytes (*Supplement V*)

- The biochemical analyses were performed in the accredited laboratory of the Department of Clinical Biochemistry, University Hospital Olomouc (CSN ISO 15189:2013; subject No. 8254; certificate No. 220/2021 valid until 9 April 2026).
- The sandwich ELISAs (CE-IVD) were used to determine: clusterin, tau protein, phosphorylated tau protein, β-amyloid $_{1\text{-}42}$, α-synuclein, phosphorylated form of neurofilament heavy chains, chromogranin A, and cystatin C. Serum cystatin C was analysed using an Atellica® CH analyser (Siemens, USA). Detection was performed spectrophotometrically or fluorometrically. Further information is provided in *Supplement V*.

5 Survey of results

Certain pathologies of the nervous system, among others, can affect metabolic pathways, thereby altering the levels of certain metabolites. Knowledge of specific metabolic profiles or patterns is essential for several reasons, for example, it may provide a basis for the discovery of new biomarkers or therapeutic targets. However, highly reliable analytical techniques are needed to monitor and map these changes (reviewed in *Supplements I* and *II*). Therefore, a sensitive method based on UHPLC–MS/MS has been developed that allows the determination of some representatives of NASs in human serum (*Supplement III*). Moreover, alterations in TRP metabolism at the level of the kynurenine, methoxyindole, kynuramine, and intestinal bacterial indole pathways have been comprehensively mapped in several degenerative proteinopathies (*Supplement IV*). Serum and CSF levels of several candidate protein biomarkers were also analysed in the same neurodegenerative cohorts (*Supplement V*).

5.1 Method development for NAS determination (*Supplement III*)

A complex method allowing simultaneous detection and quantification of nine selected steroids with neuroactive effects in human blood serum has been developed and validated. This method combines a relatively time-effective and simple purification protocol and a sensitive detection method based on UHPLC–MS/MS. The analytes included representatives of progestins (PREG, ALLO, PROG, and DHP) and androgens (T; DHT; androstenedione, ANDRO; epiandrosterone, EPIA; DHEA). Details are given in *Supplement III*.

5.1.1 Extraction and purification protocol

- A purification and extraction technique consisting of three main steps, namely serum protein precipitation, filtration, and evaporation, was proposed for the processing of blood serum samples. This simple arrangement makes the resulting sample processing relatively rapid and simple. The use of ice-cold MeOH and ACN (-20 °C) in combination without and with filtration (micro-spin filter tubes with 0.2 µm porous membranes) was tested. The best process efficiency values were achieved for ACN in combination with filtration. Filtration optimisation resulted in the selection of filters with a nylon-based membrane (other filters tested were Bio-Inert modified nylon, polytetrafluoroethylene,

and polyvinylidene fluoride). Butylated hydroxytoluene (0.05%) was added to the extraction agent to protect the analytes from oxidation. A proportion of MeOH was also added to the serum samples so that their processing corresponds to the preparation of matrix-matched calibration samples (steroid standards prepared in MeOH), see *Supplement III*.

5.1.2 UHPLC–MS/MS method

- The final reversed-phase chromatographic separation (Kinetex® Biphenyl column, 100 × 2.1 mm, 1.7 µm, 100 Å, Phenomenex, USA) of one sample was performed within 15 min with a gradient consisting of MeOH and 7.5 mmol/L aqueous formic acid at a flow rate of 0.5 mL/min. Analytes were eluted from 4.16 min (DHEA) to 10.83 min (DHP) (*Figure 3* and *Table 3* in *Supplement III*). A triple quadrupole MS in positive ESI mode was used to detect the target steroid analytes. The MS instrument operated in MRM mode with protonated $[M+H]^+$ or $[M-H_2O+H]^+$ molecules formed by the loss of water molecules as precursor ions. The values of collision energy and cone voltage were optimised to obtain specific product ions with high abundance and to ensure the highest possible sensitivity. Two MRM mass transitions were selected for each analyte, one was used for quantification and the other for confirmation. The dwell time values (0.050–0.250 ms) were set to achieve at least 15 scan points per chromatographic peak width. The MS parameters (MRM transitions, cone voltages, collision energies, dwell times, etc.) for each analyte and the corresponding internal standards are listed in *Supplement III* (*Table 3*).

5.1.3 Analytical method validation

- The developed method was validated based on the European Medicines Agency (EMA) guideline (EMA 2011). Validation experiments were performed using four quality control levels: low (LQ; 0.0569 pmol/inj.), medium (MQ; 0.18 pmol/inj.), high (HQ; 1.8 pmol/inj.), and ultra-high (UHQ; 5.7 pmol/inj.).
- The quantification of analytes was performed using matrix-matched calibration curves prepared in an artificial matrix (4% BSA in 10 mmol/L PBS, pH 7.4) spiked with unlabelled steroid standards (from 0.18 fmol/inj. to 57 pmol/inj.). Defined additions of stably isotopically labelled deuterated standards allowed the quantification of analytes

by the isotope dilution method. An overview of the individual analytes, the corresponding deuterated internal standards, and their optimised additions are given in *Supplement III* (*Table 2*). At least seven-point calibration curves with coefficients of determination (r^2) ≥0.9989 were obtained for all analytes. The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined as a signal-to-noise ratio of ≥3 and ≥5, respectively. The lowest detectable levels of some steroids were below 1 fmol/inj., while their LLOQs ranged from 0.0018 to 0.0569 pmol/inj. (see *Table 4* in *Supplement III*).

- Within- and between-run precision and accuracy were determined using sample sets of neat solutions (100% MeOH) spiked with unlabelled standards at four quality control levels (LQ, MQ, HQ, and UHQ) and a defined addition of internal standards. Within-run precision and accuracy were also determined using the participants' pool serum. Each quality control level was represented by five replicates. The measured mean concentrations for most analytes in neat solution and serum did not differ from the reference values by more than ±15%. The coefficient of variation values ranged from 0.2 to 14.1% (*Tables 5* and *6* in *Supplement III*). These results are in line with EMA recommendations (EMA 2011).
- Method recovery, matrix effect, and internal standard-normalised matrix effect were determined using blood serum from several individual participants. Two sets of serum samples were prepared: spiked at four quality control levels before and after extraction. The detailed methodology for the calculation of recovery, matrix effect, and IS-normalised matrix effect is described in *Supplement III*. Analytical method recoveries of steroids in serum samples were between 66 and 102% (*Figure 4* in *Supplement III*). However, as the accuracy and precision determination has shown, the use of internal standards compensates for these process losses. The higher standard deviation values, especially for DHEA, are probably cause by the use of serum from several participants and their individual characteristics. The strongest matrix effect in terms of ion suppression was observed for DHP. Values of the absolute and internal standard-normalised matrix effect ranged from 19 to 24% and from 27 to 33%, respectively (*Table 7* in *Supplement III*). Such a strong matrix effect may have several explanations: the elution of DHP at the end of the gradient together with a high proportion of contaminants interfering with its ionisation efficiency and the under-compensation of

matrix effect (use of an internal standard eluting at a different retention time). However, the quantification of the analytes is accurate and precise (*Table 5* and *6* in *Supplement III*), which is ensured using matrix-matched calibration curves.

- Finally, the developed and validated method was successfully applied to the analysis of 16 female and male serum samples. The determined endogenous concentrations of the target analytes corresponded to the expected levels (*Table 8* in *Supplement III*).

5.2 Metabolic profiling of tryptophan-related metabolites (*Supplement IV*)

The endogenous levels of 18 TRP-related neuroactive compounds were profiled by a high-throughput and sensitive UHPLC–MS/MS method (Hényková *et al.* 2016) in time-linked serum and CSF of 100 participants. They were divided into five cohorts based on clinical diagnoses: LBD (PD + DLB), 4R-Tau (PSP + CBS), MSA, AD, and healthy controls (HC). The basic characteristics of the cohorts are shown in *Table 1*. Diagnostic criteria and more detailed demographic characteristics are provided in *Supplement IV*.

Table 1. Characteristics of the study participants (*n* = 100).

Participants group	Description	Number of participants	Sex ratio (M/F)	Age median (range)
LBD	Parkinson's disease, Dementia with Lewy bodies	31	9/22	69 (38–82)
4R-Tau	Progressive supranuclear palsy, Corticobasal syndrome	10	2/8	66 (51–83)
MSA	Multiple system atrophy	13	2/11	65 (52–80)
AD	Alzheimer's disease	25	3/22	75 (51–90)
HC	Healthy control	21	11/10	57 (37–75)

LBD, Lewy body disease; 4R-Tau, Four-repeat tauopathy; MSA, Multiple system atrophy; AD, Alzheimer's disease; HC, Healthy control

5.2.1 Alterations of tryptophan metabolism in selected neurodegenerations

- Serum and CSF levels of eight analytes were below the LOQ or LOD in all or most participants: *N*-methylserotonin, tryptamine, *N*-methyltryptamine, 5-methoxytryptamine, *N*-acetylserotonin, 6-hydroxymelatonin, melatonin, and AFMK. For this reason, these analytes were excluded from further statistical evaluations. The remaining ten analytes were successfully quantified, these are TRP, 3-OH-KYN, serotonin, KYN, 5-OH-TRP, 3-hydroxy-anthranilic acid, 5-hydroxyindole-3-acetic acid, kynurenic acid, anthranilic acid, and indole-3-acetic acid. These analytes were subjected to statistical analyses (as described in *Supplement IV*).
- The most statistically significant differences (p-values of ≤ 0.05 to ≤ 0.0001) between the study cohorts were observed for 3-OH-KYN and 5-OH-TRP in serum, and KYN in CSF. A significant increase in serum 5-OH-TRP was found in the LBD, 4R-Tau, and MSA groups compared to HC and/or AD. The serum 3-OH-KYN levels were significantly different in the LBD and AD groups, as were CSF KYN concentrations in the LBD and HC groups (*Figure 1A-C* in *Supplement IV*).
- Furthermore, it was suggested that the effect of anti-Parkinsonian treatment (levodopa and peripheral decarboxylase inhibitors) led to a significant increase in 5-OH-TRP and 3-OH-KYN levels in the treated LBD group. A similar trend, but without statistical significance, was also observed in the case of MSA (limited number of samples) (*Figure 1D-G* in *Supplement IV*). The effect of treatment could not be evaluated in the 4R-Tau group due to the limited number of patients.
- The increase in 5-OH-TRP in the LBD group may be due to substrate competition between levodopa and 5-OH-TRP at the aromatic amino acid decarboxylase (EC 4.1.1.28) (see *Figure 6*). The effect of a peripheral inhibitor may also contribute to the increase. A similar trend was also observed for MSA, but the mechanism of the increase in 5-OH-TRP may be different.
- The effect of anti-Parkinsonian treatment was also observed in the case of 3-OH-KYN (unknown mechanism). The benefit of complementary treatments targeting the synthesis of this neurotoxic metabolite has been suggested.

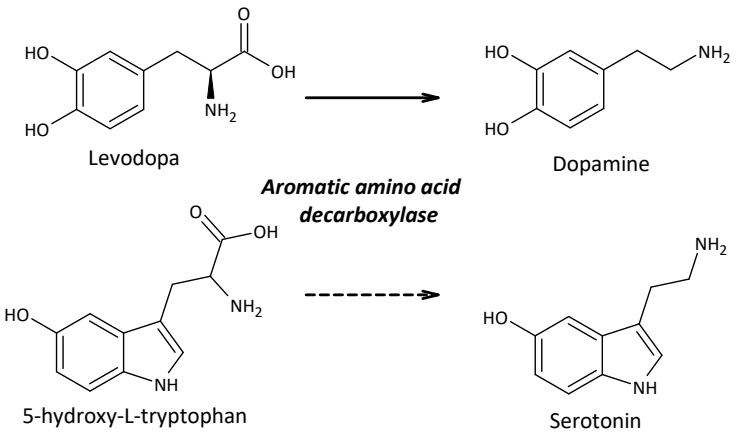

Figure 6. Metabolism of levodopa and 5-hydroxy-L-tryptophan.

- Pearson correlation analysis revealed an increased proportion of negative correlations between serum and CSF analytes in the 4R-Tau compared with the other cohorts (*Supplement IV; Figure 2*). This specific correlation pattern could be a first step in developing a reliable tool to distinguish between tauopathies (4R-Tau; PSP + CBS) and synucleinopathies (DLB, PD, and MSA). Alterations in different phases of TRP metabolism may influence the neurotoxicity of protein aggregates and thus contribute to the development of different types of neurodegenerative proteinopathies (*Supplement IV*).

5.3 Identification of serum and CSF protein biomarkers (*Supplement V*)

Selected candidate protein biomarkers (alpha-synuclein, tau protein, phosphorylated tau protein, beta-amyloid, clusterin, chromogranin A, cystatin C, neurofilament heavy chains, phosphorylated form of neurofilament heavy chains, and ratio of tau protein/beta-amyloid) were determined in serum and CSF of several selected neurodegenerative proteinopathies. The study cohorts were LBD, MSA, 4R-Tau, and HC. Diagnostic criteria, patient recruitment, and more detailed demographic characteristics of participants are provided in *Supplement V*.

- The results suggest that the determination of these specific biomarkers in blood serum, compared to CSF, does not provide any diagnostic benefit.
- The CSF collection, despite its complications (e.g. the invasiveness of the procedure for the patient), is still an important source of diagnostic information.
- The proposed panel of biomarkers in CSF could potentially be useful for the differentiation of MSA and 4R-Tau, LBD and MSA, but not LBD and 4R-Tau.

6 Conclusion and perspectives

Given the ongoing challenges associated with diagnosing neurodegenerative diseases, the pursuit of novel, highly reliable, specific, and sensitive biomarkers enabling their accurate and accelerated diagnosis is currently a significant area of research. In response to this, a comprehensive mapping of changes in TRP metabolism was conducted across several neurodegenerative proteinopathies. Furthermore, protein candidate biomarker analysis was also conducted within the same cohorts. Another highly intriguing group of substances, possessing neuroactive effects, and thus holding potential significance in terms of their involvement in pathogenesis or potential therapeutic applications, are steroid compounds. In this context, a new analytical method has been developed to enable the simultaneous profiling of several of their representatives. The most important outcomes of the work are:

- A purification and UHPLC–MS/MS detection method enabling metabolic profiling of nine selected representatives of progestins and androgens with neuroactive effects in human serum has been developed and validated.
- The determination of 18 TRP-related substances in serum and CSF in four types of neurodegenerations has been performed. Significant differences between groups were determined for serum 5-OH-TRP, 3-OH-KYN, and CSF KYN. It has been suggested that the effect of anti-Parkinsonian treatment may contribute to changes in their levels. A specific correlation pattern of TRP metabolites was found in the 4R-Tau. This observation may guide the development of tools for the differential diagnosis of tauopathies and synucleinopathies.
- In the same cohorts of patients, the levels of some protein biomarkers were determined. The suggested CSF biomarker platform could prove valuable in distinguishing between MSA and 4R-Tau, as well as between LBD and MSA.

In summary, reliable profiling of candidate molecule levels using sensitive analytical methods can help to better understand metabolic changes under physiological and pathological conditions. The knowledge gained may contribute to the discovery of new biomarkers (predictive, diagnostic, prognostic, etc.). In addition, new therapeutic strategies could be designed. However, finding sensitive and specific serum biomarkers is still a major challenge, if only because of the higher invasiveness of CSF sampling.

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8 Supplements I–V

Supplement I

Kaleta, M., Oklestkova, J., Novák, O. and Strnad, M. (2021) Analytical Methods for the Determination of Neuroactive Steroids. *Biomolecules* 11, 553.

Supplement II

Hényková, E.[2], **Kaleta, M.**[2], Klíčová, K., Gonzalez, G., Novák, O., Strnad, M. and Kaňovský, P. (2022) Quantitative Determination of Endogenous Tetrahydroisoquinolines, Potential Parkinson's Disease Biomarkers, in Mammals. *ACS Chemical Neuroscience* 13, 3230-3246.

Supplement III

Kaleta, M., Oklestkova, J., Strnad, M., Novák O. Simultaneous Determination of Selected Steroids with Neuroactive Effects in Human Serum by Ultra-High Performance Liquid Chromatography–Tandem Mass Spectrometry (In preparation).

Supplement IV

Kaleta, M., Hényková, E., Menšíková, K., Friedecký, D., Kvasnička, A., Klíčová, K., Koníčková, D., Strnad, M., Kaňovský, P., Novák O. Patients with Neurodegenerative Proteinopathies Exhibit Altered Tryptophan Metabolism in the Serum and Cerebrospinal Fluid. *ACS Chemical Neuroscience* (Submitted).

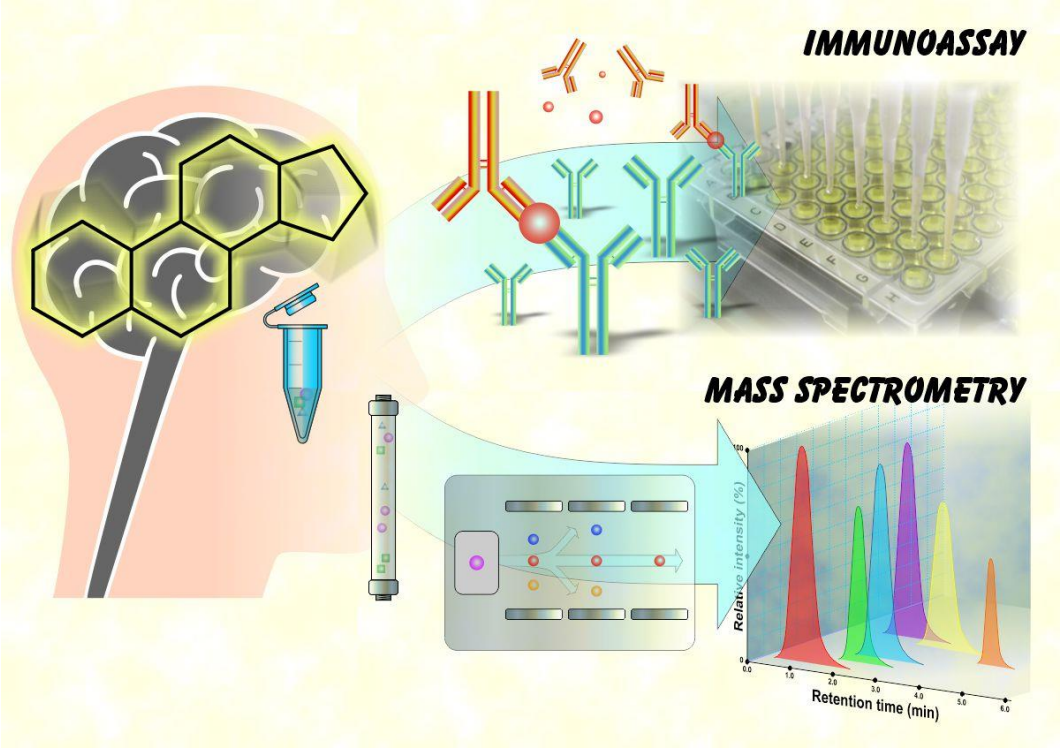
Supplement IV

Koníčková, D., Menšíková, K., Klíčová, K., Chudáčková, M., Kaiserová, M., Přikrylová, H., Otruba, P., Nevrlý, M., Hluštík, P., Hényková, E., **Kaleta, M.**, Friedecký, D., Matěj, R., Strnad, M., Novák, O., Plíhalová, L., Rosales, R., Colosimo, C., Kaňovský, P. (2023) Cerebrospinal Fluid and Blood Serum Biomarkers in Neurodegenerative Proteinopathies: A Prospective, Open, Cross-Correlation Study. *Journal of Neurochemistry* 167, 168-182.

[2] E.H. and M.K. contributed equally to the presented paper.

Supplement I

Analytical Methods for the Determination of Neuroactive Steroids



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Review

Analytical Methods for the Determination of Neuroactive Steroids

Michal Kaleta, Jana Oklestkova *, Ondřej Novák and Miroslav Strnad

Laboratory of Growth Regulators, Faculty of Science and Institute of Experimental Botany of the Czech Academy of Sciences, Palacký University, Šlechtitelů 27, CZ-78371 Olomouc, Czech Republic; michal.kaleta@upol.cz (M.K.); novako@ueb.cas.cz (O.N.); miroslav.strnad@upol.cz (M.S.)

* Correspondence: jana.oklestkova@upol.cz; Tel.: +420-585-634-853

Abstract: Neuroactive steroids are a family of all steroid-based compounds, of both natural and synthetic origin, which can affect the nervous system functions. Their biosynthesis occurs directly in the nervous system (so-called neurosteroids) or in peripheral endocrine tissues (hormonal steroids). Steroid hormone levels may fluctuate due to physiological changes during life and various pathological conditions affecting individuals. A deeper understanding of neuroactive steroids' production, in addition to reliable monitoring of their levels in various biological matrices, may be useful in the prevention, diagnosis, monitoring, and treatment of some neurodegenerative and psychiatric diseases. The aim of this review is to highlight the most relevant methods currently available for analysis of neuroactive steroids, with an emphasis on immunoanalytical methods and gas, or liquid chromatography combined with mass spectrometry.

Keywords: immunoassay; mass spectrometry; metabolomics; neuroactive steroids; steroid

Citation: Kaleta, M.; Oklestkova, J.; Novák, O.; Strnad, M. Analytical Methods for the Determination of Neuroactive Steroids. *Biomolecules* **2021**, *11*, 553. https://doi.org/10.3390/biom11040553

Academic Editor: Hana Kubova

Received: 10 March 2021
Accepted: 8 April 2021
Published: 9 April 2021

1. Introduction

Neuroactive steroids (NASs) can be characterized as substances of steroid origin that can have effects on the nervous system [1]. They include hormonal steroids which originated in the peripheral glands, steroids locally synthesized by neurons and glial cells, and synthetic steroids that modify the activity of the nervous system [2].

Their core structure, as for other steroids, is represented by sterane or cyclopentanoperhydrofenanthrene [3] (Figure 1). Members of this group, such as progesterone, estrogens, testosterone, dehydroepiandrosterone (DHEA), or cortisol, are involved in shaping the structure and function of the central nervous system throughout the life cycle [4]. The nervous system is affected by both endogenously synthesized NASs and steroids of exogenous origin [5]. The first location for NAS steroidogenesis is the peripheral endocrine gland. However, the biosynthesis of these substances can also occur directly in the central and peripheral nervous systems, based on which this specific subgroup is referred to as neurosteroids [6]. Steroids of exogenous origin include substances prepared synthetically. The regulation of many processes in the body is based on the ability of NASs to interact with different types of receptors [7]. In particular, these are γ-aminobutyric acid (GABA) receptors, N-methyl-D-aspartate (NMDA) receptors, voltage-gated calcium channels, voltage-dependent anion channels, serotonin receptors, microtubule-associated protein 2, and others.

Many metabolites of sex hormones and some stress hormones act in the central nervous system through the so-called nongenomic mechanism, the effect of which is manifested within a period ranging from a few milliseconds to seconds [8] (Figure 2). These rapid nongenomic effects are made possible due to the interaction of NASs with ion channels and membrane receptors [9]. In contrast, when steroids interact with nuclear or cytoplasmic receptors, the resulting effect occurs after a longer period of time [10]. This is because these intracellular receptors function as transcription factors and are involved

in gene expression [11]. NASs can thus affect the function of neurons, as well as other cells of the brain, such as astrocytes, microglia, oligodendrocytes, and endothelial cells [1]. NASs are likely involved in the regulation of neurogenesis, neuron survival, neuritogenesis, glial cell differentiation, myelin formation, and synaptic plasticity. Their neuroprotective effects and their ability to suppress nerve tissue inflammation are also described. For a wide range of neurological and psychiatric diseases, such as schizophrenia, epilepsy, depression, or multiple sclerosis, these substances play an important role in their pathology and therapy [11]. Hormonal differences between men and women are noticeable under both physiological and pathological conditions [1]. It has already been shown that neurodegenerative diseases, such as multiple sclerosis, can affect the levels of circulating NASs [12]. Monitoring these changes in blood plasma or cerebrospinal fluid (CSF) can serve as a warning signal (biomarkers) to draw attention to pathological processes taking place in the nervous system.

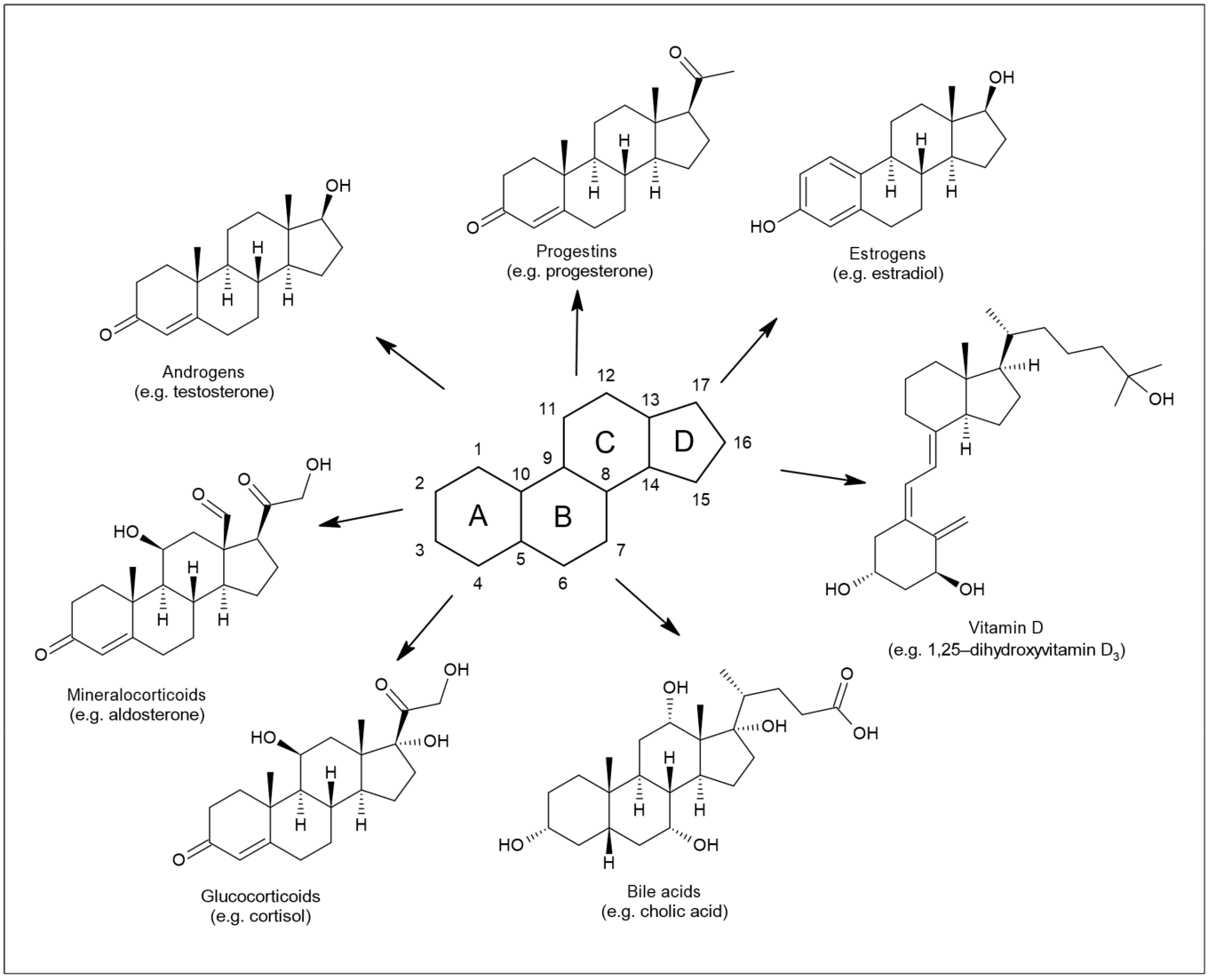

Figure 1. Structure of sterane and seven principal classes of steroid substances.

Knowledge of NAS formation and their correct detection can be used to prevent and treat some neurodegenerative and psychiatric diseases. The aim of this article is to provide an overview of the most important methods for NAS analysis, with an emphasis on immunoanalytical methods and gas (GC), or liquid chromatography (LC) combined with mass spectrometry (MS).

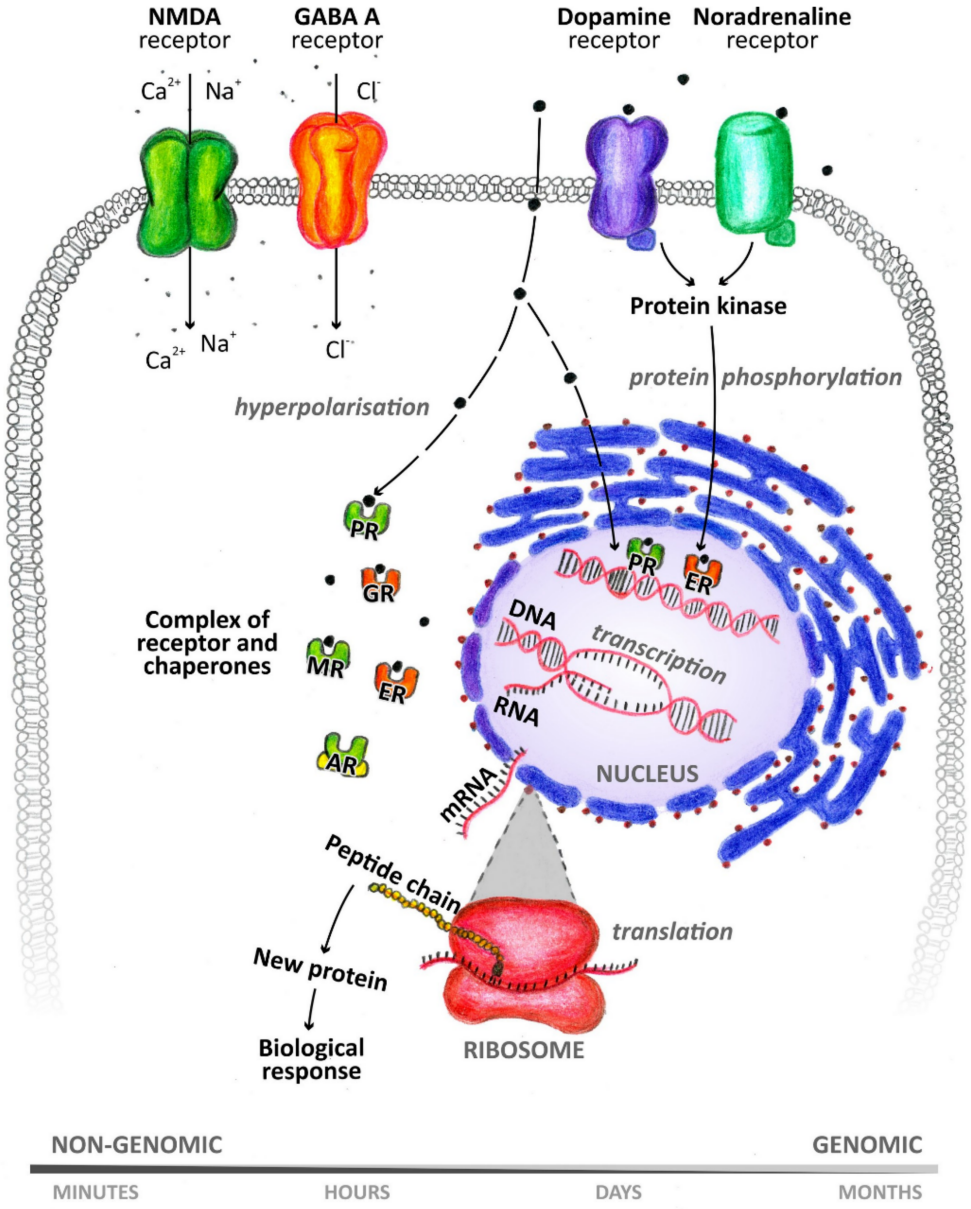

Figure 2. Genomic and nongenomic mechanism of neuroactive steroid action. PR: progesterone receptor, GR: glucocorticoid receptor, MR: mineralocorticoid receptor, ER: estrogen receptor, AR: androgen receptor.

2. Determination of Neuroactive Steroids

Efforts to quantify steroid hormones using colorimetry-based methods have existed since the 1930s [13–15]. One of the first methods used to define and quantify hormonal activity was also a bioassay [16]. The so-called whole animal in vivo bioassays used before the introduction of radioimmunoassays (RIAs) were arduous and insensitive [17]. Despite large advances in molecular–genetic methods (e.g., PCR, DNA sequencing), determining the metabolic profile of NASs is still important [18]. The information obtained can be used to diagnose, but also to monitor, the course of a disease. Immunoassay- (IA) and MS-based methods are currently used to monitor steroid hormone levels [19]. They provide higher sensitivity, specificity, and reliability. RIAs and direct immunoassays (DIA) dominate among IA approaches.

2.1. Types of Biological Matrices

To determine steroid hormones in clinical practice, blood serum and plasma samples are mainly used [20–25]. Moreover, saliva can be an alternative biological material. The advantages of this type of sample are that it is noninvasive, less stressful, and easier to collect compared to blood. Saliva sampling can also be performed in a home environment. In addition, there is a correlation between levels of unconjugated steroid hormones in saliva and levels of their unbound fraction in blood serum. The final levels may differ, which is a consequence of steroid metabolism in the salivary glands. The ratio of cortisol concentrations in saliva and blood serum is around 1:20 and, for testosterone and estradiol, it approximates to 1:90. Furthermore, steroids can be analyzed in other biological matrices, such as urine, CSF, hair, or nails [26–32]. Urinary steroid profiling has been an integral part of the diagnosis of steroid metabolism disorders for several decades (since the 1960s) [33]. Great interest is focused on the analysis of steroids in human CSF [12,32,34–36]. CSF is the only matrix that can be obtained from living donors and it allows for the monitoring of brain metabolism. As mentioned above, the nervous system is a source of neurosteroids. Central neurosteroids are studied as potential biomarkers of various cognitive disorders (e.g., dementia, depression). Extensive research in and use of this matrix are complicated by its limited availability, due to the complexity of collection (usually lumbar puncture), the limitations of its indication in healthy individuals (very small participant groups), as well as its limited sample amount for analysis. Due to the trace levels of neurosteroids in CSF, great demands are also placed on analytical techniques. The interested reader is referred to the cited reference for detailed description of CSF analysis [34].

2.2. Factors Affecting Steroid Hormone Levels

The results of NAS analysis are affected by several important factors, such as age, sex, and, of course, sampling time [19]. A natural decrease in the levels of certain steroids in relation to male age has been found. A similar trend can be observed for women; however, hormonal changes also occur during the menstrual cycle. A decrease in testosterone levels with increasing age in men and women has also been determined in saliva [24]. Levels of some steroid hormones in humans fluctuate during the day (e.g., cortisone, cortisol, corticosterone, 11-deoxycortisol, androstenedione, 17α-hydroxyprogesterone, DHEA, testosterone) [37,38]. Therefore, it is important to take samples under standardized conditions. Boyce and co-workers (2004) published different reference ranges for serum testosterone levels in men depending on the time of day [39]. The range for morning testosterone concentrations is 10.07–38.76 nmol.L^{-1} for men under 40 years, and 7.41–24.13 nmol.L^{-1} for men over 40 years of age. The evening ranges are then 6.69–31.51 nmol.L^{-1} and 6.46–21.93 nmol.L^{-1}, respectively. Salivary testosterone levels for men and women even differ depending on the season [24]. Keevil and co-workers (2017) found the lowest levels of salivary testosterone for men, and the highest for women, in the summer (June–August). In addition to age, sex, and time of day, total testosterone levels are also affected by various pathological conditions [40].

3. Analysis of Neuroactive Steroids by Immunoassays

3.1. History of Immunoassays

The year 1959 was ground-breaking because Rosalyn S. Yalow and Solomon Berson published their immunoanalytical method for determining insulin in human blood plasma using radioisotope antigen labelling [41]. The discovery of RIA revolutionized the possibilities of determining the levels of hormones, drugs, vitamins, viruses, tumor antigens, etc. [42,43]. In 1969, Abraham described an RIA-based method allowing 17β-estradiol to be determined [44]. After this first RIA, applications allowing the determination of estradiol and IAs were developed to determine other steroid hormones, such as testosterone or progesterone [45].

The advantages of RIAs include their accuracy and reliability [19]. Radioactive markers, such as ^{125}I, ^{32}P, or ^{14}C, are used as indicators in this case [46]. Because radioactivity is

used, it is necessary to ensure the safety of laboratory personnel, to establish specialized facilities, and to ensure the disposal of radioactive waste [47]. Although this method of analysis of both steroids and sterols is highly sensitive, it is not specific enough [48]. In addition, it is necessary to define the established analytes before self-analysis.

3.2. Preparation of Antibodies

Similar to other low molecular weight substances, steroids are not immunogenic, which is contrary to chemical compounds with a molecular weight over 1000 kDa that are usually immunogenic [46,49]. As with all other low molecular weight haptens, the immunogenicity of steroids can be increased by conjugation to a high molecular weight carrier (usually a protein carrier such as serum albumin, ovalbumin, or keyhole limpet hemocyanin). It is generally accepted that, in the preparation of highly specific antisera, the carrier protein should be conjugated to the steroid molecule via sites on its cyclohexane rings B or C [18]. The location of the chemical bond between the high molecular weight carrier and the steroid molecule most significantly affects the specificity of the antiserum obtained.

3.3. Enzyme and Direct Immunoassay

Over time, methods using indicators other than radioisotopes, in particular enzymatic, fluorescent, or chemiluminescent labeling, have also emerged [45]. At the turn of the 1960s and 1970s, the idea arose to use enzymes (e.g., alkaline phosphatase, glucose oxidase, and horseradish peroxidase) to label antigens or antibodies, which proved to be feasible and served as a basis for the development of the enzyme immunoassay (EIA) [47]. However, compared to RIA, sensitivity may be reduced [43].

In the 1980s and 1990s, IAs spread to clinical and laboratory practices, which was consistent with efforts to simplify them, increase sample throughput, and lower the price [50]. Due to the health risk associated with the use of radioisotopes, as well as time requirements and complexity, RIAs in clinical laboratories have been gradually displaced by DIA methods using enzyme labeling [19]. The advantage of this method lies in its security, lower financial costs, commercial convenience, and the possibility of automation. Although testing on the analyzer platform is simple, fast, high capacity, commercially friendly, automated, and affordable, the introduction of DIAs has been associated with a deterioration in testing performance [51]. To increase specificity, more efforts are needed to design individual kits and antibodies. As a result of omitting the extraction step and chromatography in automated IAs, specificity decreased [45].

3.4. Antibody Specificity

Routine diagnostic laboratories mainly use immunoanalytical techniques [33]. However, there are limitations to their use. Antibody specificity is a property that expresses its ability to distinguish between the antigen against which it was produced and any other antigens present [52]. The specificity of an antibody can be defined by cross-reactivity, which indicates the ability of the antibody to cross-react with antigens other than the immunogen. Thus, the results provided by IAs may be overestimated if the level of cross-reactivity is high [53]. Cross-reactivity is usually a result of the presence of structurally similar analytes, such as other endogenous substances, pharmaceuticals, and natural products, in addition to metabolites of these compounds, which may cross-react with antibodies [54]. For example, many direct platforms created for testosterone analysis interfere with dehydroepiandrosterone sulfate [55,56]. Interference with the synthetic steroid, mifepristone, used as a contraceptive, has been observed in some commercially available EIAs for the determination of estradiol and testosterone [57]. False overestimation of the result may occur even in the presence of substances with low cross-reactivity, but at a concentration higher than the concentration of the analyte. For this reason, an extraction step and/or chromatographic separation can be included before IAs, ultimately making these techniques more accurate

and sensitive compared to DIAs [40]. Extraction and chromatographic separation allow removing interfering substances and separation of cross-reacting steroids from the analyte.

3.5. High-Dose Hook Effect

Another problem associated with IA may be the so-called high-dose hook effect, which leads to a false undervaluation of the analysis results [58–61]. This effect has been observed in the "sandwich" IAs. There is an interference between antigens in high concentration and IA. In some cases, the concentration of the analyte reaches a certain point, the system saturates, and the formation of "sandwiches" is prevented, thus the signal reduces. This effect has already been observed in the steroid hormones aldosterone, testosterone, and 17-hydroxyprogesterone. Insufficient recognition of this phenomenon may have a negative effect on the patient because it can lead to wrong diagnosis and improper therapy. This problem can be solved by a change of ratio between antigen and antibody, either by modifying IA or diluting the analyzed sample.

3.6. Positives of Immunoassays

IAs are still a widely used method for the quantification of steroid hormones, mainly due to the high throughput of samples, simple and fast execution, and relatively low cost [53,62]. IAs such as RIA and the enzyme-linked immunosorbent assay (ELISA) are commonly used in laboratories for the determination of estrogen metabolites in blood or urine because of their efficiency and low cost [63]. Commercially available kits, which can be used manually and also enable automation, are often used in different laboratories [64]. Unlike MS, working with IAs does not require highly qualified personnel, and their setup and execution is relatively easy [18]. It is important to ensure that IAs are only used for the purposes for which they were originally developed. It is also necessary to take their limits into account.

3.7. Limitations of Immunoassays

The biggest limitation of these methods is that they focus on only one analyte, meaning that each analyte requires its own IA [17]. If it is necessary to analyze more steroids in a sample, it is necessary to use the appropriate IA for each analyte, which is associated with higher financial and time costs and, of course, higher consumption of the sample [17,65]. Therefore, each sample must be aliquoted for individual testing.

Despite the widespread use of IAs, the results that these methods provide may sometimes be misleading [58]. A general problem with IAs is their lack of sensitivity and specificity of antibodies, in addition to their sensitivity to matrix effects and interferences [19]. The specificity of IAs is also questionable, especially when determining low levels of steroid hormones [66]. There is no sufficient congruence between the different platforms because different IAs use antibodies directed against different epitopes to quantify the same analyte [58]. Another problem may be the presence of endogenous autoantibodies or antibodies. Unfortunately, commercially available tests are not properly validated by the manufacturer, especially concerning their sensitivity, accuracy, precision, and specificity, and can thus provide unreliable results [64]. In their paper, Stanczyk and co-workers (2003) examined the reliability of commercial diagnostic IAs that are used to quantify serum testosterone and estradiol levels. This study evaluated nine commercial IA kits used for estradiol determination working on the principles of RIA, EIA, and chemiluminescent IA (CIA). The method used in most cases was DIA without the purification step. To determine the reliability of testosterone determination kits, four different direct RIAs and CIAs were tested. The results were compared with those obtained using conventional RIA, which included an extraction step and chromatographic separation. In general, the authors noted large differences in the determined levels of these hormones in the samples when they used kits from different manufacturers to quantify them.

Faupel-Badger and co-workers (2010) compared the determination of selected steroid hormones using indirect RIA and ELISA with LC–MS/MS (liquid chromatography–tandem

mass spectrometry), and also observed these discrepancies [63]. In this work, urine samples from premenopausal and postmenopausal women were used to analyze steroid hormones. The results showed that the absolute concentrations of estrone, estradiol, estriol, 2-hydroxyestrone, and 16α-hydroxyestrone, which were provided using immunoanalytical methods, were 1.6–2.9 higher in premenopausal women and 1.4–11.8 times higher in postmenopausal women than the concentrations determined by LC–MS/MS. This overestimation may be due to the cross-reactivity with other estrogen metabolites. LC–MS/MS measurements highly correlated (Spearman r (rs) = 0.8–0.9) with RIA and ELISA measurements in premenopausal women. However, only a slight correlation was observed in postmenopausal women (rs = 0.4–0.8).

The use of these tests to determine low levels of steroid hormones that are typical for postmenopausal women and children can also be problematic [33]. According to Taieb and co-workers (2003), the use of IAs to determine the low and very low testosterone concentrations (0.17–1.7 nmol.L^{-1}) expected in women and children is not sufficiently reliable [67]. In their study, they compared the determination of testosterone in the blood serum of 50 men, 55 women, and 11 children using different DIAs (eight non-isotopic methods and two RIAs) and isotope-dilution GC–MS. A total of 7 out of 10 IAs provided an average of 46% higher testosterone levels in women's samples compared to GC–MS analysis. In men, by contrast, IAs results were, on average, 12% lower.

Huhtaniemi and co-workers (2012) compared the determination of testosterone and estradiol in men's blood serum using a commercially available electrochemiluminescent IA platform (ECLIA) (Roche Diagnostics E170) with GC–MS determination [62]. Their results indicated that the testosterone concentrations measured by IA showed a high correlation with the concentration determined by MS over a wide concentration range. In the case of the hypogonadal range (<11 nmol.L^{-1}), the correlation was less significant; however, this IA platform is sufficient to detect the subnormal testosterone concentrations observed in men with hypogonadism. Overall, weaker correlations between IA and MS were observed in estradiol assays. IAs in this case are only suitable for the detection of high estradiol concentrations in men (>120 pmol.L^{-1}).

4. Analysis of Neuroactive Steroids by Mass Spectrometry

In recent years, MS has also become a method used for steroid analysis due to its high sensitivity and specificity [45]. However, IAs (those with satisfactory specificity and sensitivity) should not be completely replaced, because both approaches may complement each other. Immunoanalytical methods are still preferred in steroid analysis, mainly due to their simplicity, lower costs, and availability of commercial kits and reagents that do not require special staff skills [51]. The implementation of MS is very expensive, technically demanding, and requires special instrumentation. However, manufacturers have made significant efforts to develop more user-friendly MS technologies that can also be used in clinical laboratories, and which are able to perform routine analyses with high robustness and throughput.

Steroid analysis often uses chromatographic methods which, however, yield only limited information about separated compounds such as retention times [18]. For correct characterization, these techniques should be coupled with a suitable detection system. The ideal tool that allows analysis of a wide spectrum of compounds is the MS detector. The unequivocal advantage of quantification of steroid hormones by MS is the ability to analyze several analytes simultaneously in a single injection with high selectivity, sensitivity, accuracy, and precision [66].

The combination with chromatography is particularly advantageous due to its versatility and high separation strength [68]. Another advantage is the availability of various chromatographic techniques and the number of separation mechanisms that can be used. Better separation power can be achieved using comprehensive two-dimensional chromatographic technologies (GC × GC, LC × LC).

4.1. Internal Standards

Quantitative MS analyses are mainly based on the use of internal standards, which are compounds of either similar structure, analyte analogues, or stable isotope-labeled (SIL) standards bearing, for example, ^{13}C, ^{15}N, ^{17}O, or ^{2}H [69,70]. The use of SIL standards has become increasingly dominant in recent years. In general, the use of internal standards makes it possible to compensate for analyte losses during sample processing and purification [71]. They also eliminate the variability of injected volumes and the MS signal response due to the effect of sample matrices on ionization. Moreover, loss of some analytes may occur, e.g., due to incomplete extraction, derivatization protocol, or degradation of analytes during storage [72]. During daily operation, fluctuations in the temperature and pressure of the ion source can also affect the MS ionization efficiency of the MS analyzer [69].

However, even the use of SIL standards is not always ideal [73]. SIL internal standards are more costly to prepare and may not be commercially available in all cases. Some deuterated internal standards also showed discrepancies in retention times and recoveries of analytes versus SIL [74]. The purity of the SIL standards used is also important [69]. If they are not available or are too expensive, the use of structural analogues may be an alternative solution [69,70].

4.2. Gas Chromatography–Mass Spectrometry

4.2.1. Introduction and History of GC–MS

History of the use of GC for steroid separation dates back to the early 1960s [75]. In 1964, Eneroth and his team published a paper focused on identification and quantification of neutral steroids in human feces using an analytical tool combining GC and MS [76]. Because both the ion source and the MS analyzer required a high vacuum, the MS was originally combined with GC separation due to the gas phase operation and the relative ease of coupling [65].

4.2.2. Gas Chromatography

GC is a separation technique which separates components of a mixture based on their different affinity to stationary phase of chromatography column [77,78]. Due to the relatively high molecular weight of steroids, a high temperature is required (usually above 200 °C) during gas chromatographic separation [79]. After evaporation, the sample is batched into the mobile phase, in this case, carrier gas, most commonly helium or hydrogen [77,78]. Separated components exiting the chromatography column are then detected using a detector (e.g., mass spectrometer). The chromatography columns made of fused silica allow an easy coupling with MS. These columns with conventional stationary phases, based on polysiloxane or polyethylene glycol, are still commonly used [80].

4.2.3. GC–MS Sample Preparation: Hydrolysis and Derivatization

In the case of analysis of polar, thermolabile, and/or nonvolatile analytes, it is necessary to use a derivatization step [81]. Steroids are nonvolatile compounds and can decompose during analysis due to high temperatures [65]. Derivatives obtained by chemical modifications are more hydrophobic, volatile, and temperature-stable compared to original steroids. Prokai-Tatrai and co-workers (2010) developed a gas chromatography–tandem mass spectrometry (GC–MS/MS) method for the analysis of 17β- and 17α-estradiol and estron in human blood serum [25]. Sample preparation included liquid–liquid extraction (LLE) and one-step derivatization with *N*-(trimethylsilyl)imidazole. Nilsson and co-workers (2015) chose a derivatization technique based on steroid oximation and esterification to profile seven sex steroid metabolites in rodent blood serum [66]. These modifications were made to achieve an exceptionally high sensitivity using a triple quadrupole operating in selective multi-reaction monitoring (MRM) mode.

During derivatization, chemical changes in analytes occur, associated with changes in their physical–chemical properties [82]. The derivatization step improves the volatility and thermal stability of the analytes [48,83]. This step leads to a better chromatographic

separation and an increase in the sensitivity of the method [82]. Derivatization does not only apply to GC–MS, because it can also be used in the preparation of samples for LC–MS/MS [32,53]. Despite these positives, there may be discrimination of analytes in the sample during derivatization (different derivatization efficiencies, loss of compounds), contamination, and the formation of by-products [80]. Moreover, derivatization can also lead to isomer mixtures of derivatives [65].

The low throughput of GC–MS is caused by time-consuming sample preparation [80]. The need to examine a large number of samples, e.g., in population studies, is a problem because this method is characterized by higher time demand due to the need for derivatization, and also requires higher sample volumes [53].

If steroid conjugates (e.g., sulphates, glucuronides) are present, enzymatic or chemical hydrolysis of charged groups can also be performed [65]. The GC–MS tools require cleavage of the sulphate group, in addition to subsequent derivatization [84,85]. In the case of GC–MS analysis of sulphate conjugates, increases in signals may occur due to the presence of other conjugates, especially glucuronides. This is caused by the fact that commercially available sulfatases often also show glucuronidase activity; furthermore, chemical hydrolysis of the sulphate group is also not specific. However, the LC–MS technique allows for the analysis of intact sulphate steroid conjugates without any modification.

4.2.4. GC–MS Ionization Techniques

The GC system coupled with MS is one of the most universal, standard, and used analytical tools [86,87]. This approach has been used for the analysis of steroid metabolites for several decades. Due to the high reproducibility, sensitivity, and availability of mass spectrum databases, GC–MS and GC–MS/MS techniques have often been used in these studies [72]. The most common ionization technique applied in metabolic studies based on GC is electron impact ionization (EI) [81,88,89]. This hard ionization technique can help to achieve the reproducible fragmentation of molecules [18,81]. Using EI and GC–MS, several spectral libraries and databases are available. Chemical ionization (CI) is another ionization technique optimal for steroid analysis, and it is commonly used due to less fragmentation of analyte molecules and higher occurrence of parent ions. Polet and co-workers (2016) used CI in their study focused on the analysis of anabolic steroids [90]. The abovementioned EI is often used in steroid analysis [88,91–94]. For example, Hill and co-workers (2019) described a method for the determination of 100 endogenous steroids in human blood serum by EI combined with GC–MS/MS [95].

4.2.5. GC–MS and Their Applications

GC–MS is a tool that can be used in both targeted and untargeted analysis approaches [80]. Compared to LC–MS, this analytical technique provides higher sensitivity, resolution, reproducibility, reliability, and relatively low cost [72]. For example, profiling of steroids in urine by GC–MS and GC–MS/MS is a suitable tool for the discovery of new steroids, their characterization, and the acquisition of new knowledge useful for the diagnosis of metabolic disorders [51,93]. Due to its excellent chromatographic resolution, GC–MS allows for the identification of new therapeutic metabolites [45].

GC–MS systems work in both full scan mode and selected ion mode (SIM) [33]. GC–MS in scanning mode is a suitable tool for the untargeted profiling of steroid hormones, the discovery of new compounds, and the study of metabolic pathways. Spectral databases are appropriate tools for identifying unknown compounds [18]. Using SIM acquisition, GC–MS analysis can achieve higher sensitivity because of noise reduction [65]. Due to the selectivity and higher sensitivity of this mode, it is preferably used for quantification [18]. However, high background noise is a problem when analyzing very low concentrations of analytes using the classical single-stage GC–MS [96,97].

Further development has recently led to the connection of GC with tandem mass spectrometers [95]. GC–MS/MS can be used to eliminate background interference and increase overall sensitivity and specificity [96,97]. For example, this approach can be used

for multicomponent determination of several dozen endogenous steroids [95]. Hill and co-workers (2019) developed and validated the GC–MS/MS method for the quantification of 58 unconjugated steroids and 42 polar steroid conjugates, including neuroactive or immunomodulatory steroids. Such steroid profiling in male and female blood samples, including the blood of pregnant women and the umbilical cord, can be useful in quickly diagnosing various pathologies, identifying their causes, or seeking new therapy options.

Other studies also used the GC–MS/MS for analysis of circulating steroids in humans or other vertebrates [91,92]. Hansen and co-workers (2011) described an optimized and validated GC–MS/MS method for determining pregnenolone, progesterone, DHEA, androstenedione, testosterone, 5α-dihydrotestosterone, estrone, 17α-estradiol, and 17β-estradiol in blood plasma and serum of certain vertebrates [91]. Moreover, the triple quadrupole with EI operating in selected reaction monitoring (SRM) mode allowed extremely low background noise when analyzing biological samples. Applying different MS analyzers, such as ion traps, the use of a scan mode enabling MS/MS monitoring is also possible [96,97].

Finally, Kanceva and co-workers (2015) used a GC–MS system to study the relationship between levels of certain steroids and multiple sclerosis, one of the most common neurological diseases [88]. For this purpose, they analyzed steroids and polar conjugates of steroids (51 in total) in 12 patients with multiple sclerosis who were untreated with steroids, and 6 women as a control group. In patients with multiple sclerosis, they observed a significant increase in circulating levels of C21 steroids (e.g., pregnenolone), their polar conjugates (e.g., pregnenolone sulfate), and some bioactive C19 steroids (e.g., androstenedione). This work shows the importance of simultaneous targeted profiling of a broad spectrum of steroids using GC–MS methods. An accurate determination of circulating hormone levels can help us understand their effect on nervous system functions, for example, on their disruption of the balance between neuroprotection and excitotoxicity.

4.3. Liquid Chromatography–Mass Spectrometry

4.3.1. Introduction and History of LC–MS

The combination of LC or supercritical fluid chromatography (SFC) with MS took place a few decades later (the 1980s) than GC–MS due to demanding technical requirements [68]. In the field of steroid analysis, the LC–MS technique has significantly expanded in recent years, mainly because it allows high sample throughput to be achieved [18]. Although this technique is suitable for a quick targeted analysis of conjugated and unconjugated steroids, its use in untargeted approaches is less appropriate. Due to existing factors that can significantly impair its specificity, the LC–MS tool in still used less frequently in untargeted analysis (e.g., lower chromatographic resolution compared to GC, higher susceptibility of soft ionization to matrix effects compared to EI).

The abovementioned SFC is not a new separation technique because it has existed since the 1960s [98,99]. Technological progress and the introduction of commercial ultra-high-performance supercritical fluid chromatography (UHPSFC) tools at the beginning of the millennium contributed to its more comprehensive application [68,98,99]. Many benefits are associated with SFC, such as a reduction in the consumption of harmful organic solvents (so-called green analytical chemistry), the robustness of advanced techniques, reproducibility, selectivity, sensitivity, and its usefulness in the separation of thermally unstable, nonvolatile, and chiral compounds [99]. Compared to other separation techniques, better separation of enantiomers and isomers can be achieved with SFC [100]. Due to the possibilities offered by commercial UHPSFC systems, both in the choice of different organic modifiers and types of stationary phases, this approach is highly versatile and selective [98]. These properties make UHPSFC useful for the separation of steroids, in addition to other compounds sharing similar structures and mass spectra.

4.3.2. Liquid Chromatography

Reverse phase (RP) chromatographic separation is widely used in steroid analysis [79] (Figure 3 shows an example). Compared to the normal phase, RP chromatographic separation is more effective due to the hydrophobicity of unconjugated steroids [65]. The best tool for separating a number of steroids is elution in gradient mode [79]. Compared to GC–MS, LC–MS achieves lower chromatographic resolution [33,81]. Better chromatographic efficiency and sensitivity can be achieved using columns with particles smaller than 2 µm [101]. The use of such small particles is associated with high back pressures and has become possible by the introduction of ultra-high-performance liquid chromatography (UHPLC) technology that is able to withstand them [102]. Another option offering similar chromatographic separation, but at lower pressures and without the need for UHPLC instrumentation, is the particle-packed columns based on so-called fused core particle technology. This alternative can have almost the same efficiency as the above-mentioned particles, but they are larger compared to them and, therefore, no high back pressures are generated [101]. This makes this technology compatible with conventional high-performance liquid chromatography (HPLC) systems.

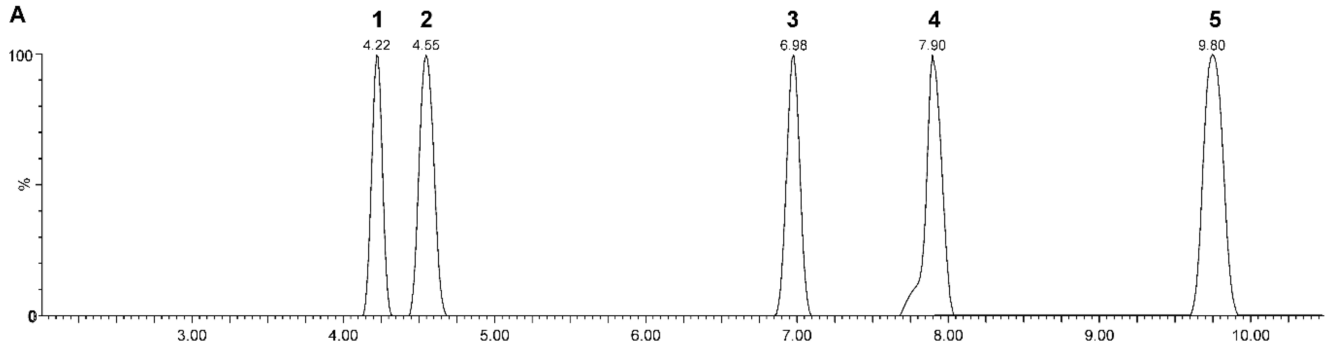

Figure 3. Reversed-phase chromatographic separation (Kinetex Biphenyl column, 2.1 × 100 mm; 1.7 µm) of selected neuroactive steroids by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Figure 3 shows the merged chromatograms for the steroid standards mixture: 1—dehydroepiandrosterone, 2—testosterone, 3—pregnenolone, 4—allopregnanolone, and 5—progesterone. Retention times (min) are indicated above the peak.

4.3.3. Ionization Techniques Used in LC–MS

The first important step in connecting LC and MS was the introduction of atmospheric pressure ionization techniques (APIs) [103]. Because LC–MS methods are API-compatible, they can be used for analysis of intact conjugates [48]. Commonly used ionization techniques for LC–MS detection of steroid substances are electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and atmospheric-pressure photoionization (APPI) [97]. ESI is also suitable for the analysis of polar molecules and steroid conjugates. When analyzing less polar substances, chemical modification may be required to increase ESI sensitivity to the level of APPI and APCI ionization techniques. APPI and APCI ionization techniques are more suitable for unconjugated nonderivatized steroids. Moreover, APCI provides more selective ionization and lower matrix effects for some substances [102].

4.3.4. Matrix Effects

In connection with LC–MS analysis, so-called matrix effects are often mentioned [104]. Although the LC–MS/MS system is highly selective and sensitive, matrix effects are its biggest problem [101]. A common cause of matrix effects is compounds being present in the matrix that co-elute with the analytes and disrupt their ionization [105]. Changing ionization efficiency can lead to ion suppression or ion enhancement. The ionization process may be disrupted by both organic and inorganic substances of endogenous origin

which come from the sample and are, therefore, present in the final extract (e.g., salts, urea, lipids, and peptides), but also substances of exogenous origin, i.e., those that enter the sample during the preparation process [106]. The biggest problem in this respect is the analysis of extracts of complex matrices using the ESI ionization technique [105]. APPI and APCI techniques are less susceptible to matrix effects. As a result, co-elution of the analyte with interfering components can lead to a negative impact on the accuracy, precision, and sensitivity of the LC–MS method [101]. To eliminate, reduce, or at least compensate for matrix effects, it may be beneficial to pay attention to the adjustment of the sample's quantity, the preparation of the sample, the modification of chromatography conditions (e.g., optimization of the mobile phase, change of column parameters), the optimization of MS (choice of ionization technique, the polarity of ionization), and the selection of possible calibration techniques (e.g., external matrix-matched calibrators, internal standards, and standard addition).

4.3.5. Sample Preparation for LC–MS

In methods based on LC–MS, preparation of the sample before analysis is essential [102]. The most common techniques used for sample preparation include protein precipitation, solid-phase extraction (SPE), and liquid–liquid extraction (LLE). Extraction of steroids from biological matrices, such as blood plasma, serum, urine, or saliva, is performed in order to remove interfering substances from the samples, increase the sensitivity of the methods, and protect the instrumentation, and, in particular, to extend the life of the chromatographic columns [107]. Due to the precipitation of proteins with methanol or acetonitrile, the steroids are released from binding to their carrier proteins.

The offline SPE allows for the removal of interfering substances of the matrix and concentrates the analytes [29]. Naldi and co-workers (2016) designed and validated a fully automated method combining an online SPE with LC–MS/MS for simultaneous analysis of the free and conjugated forms of selected steroids in urine and various types of water (sewage water, river water, etc.). A similar arrangement may be seen in works concerned with the analysis of other matrices; for example, human saliva or plasma [53,108]. Online SPE has several advantages compared to the offline approach [29,53]. These include working with smaller sample volumes, reduced risk of procedural errors, better repeatability and reproducibility, reduced sample preparation time, and increased sample throughput. The development of online SPE, however, is not always easy and may be associated with a number of problems, such as the incompatibility of SPE sorbents and analytical column and broadening of peaks. Li and co-workers (2018) developed a new method for quick, highly sensitive, specific, and simultaneous determination of estrone, estradiol, and estriol in human saliva [21]. This method combines LC–MS/MS with a miniaturized and high-throughput SPE on a hydrophilic-lipophilic-balanced microplate with 96 wells. Miniaturized SPE allows the purification of samples and analyte enrichment without the need for derivatization, evaporation, or LLE. Advantages of this approach are also reduction in solution consumption and manual handling time. The sensitivity of this method is 1 $pg.mL^{-1}$ and it allows the quantification of trace estrogen levels.

4.3.6. LC–MS Applications in Steroid Analysis

In recent years, LC–MS/MS has become the main technique for the analysis of steroid hormones [33]. The progressive improvement of LC systems and the introduction of MS/MS led to gradual replacement of the GC–MS techniques [51]. The clear advantages of LC–MS/MS include the speed of analysis, specificity, possibility of automation, and easy and time-saving sample preparation [18,33,102]. For high sample throughput and fast processing time, the LC–MS/MS methods are particularly attractive for clinical laboratories [51]. However, the expansion of LC–MS/MS systems and their routine use are complicated mainly by high acquisition costs and higher technical complexity [102]. No hydrolysis of conjugates or chemical derivatization are required [33]. Compared to IA, the determination of steroid hormones using LC–MS/MS requires smaller sample

volumes [102]. Furthermore, chemical derivatization can improve ionization, increase sensitivity, and, consequently, reduce the limits of quantifications (LOQs) [19]. Including the derivatization step may also help in determining the location of functional groups on the steroid molecule because derivatization usually supports unique MS fragmentation [33].

MS/MS-based analysis achieves higher selectivity and sensitivity than using only a single mass analyzer [104,107]. One of the most common tandem mass analyzers is the triple quadrupole. This instrument is the most widely used for steroid hormone quantification due to its versatility and sensitivity. The triple quadrupole allows different working modes, such as full scan and product ion mode. [104]. The scanning mode is referred to as an SRM or MRM transition, in which only pre-selected ions are detected. The MRM mode allows the simultaneous quantification of several analytes in a single experiment. Other compounds (unselected analytes) presenting a chemical background of the sample are not detected, which is the biggest disadvantage of this approach [68]. The MRM mode provides the highest sensitivity and selectivity with regard to these favorable qualities [104]. This mode is one of the most reliable tools for confirming the presence of specific compounds in samples. The SIM mode can be also used; however, it is characterized by lower sensitivity and selectivity. A combination of LC methods and triple quadrupole MS/MS can provide qualitative and quantitative information for many analytes in the same sample (multiplexing) and in one analytical run [107].

Keevil and co-workers (2017) used an LC–MS/MS instrument operating in positive ionization mode to analyze testosterone in saliva [24]. Sample preparation included the addition of an internal standard and LLE using methyl-tert-butyl ether. This analytical tool is more specific and sensitive compared to IA. The UHPLC–MS/MS system was also used to determine neurosteroids and steroids with immunomodulatory effects in human CSF and blood plasma [32]. MS detection was performed using triple quadrupole-MS with positive ESI working in MRM mode. In this study, free DHEA, its selected metabolites, namely 7α-hydroxy- and 7β-hydroxy-DHEA, 7-oxo-DHEA, and 16α-hydroxy-DHEA, in addition to cortisol and cortisone, were quantified to better understand degenerative diseases and, in particular, to monitor their development and progression. Furthermore, Caruso and co-workers (2014) analyzed NASs in blood plasma and CSF by UHPLC–MS/MS with APCI ionization working in the positive mode [12]. The study showed differences in levels of steroid hormones between 26 men diagnosed with multiple sclerosis (avg. age of 34 years), specifically in the relapsing-remitting form, and in 12 samples representing the control group (avg. age of 29 years). For example, pregnenolone, progesterone, and 5α-dihydrotestosterone levels were increased, and in 5α-dihydroprogesterone and allopregnanolone, levels were decreased in patients with multiple sclerosis compared to the control group. In another study, chromatographic separation of the samples performed on a PR-C18 column was combined with an MS/MS analyzer using the positive mode of APCI and SRM transitions [109]. A simple and specific method has been developed for simultaneous determination of selected NASs, namely cortisone, cortisol, DHEA, estradiol, progesterone, pregnenolone, and testosterone. Sample preparation prior to analysis employed LLE using an ethyl acetate extraction procedure for the seven NASs from blood plasma and brain tissue of laboratory rats. The results suggest that the observed differences in the levels of some endogenous NASs may have potential as biomarkers usable for the diagnosis or treatment of depression.

The analysis of steroid substances is not limited only to blood serum and plasma, and other matrices are also used. Nguyen and co-workers (2011) developed and validated a method for the simultaneous quantification of several estrogen hormones, namely estriol, estrone, 17α-estradiol, and 17β-estradiol in human CSF, based on the heart-cutting two-dimensional LC–MS/MS system [36]. The sample preparation included LLE with determined extraction recoveries between 91% and 104%, and subsequent derivatization with dansyl chloride. By including the derivatization step, an increase in sensitivity limits was achieved. The accuracy and precision of this method was more than 86% for 17β-estradiol and 17α-estradiol and 79% for estriol and estrone. However, to improve detection limits,

the ion trap instrument used should be replaced with a more sensitive means of detection, such as a triple quadrupole. Finally, the developed method was applied to analyze estrogen in patients suffering from ischemic trauma.

Gao and co-workers (2015) developed a highly sensitive, selective, and fast online SPE LC–MS/MS method to determine estradiol and some other steroid hormones (cortisol, cortisone, testosterone, progesterone, corticosterone, DHEA) [53]. All analyzed hormones, except for DHEA, had a LOQ lower or equal to 5 $pg.mL^{-1}$. The LOQ for DHEA was 10 $pg.mL^{-1}$. The authors finally used this method to determine selected steroid hormones in saliva samples, in which no estradiol levels were detected when using a routine IA.

4.3.7. SFC–MS Applications in Steroid Analysis

One of the first supercritical fluid chromatography–mass spectrometry (SFC–MS) applications in steroid analysis is associated with the end of the last century [110]. Tuomola and co-workers (1998) applied a packed column SFC combined with APCI–MS to determine androstenone in porcine fat. Fat sample processing was simple and included only dichloromethane extraction. Xu and co-workers (2006) described a method for the separation and quantification of 15 structure-related estrogen metabolites (e.g., estrone, estradiol, estriol, 4-methoxyestron, 2-hydroxyestradiol) by a packed column using SFC–MS/MS in less than 10 min [111]. Chromatographic separation was performed on a cyanopropyl silica column connected in series with a diol column. The mobile phase was carbon dioxide with a linear gradient of methanol. This analytical approach is several times faster compared to RP–HPLC–MS/MS analysis of the same group of analytes. The SFC–MS is therefore more suitable for the analysis of larger sets of samples. Furthermore, Doué and co-workers (2015) developed and validated UHPSFC–MS/MS for analysis of several conjugated urinary steroids, specifically glucuronide and sulfate steroids in bovine urine samples [112]. Their analysis is a suitable tool for the monitoring of anabolic steroid misuse (e.g., food industry, doping). The optimization of several SFC conditions (e.g., stationary phase, addition of modifiers, back pressure, column temperature) resulted in 2 different approaches enabling the analysis of 8 glucuronide and 10 sulfate steroids. Furthermore, UHPSFC–MS/MS provided better sensitivity and repeatability in less run time.

Other studies also used SFC–MS for analysis of steroid substances [100,113,114]. Kock and co-workers (2018) described a novel UHPSFC–MS/MS method with positive ESI for simultaneous determination of 19 steroids (from androgen, estrogen, progestogen, and glucocorticoid classes) in human plasma within 5 min [100]. Other biological materials, such as CSF or urine, are applicable for the profiling of endogenous steroids or the screening of doping agents, respectively [113,114].

4.3.8. Ion Mobility

Among steroids, a large number of isomers and isobaric compounds with different biological effects can be found [115]. A common problem with LC methods is their limited resolution of structurally similar substances [116]. To achieve their separation, it is necessary to extend the run time of chromatographic separation. Some isomers, especially stereoisomers, may be subject to similar fragmentations, complicating the rapid identification and quantification of steroids by MS/MS. An additional dimension of separation can be provided by complementing the MS system with ion mobility spectrometry (IMS), which allows further differentiation of isomers or isobaric compounds [68]. IMS can be easily combined with existing GC–MS or LC–MS methods [116]. In IMS–MS techniques, time separators such as drift tube ion mobility spectrometry (DTIMS), traveling wave ion mobility spectrometry (TWIMS), and spatial separators, such as differential ion mobility spectrometry (DMS), also referred to as high-field asymmetric ion mobility spectrometry (FAIMS), are most commonly used for steroid analysis [117].

According to the study of Chouinard and co-workers (2017), DTIMS holds considerable potential for improving the analysis of isomer forms of steroids [116]. Rister and Dodds (2020a) used the LC–MS system containing TWIMS for the analysis of steroid hormone

isomers [115]. The results showed that a combination of LC and IMS–MS systems can lead to an increase in the resolution of steroid isomers compared to using only LC or IMS. Applying LC–IMS–MS can also achieve faster analysis of steroid isomers compared to simple LC–MS. Moreover, IMS also provides a CCS (ion-neutral collision cross-sections) parameter that can be used together with retention time and *m/z* as an adjunct in identifying analytes.

Finally, the use of analytical techniques combining the LC–MS/MS system with the DMS also enhance the performance of the determination of endogenous steroids in human blood serum and plasma [118]. The inclusion of DMS has led to an increase in the specificity of the analysis, which makes it possible to simplify sample preparation, reduce chromatographic separation time, and increase analysis speed. Ray and co-workers (2015) developed and validated a highly sensitive and specific method for determining corticosterone, 11-deoxycortisole, 11-deoxycorticosterone, 17-hydroxyprogesterone, and progesterone. Because the pairs corticosterone and 11-deoxycortisole, and 11-deoxycorticosterone and 17-hydroxyprogesterone, are isomer pairs, their distinction by LC–MS/MS is complicated due to similar fragmentation and chromatographic retentions. Combining chromatographic separation and DMS increased isomer resolution and reduced background noise.

Recently, the use of IMS as a single separation technique for the analysis of steroids without the inclusion of chromatographic separation has been the subject of interest [117]. Analysis of steroids using IMS–MS without chromatography would significantly reduce the time of acquisition and sample preparation.

4.4. Metabolomics, Targeted and Untargeted Mass Spectrometry Analysis

In recent years, the field of metabolomics has been of great interest, and has helped us in further understanding metabolic mechanisms under physiological and pathological conditions [119]. This field focuses on comprehensive analysis of intracellular and extracellular metabolites in biological fluids, cells, tissues, and organisms [72,120]. Studying only a few steroids, or a comprehensive monitoring of steroid metabolome (so-called steroidome), can lead to the discovery of new steroids, steroid pathways or biological markers that may be useful for diagnosis, monitoring, prevention, or prediction of disease risk, as well as drug development [119,121]. Steroid metabolome studies may result in the development of more sophisticated approaches to screening or diagnosing a number of endocrine diseases [122,123]. Monitoring differences in steroidome in healthy subjects and patients may contribute to the discovery of candidate steroid biomarkers for schizophrenia, but also for other psychiatric disorders (e.g., mood, anxiety disorders) [124–127]. These findings can, of course, improve the quality of life of patients, because changes in the level of metabolites are often associated with a number of diseases and often occur before the clinical manifestation of a disease [72].

However, the analysis of steroid profiles with chromatography techniques, coupled with MS, is an analytical challenge due their large dynamic range, their extraction from complex biological matrices, or the selectivity of the analytical techniques [121]. Due to the large variability of metabolites in terms of their chemical diversity, polarity, molecular weight, and concentration range, a single analytical tool and sample preparation protocol cannot be used within the framework of an untargeted approach, because no sampling strategy or analytical technique can cover all the metabolites present [81]. By contrast, targeted analysis, in which specific groups of metabolites are analyzed, is often sufficient with a single strategy. Targeted analysis is carried out based on a certain hypothesis and focuses on predefined analytes; in contrast, untargeted analysis is global and does not focus on specific analytes or hypotheses [80]. Both approaches can be combined.

In their paper, Palermo and co-workers (2017) presented an untargeted metabolomics approach based on UHPLC–MS/MS for the study of the urinary steroidal profile [128]. This proposed workflow is able to detect up to 3000 metabolites of steroid origin using high-resolution mass spectrometry. The study of urinary steroids is an approach that can be used to monitor various pathological conditions and to detect the illicit use of anabolic steroids. Targeted and untargeted approaches can be combined, and they can provide a more

comprehensive view of the issue [129]. An example is the isotope dilution-based targeted and untargeted profiling of carbonyl neurosteroids and steroids. This hybrid method allows absolute quantification of pregnenolone, progesterone, 5α-dihydroprogesterone, 3α,5α-tetrahydroprogesterone, and 3β,5α-tetrahydroprogesterone, and relative quantification of other carbonyl-containing steroids in animal models.

In-depth views of the different aspects of steroidomics can be found in a number of existing publications [121,130,131].

4.5. Validation of Bioanalytical Method

Validation of the method should demonstrate that the method is sufficiently reliable for determining the selected analyte in a particular biological matrix [132]. According to the European Medicines Agency (EMA) guideline, the validation of a method should include, for example, the determination of calibration range, accuracy, precision, and matrix effect. Method validation can also be carried out based on the Food and Drug Administration (FDA) guidelines [133].

5. Conclusions

Analytic methods summarized in Table 1 allow the monitoring of the differences between the levels of neuroactive steroids in different physiological conditions and pathological conditions. They may represent a useful instrument in deepening knowledge of physiological mechanisms and pathophysiology of some diseases. Monitoring changes in steroid hormone levels can be an effective tool in the search for new biological markers useful in monitoring, preventing, or predicting disease risks. The knowledge gained from metabolite studies can also increase our understanding of the pathophysiology of certain diseases and provide new insights into the possibilities of their diagnosis, or even treatment, which will contribute, for example, to the development of new drugs and procedures. All of the steroid analysis presented herein, whether methods based on immunoassay or mass spectrometry, have their advantages and disadvantages. It is especially important to know the virtues but also the limits of such analytical methods, and to consider their use for the intended purposes, accordingly, to obtain reliable results. Individual approaches to the analysis of steroids may complement each other, thus providing specific pieces of information and allowing us to compile a comprehensive picture of the issue.

Table 1. Summary of selected analytical approaches used for neuroactive steroids analyses. IA: immunoassay; LC–MS(/MS): liquid chromatography–(tandem) mass spectrometry; SFC–MS(/MS): supecritical fluid chromatography–(tandem) mass spectrometry; gas chromatography–(tandem) mass spectrometry; IMS: ion mobility spectrometry; CSF: cerebrospinal fluid.

Analytical Method	Sample Type	Class of Analytes	Reference
IA	saliva, serum	glucocorticoids, mineralocorticoids	[22]
	serum, CSF	androgens, progestins, estrogens	[35]
	serum	androgens	[39]
	plasma	estrogens	[44]
	plasma, serum	androgens, progestins, estrogens, glucocorticoids	[54]
	serum	androgens	[55]
	serum	androgens	[56]
	plasma	androgens, estrogens	[57]
	serum	androgens, estrogens	[62]
	urine	estrogens	[63]
	serum	androgens, estrogens	[64]
	serum	androgens	[67]
	serum, CSF	progestins, glucocorticoids	[89]
	plasma	progestins, estrogens, glucocorticoids	[126]

Table 1. *Cont.*

Analytical Method	Sample Type	Class of Analytes	Reference
GC–MS(/MS)	serum	estrogens	[25]
	urine	androgens, progestins, glucocorticoids	[27]
	serum	androgens, estrogens	[62]
	serum	androgens, progestins, estrogens	[66]
	serum	androgens	[67]
	feces	neutral fecal steroids	[76]
	serum	androgens, progestins, estrogens	[88]
	serum, CSF	androgens, progestins	[89]
	plasma, serum	androgens, progestins, estrogens	[91]
	plasma	androgens, progestins	[92]
	plasma	androgens, progestins, estrogens	[94]
	serum	androgens, progestins, estrogens, glucocorticoids	[95]
	serum	androgens, progestins, glucocorticoids	[125]
	plasma	androgens, progestins, estrogens	[126]
LC–MS(/MS)	serum	androgens, progestins, glucocorticoids	[4]
	plasma, CSF	androgens, progestins, estrogens	[12]
	saliva	estrogens	[21]
	saliva	androgens	[24]
	scalp hair	androgens, progestins, glucocorticoids	[26]
	finger nails	androgens, progestins, glucocorticoids, mineralocorticoids	[28]
	water matrices, urine	estrogens	[29]
	urine	androgens	[30]
	urine	androgens	[31]
	plasma, CSF	androgens, glucocorticoids	[32]
	CSF	estrogens	[36]
	serum	androgens, progestins, glucocorticoids	[37]
	plasma	androgens, progestins, glucocorticoids	[38]
	saliva	androgens, progestins, estrogens, glucocorticoids	[53]
	urine	estrogens	[63]
	serum	androgens, progestins, estrogens	[84]
	serum	progestins, androgens	[85]
	serum	androgens, progestins, glucocorticoids, mineralocorticoids	[108]
	plasma, brain tissue	androgens, progestins, estrogens, glucocorticoids	[109]
	plasma	androgens, progestins, estrogens, glucocorticoids, mineralocorticoids	[123]
	urine	>3000 individual metabolic features	[128]
	brain tissue	carbonyl steroids	[129]
SFC–MS(/MS)	plasma	androgens, progestins, estrogens, glucocorticoids, mineralocorticoids	[100]
	fat	androgens	[110]
	urine, serum	estrogens	[111]
	urine	androgens, estrogens	[112]
	CSF	androgens, progestins, estrogens, glucocorticoids	[113]
	urine	androgens	[114]
(LC–)IMS–MS(/MS)	standard solutions	androgens, glucocorticoids, mineralocorticoids	[115]
	standard solutions	androgens, progestins, estrogens, glucocorticoids, mineralocorticoids	[116]
	serum, plasma	progestins, glucocorticoids	[118]

Author Contributions: M.K., J.O. and O.N. were involved in the writing of this article. M.S. reviewed the structure of the article. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by ERDF project "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827) and Palacky University Olomouc (IGA_PrF_2021_011).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors would like to thank Ota Blahoušek for drawing the figures using CorelDRAW 2017.

Conflicts of Interest: The authors declare no conflict of interest.

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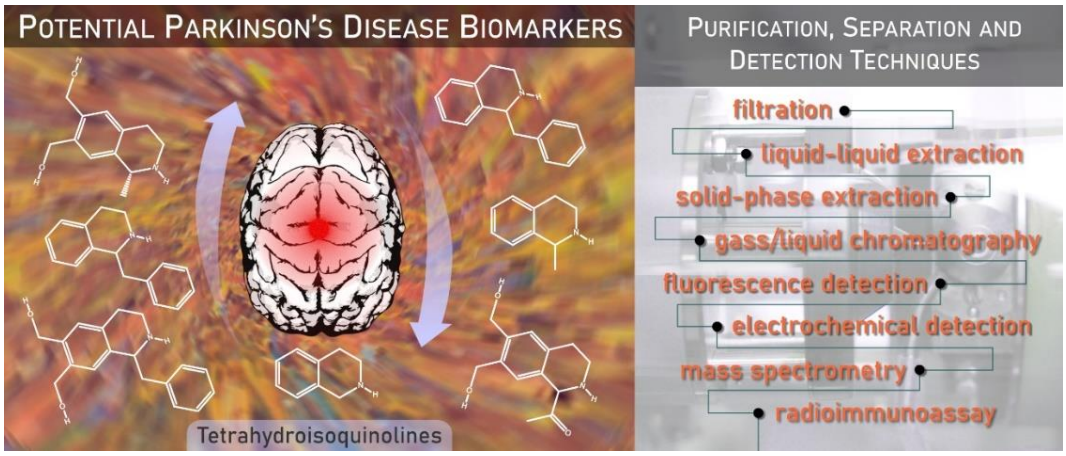
Supplement II

Quantitative Determination of Endogenous Tetrahydroisoquinolines, Potential Parkinson's Disease Biomarkers, in Mammals



ACS Chemical Neuroscience

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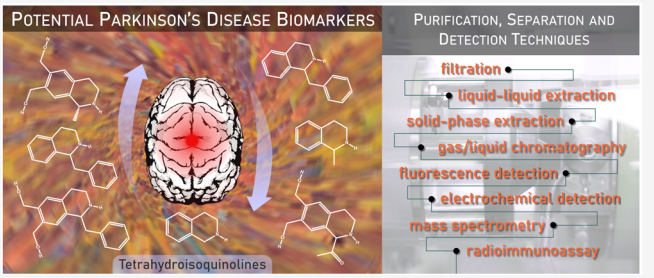
Quantitative Determination of Endogenous Tetrahydroisoquinolines, Potential Parkinson's Disease Biomarkers, in Mammals

Eva Hényková,[‖] Michal Kaleta,*[,‖] Kateřina Klíčová, Gabriel Gonzalez, Ondřej Novák, Miroslav Strnad, and Petr Kaňovský

Cite This: *ACS Chem. Neurosci.* 2022, 13, 3230−3246 Read Online

ABSTRACT: Current diagnostic options for Parkinson's disease are very limited and primarily based on characteristic clinical symptoms. Thus, there are urgent needs for reliable biomarkers that enable us to diagnose the disease in the early stages, differentiate it from other atypical Parkinsonian syndromes, monitor its progression, increase knowledge of its pathogenesis, and improve the development of potent therapies. A promising group of potential biomarkers are endogenous tetrahydroisoquinoline metabolites, which are thought to contribute to the multifactorial etiology of Parkinson's disease. The aim of this critical review is to highlight trends and limitations of available traditional and modern analytical techniques for sample pretreatment (extraction and derivatization procedures) and quantitative determination of tetrahydroisoquinoline derivatives in various types of mammalian fluids and tissues (urine, plasma, cerebrospinal fluid, brain tissue, liver tissue). Particular attention is paid to the most sensitive and specific analytical techniques, involving immunochemistry and gas or liquid chromatography coupled with mass spectrometric, fluorescence, or electrochemical detection. The review also includes a discussion of other relevant agents proposed and tested in Parkinson's disease.



KEYWORDS: *Tetrahydroisoquinolines, biomarker, mammals, fluids, tissues, quantitative analysis*

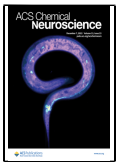
1. INTRODUCTION

Parkinson's disease (PD) afflicts 10 million people globally.[1] Characteristic clinical symptoms of this aging-related movement disorder include muscular rigidity, bradykinesia, postural imbalance, and resting tremor, generally called parkinsonism. Risks of developing PD are thought to be influenced by numerous genetic and other factors, endogenous or environmental, some of which have not been identified but may be associated with toxins, drugs, infections, and diet.[2] All of these factors may interactively increase probabilities of the typical late onset of idiopathic PD.

Diagnosis of PD is based primarily on clinical symptoms and is complicated, and misdiagnosis is common in early stages.[3,4] Currently, final confirmation is based solely on autopsy results. Clearly, identification of reliable biomarkers that play important roles in its pathogenesis[5−8] would greatly facilitate its early diagnosis. Specific markers enabling sufficiently early detection to initiate treatment before irreversible neuronal loss and appearance of the clinical syndrome would be particularly valuable.[5−7] Moreover, trustworthy diagnostic markers could be useful for distinguishing PD from atypical Parkinson's syndromes. No single biomarker is likely to meet all the criteria for accurate diagnosis and monitoring the progression of PD, so a combination of multiple biomarkers will probably be needed.[8] No specific biomarkers can be recommended in clinical practice as yet, but there are some promising candidates.[9] These include tetrahydroisoquinoline derivatives (TIQs), a group of neurotoxins/neuroprotectants that are putatively involved in PD's multifactorial etiology.[10−15]

The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which has structural similarity to TIQs, can cause parkinsonism prompted a TIQ-based hypothesis of PD in the 1980s.[10−13] Hence, the toxicity of endogenous MPTP-like compounds that may participate in PD-associated neurodegeneration to cultured dopaminergic neurons has received intense attention.[14] Aggregates of TIQs have been shown to accumulate chronically in dopaminergic neurons and induce loss of substantia nigra pars compacta neurons with emergence of parkinsonism. Although TIQs are apparently less

Received: August 30, 2022
Accepted: November 3, 2022
Published: November 14, 2022



https://doi.org/10.1021/acschemneuro.2c00516
ACS Chem. Neurosci. 2022, 13, 3230−3246

neurotoxic than MPTP, their potential long-term effects in idiopathic PD or other neurodegenerative diseases gradually progressing over years or decades warrant rigorous examination.

There are several approaches for evaluating TIQs' endogenous levels, involving numerous analytical methods based on immunochemistry, such as radioenzymatic assays or radioimmunoassays (REA, RIA) or gas (GC) or liquid chromatography (LC) coupled with mass spectrometric (MS), fluorescence detection (FD), or electrochemical detection (ED) (Table S1). Developments of increasingly selective and sensitive analytical platforms have allowed quantitative analyses of TIQ analytes in plasma, cerebrospinal fluid (CSF) and brain tissues associated with PD's pathophysiology or pathogenesis. Moreover, numerous studies have been published on TIQs' occurrence in mammals (Table S1), but few have provided detailed summaries of the analytical techniques used to determine TIQ derivatives. Thus, this critical review is intended to provide the first thorough coverage of available analytical methods from sample pretreatment to detection of TIQs' levels in mammalian bodily fluids and tissue. A further objective is to summarize results of quantitative analyses of TIQs and their distributions in mammals.

The review is based on a systematic search of literature compiled in the Web of Science database using combinations of the following search terms: (tetrahydroisoquinoline) AND (salsolinol) AND (quantitative analyses) AND (separation) AND (determination) AND (detection) AND (identification) AND (gas chromatography) AND (liquid chromatography) AND (capillary electrophoresis) AND (electrochemical detection) AND (mass spectrometry) AND (biological samples) AND (mammals). Included articles had to report original studies on identification, detection, or quantitative determination of TIQs in mammalian biological fluids and tissues. Articles were excluded if only abstracts were available. Some sections were not in English. They reported determination of exogenously supplied compounds (e.g., after iv administration, food digestion, or drug administration) or did not clearly present endogenous levels. No time-related restrictions were imposed, and the sources of analyzed materials could be healthy people (or other mammals) and/or patients with PD diagnosis (regardless of stage of the disease).

2. OVERVIEW OF TETRAHYDROISOQUINOLINES

2.1. MPTP's Cellular Mechanism of Neurotoxicity. PD is associated with selective loss of dopaminergic neurons located in substantia nigra with projections to the striatum. As the progression of the disease continues, PD becomes widespread affecting other nuclei as described in, for example, Parkinson's disease dementia. However, afflictions of extranigral structures are also present in the vast majority of postmortem PD brains.[16−20] Another pathological hallmark is the presence of cytoplasmic inclusions containing presynaptic protein α-synuclein (α-SYN), known as Lewy bodies, in surviving neurons.[21−23] The exact cause and molecular mechanism of the death of dopaminergic neurons in PD are still unknown, but possible exogenous and/or endogenous toxic compounds are putatively related to PD's pathobiology.[10,14,24−26] Hao et al. (1995) found that inhibition of the function and growth of rat dopaminergic neurons in culture caused by TIQs can be completely prevented by selegiline, a monoamine oxidase type B (MAO-B) inhibitor.[14] Accumulated neurobiological evidence since then has indicated that isoquinoline derivatives may be important neurotoxins that contribute to dopaminergic cell death in PD,[24,27] and the cellular mechanism of MPTP-elicited neurotoxicity in PD has been extensively studied.

As a highly lipophilic proneurotoxin, MPTP rapidly crosses the blood−brain barrier (BBB) into the brain. It is subsequently converted in the brain to 1-methyl-4-phenyl-2,3-dihydropyridinium ion ($MPDP^+$) in a reaction catalyzed by MAO-B, localized in the outer membrane of mitochondria of glial cells. Oxidation of $MPDP^+$, probably spontaneous, leads to production of 1-methyl-4-phenylpyridinium (MPP^+), which is actively transported into presynaptic dopaminergic nerve termini of nigrostriatal dopaminergic neurons through the plasma membrane dopamine transporter. MPP^+, the active form of the neurotoxin, accumulates within the inner mitochondrial membrane. It can kill dopaminergic neurons by inhibiting mitochondrial complex I (NADH ubiquinone reductase), which alters mitochondrial transition pores' permeability, thereby interrupting electron transport, reducing adenosine triphosphate formation, releasing reactive oxygen species (ROS), and inhibiting oxidative phosphorylation.[28] MPP^+ also decreases DA synthesis by acutely inactivating the tyrosine hydroxylase system.[29−31] The decrease in DA pools resulting from inhibition of activity should also be considered an effect of the enzyme inhibition caused by TIQs.[31−33] In sum, MPP^+ accumulation and complex interactions may trigger apoptotic death of nigrostriatal dopaminergic neurons and cause PD-like symptoms.

2.2. Toxicity of TIQ Family Members. There are still questions regarding TIQs' neurobiological activities. Both neuroprotective and neurotoxic dose- and time-dependent effects, *in vitro* and *in vivo*, have been proposed,[34] but TIQs' contributions to PD's pathogenesis have not been fully demonstrated. Systematic administration of various TIQs has led to acute parkinsonism (and other monitored pathobiochemical changes) in various animals including mice, rats, and non-human primates. However, there has been no cohesive study and convincing anatomical demonstration of cell death in the substantia nigra pars compacta of animals exposed to TIQs.[32,33,35−38] Moreover, limited neuronal cell death has been observed in animals treated with some TIQs.[39,40] For such reasons it has been suggested that TIQs may alter certain biochemical traits that may only lead to parkinsonism in animals in conjunction with other factors. However, the failure to detect neuronal cell death caused by TIQs might be due to use of insufficiently sensitive markers or methodological issues.

Endogenous TIQs are poor substrates for MAO in comparison to exogenous MPTP,[39] prompting conclusions that TIQs are not strong neurotoxins under physiological conditions. However, accumulative effects of endogenous TIQs should also be considered. Moreover, *in vitro* experiments have shown that enzymatically N-methylated, benzylated, and further oxidized TIQs may be highly neurotoxic. Neuronal or glial enzymes may convert relatively innocuous catechol and non-catechol TIQs into more potent neurotoxins. For example, 1,2,3,4-tetrahydroisoquinoline (TIQ) can be methylated to either 2-methyl-1,2,3,4-tetrahydroisoquinoline (*N*-Me-TIQ) or 1-methyl-1,2,3,4-tetrahydroisoquinoline (1-Me-TIQ) after crossing the BBB and higher activities of *N*-methyltransferase, a widely expressed cytosolic enzyme, have been detected in PD patients than in controls.[41] Generally, N-

https://doi.org/10.1021/acschemneuro.2c00516
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methylated derivatives are considered more toxic than their nonmethylated parent compounds. Subsequent oxidation catalyzed by MAO, which may be critical for TIQs' neurotoxicity, converts *N*-Me-TIQ to the more toxic *N*-methylisoquinolinium ion $NMIQ^+$. Salsolinol (SAL) can be methylated by *N*-methyltransferase to (1*R*)-1,2-dimethyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol ((*R*)-*N*-Me-SAL), and consequent oxidation leads to 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$) while the 1,2,3,4-tetrahydroisoquinoline-6,7-diol (nor-SAL) pathway leads to 2-methyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol (*N*-Me-nor-SAL) and *N*-methyl-6,7-dihydroxyisoquinolinium (*N*-methylnorsalsolinium) ion. A biosynthetic pathway of benzylated TIQs (Bn-TIQs), yielding 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1-Bn-TIQ), 1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline (3′,4′-DHBnTIQ), and 1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol (6,7-DHBnTIQ), via condensation may also be hypothetically present in the brain.[42] Of the various TIQs in the brain, SAL, *N*-Me-nor-SAL, 1-Bn-TIQ, and *N*-Me-SAL have been most extensively investigated and are regarded as the most likely enzymatically formed PD-causing neurotoxins.[43,44] Accordingly, higher levels of these TIQs have been found in the brain and/or CSF of PD patients than in healthy controls.[14,24,27,38] Interestingly, no significant general relationship has been found between isoquinoline derivatives' lipophilicity and their inhibition of respiratory chain complex I inhibition in rat brain.[28] However, the (*R*)-enantiomers are considerably more toxic (more than 10-fold in DNA fragmentation assays) than the (*S*)-enantiomers.[45]

1-Bn-TIQ has been identified as the most toxic TIQ to dopaminergic neurons in *in vitro* cultures.[46,47] Experimental evaluation of its effects on human dopaminergic SH-SY5Y cells in culture[43] has shown that it can significantly reduce [^{3}H]-DA uptake and increase lipid peroxidation. SH-SY5Y cells' viability and antiapoptotic protein B-cell lymphoma 2, glutathione, and adenosine triphosphate levels were decreased in the cited study, while expression of the proapoptotic protein Bax and formation of active caspase-3 increased. Further, 1-Bn-TIQ administration increased levels of α-SYN and ROS. It can also cause endoplasmic reticulum (ER) stress in SK-N-SH neuronal cells in culture[48] and induce symptoms of parkinsonism in animal models (accompanied by reductions in levels of DA and DA metabolites), and it is selectively toxic to dopaminergic neurons of the nigrostriatal regions.[49] In addition, it causes morphological changes specifically in tyrosine hydroxylase-positive neurons.

Increased SAL levels in the CSF of PD patients have been observed in several studies, and SAL is putatively toxic to dopaminergic neurons.[50−52] SAL can inhibit TH, MAO-B, and mitochondria complexes I and II in dopaminergic neurons.[53] Rapid intracellular adenosine triphosphate and glutathione depletion, accompanied by increases in ROS production, has been observed following treatment with SAL, leading to necrosis of cultured dopaminergic cells. Furthermore, SAL can activate ER stress signaling pathways in dopaminergic SK-N-SH neuronal cells.[48] It has been suggested that ROS and ER stress is widely involved in SAL-mediated neurotoxicity in dopaminergic neurons. In addition, a N-methylated derivative of SAL, (*R*)-*N*-Me-SAL, can accumulate in the nigrostriatal region of the brain and cause apoptosis in dopaminergic neurons via caspase-3 activation and nuclear fragmentation.[41] Parkinsonism, together with reduction of tyrosine hydroxylase-positive neurons, has been observed in rats administered (*R*)-*N*-Me-SAL.[38] The apoptotic cell death caused by (*R*)-*N*-Me-SAL can be reportedly prevented by the MAO inhibitors deprenyl and rasagiline.[41,54]

The most potent TIQ derivative for reducing tyrosine hydroxylase activity, among those tested by Maruyama et al. (1993), is *N*-Me-nor-SAL,[29] and the only potential neuroprotective derivative described to date is 1-Me-TIQ.[55−58] 1-Me-TIQ has been detected in food[59] and brain tissue.[60] It may prevent neurotoxic effects of MPP^+ and other TIQ derivatives by shielding complex I.[61] Accordingly, it has been proposed that reduction in the levels of 1-Me-TIQ synthesizing enzyme in substantia nigra and striatum may play key roles in the pathogenesis of idiopathic PD activity in aged rats.[62]

2.3. Exogenous Occurrence of TIQs and the Blood−Brain Barrier. Assuming that some neurotoxins with chemical similarity to MPTP have a wide spectrum of psychopharmacological and behavioral effects and may be related to PD, distributions of a group of TIQs have been thoroughly investigated in both plants and animals. An abundant natural family of these heterocyclic compounds has been detected at high concentrations in many common edible plants. These include fruits (banana, grape, pear, peach), vegetables (leaf lettuce, celery, sweet potato, green bean, cherry), mushrooms, milk and milk byproducts, wine, cocoa, and chocolate (in ng/g wet weight, pmol/mL, amounts).[59,63−66] Several TIQs present in food and beverages have been shown to have quite potent nonspecific toxic effects on cultured nigral neurons, including apoptosis.[67,68]

According to Ohta et al. (1990), orally administered or consumed exogenous TIQs are generally the major contributors to their plasma levels.[69] Although TIQs are metabolized in the liver to 4-hydroxy-TIQs by debrisoquine hydroxylase (P-450 CYP2D6), several lines of evidence suggest that some exogenously administered TIQs can easily cross the BBB and accumulate in different regions of the brain. Notably, TIQ and its methylated and benzylated derivatives have proven abilities to cross the BBB and accumulate in rat brain after intraperitoneal injection.[64,70−73] Until recently there was no definitive evidence that another TIQ derivative, SAL, could or could not cross the BBB.[74] Neither Origitano et al. (1981) nor Song et al. (2006a) found any evidence that levels of (*R*/*S*)-SAL in the brain were increased by these compounds' intraperitoneal administration, prompting conclusions that SAL could not be transported across the BBB.[73,75] However, more recently Quintanilla et al. (2014) showed that systemically administered SAL, at 10 mg/kg, could cross the BBB in sufficient amounts for detection *in vivo* by microdialysis of neostriatum, reaching an estimated concentration of 100 nmol/L in the dialysate.[74] Moreover, exogenous SAL can alter laboratory animals' behavior, indicating that neuronal or glial enzymes could convert it to a potent neurotoxin and hence alter central dopaminergic pathways. Therefore, in principle, consumption of edibles that contain TIQ derivatives that may potentially be converted to neurotoxins should be considered risk factors that may detrimentally interact with predisposing genetic factors. In addition, inflammatory and neurodegenerative processes in PD may adversely affect BBB functions and cause the leakage of neurotoxic TIQs from plasma to the brain. Chronically administered exogenous TIQs seem to accumulate in the brain and participate in induction of nigrostriatal dopaminergic neurons' apoptosis.[64,70]

2.4. Endogenous TIQs Synthetic Pathways. Both enzymatic and nonenzymatic synthesis of TIQs has been

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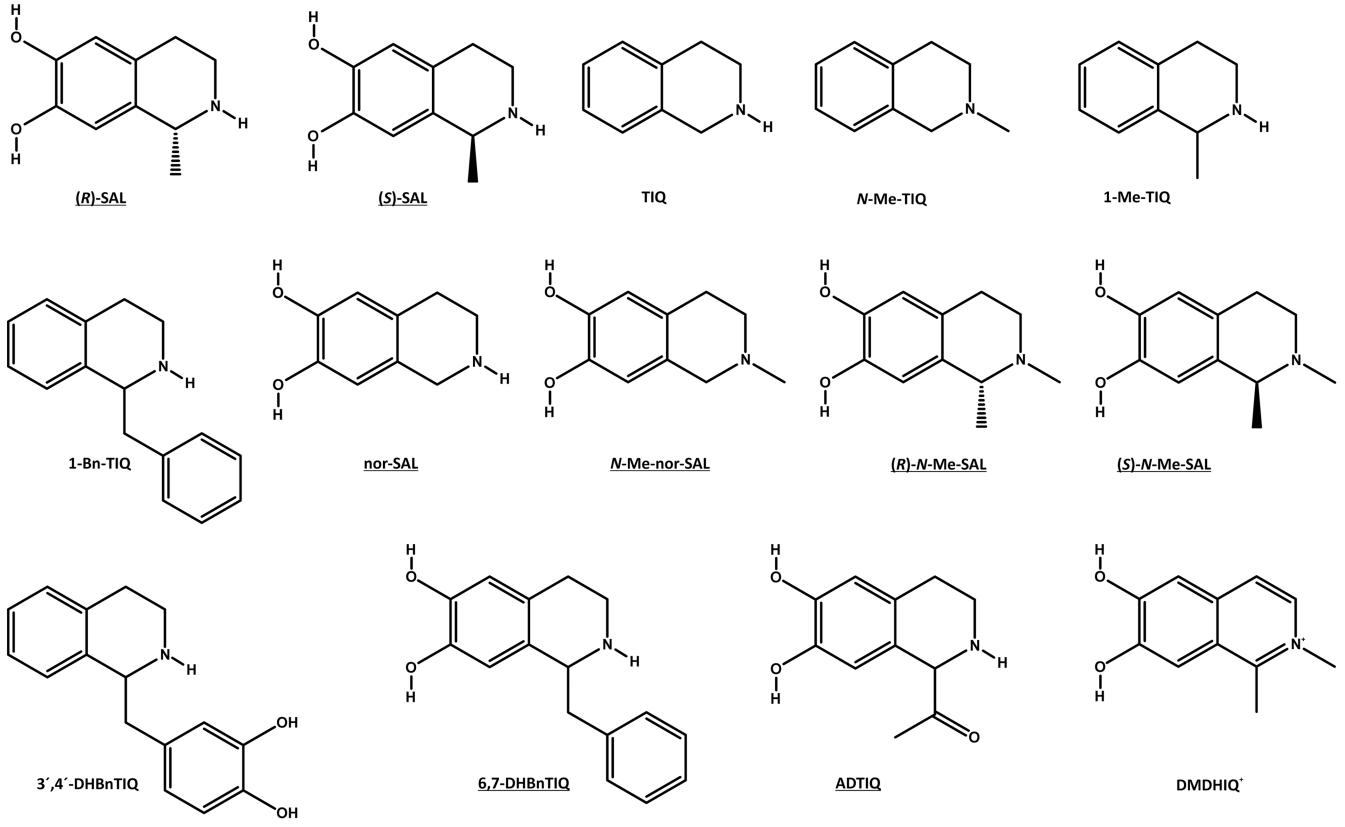

Figure 1. Chemical structures, IUPAC names (according to the PubChem database) and abbreviations of the catechol (underlined) and non-catechol tetrahydroisoquinolines analyzed in retrieved articles. (*R*)-SAL, (1*R*)-1-methyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol; (*S*)-SAL, (1*S*)-1-methyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol; TIQ, 1,2,3,4-tetrahydroisoquinoline; *N*-Me-TIQ, 2-methyl-1,2,3,4-tetrahydroisoquinoline, 2-methyl-3,4-dihydro-1*H*-isoquinoline; 1-Me-TIQ, 1-methyl-1,2,3,4-tetrahydroisoquinoline; 1-Bn-TIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline; nor-SAL, 1,2,3,4-tetrahydroisoquinoline-6,7-diol; *N*-Me-nor-SAL, 2-methyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol; (*R*)-*N*-Me-SAL, (1*R*)-1,2-dimethyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol; (*S*)-*N*-Me-SAL, (1*S*)-1,2-dimethyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol; 3′,4′-DHBnTIQ, 1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline; 6,7-DHBnTIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol; ADTIQ, 1-acetyl-6,7-dihydroxyl-1,2,3,4-tetrahydroisoquinoline; DMDHIQ$^+$, 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion.

detected in plants and animals. They can be formed endogenously by well-known Pictet−Spengler condensation of biogenic amines with aldehydes or α-keto acids. Such synthesis of catechol-bearing TIQs under physiological conditions has been reported by several authors.[24,63,76,77] Condensation of DA with acetaldehyde generates SAL, whereas reaction of DA with phenylacetaldehyde leads to formation of 6,7-DHBnTIQ. In contrast, spontaneous reactions of 2-phenylethylamine (PEA) leading to non-catechol TIQs seem unlikely because PEA does not cyclize with aldehydes under physiological conditions.[78] However, a 1-Me-TIQ synthesizing enzyme has been identified in rat brain.[79] Therefore, it has been assumed that non-catechol TIQ derivatives are formed exclusively enzymatically,[42,47] while catechol TIQs may be synthesized both enzymatically and nonenzymatically.[79] Stereoselective enzymatic synthesis of SAL would only generate the (*R*)-enantiomer, whereas endogenous formation of SAL by Pictet−Spengler condensation yields equal amounts of *R*/*S* enantiomers.[80] Only the (*R*)-enantiomer of the *N*-methyl-salsolinol is putatively synthesized enzymatically in the brain. Interestingly, however, endogenously formed nor-SAL may be generated solely by condensation reaction.[81] Chemical structures of the catechol and non-catechol TIQs identified in mammalian fluids and tissues in reviewed articles are summarized in Figure 1.

3. OTHER NATURAL PD TOXINS

Outside of TIQs as neurotoxic/neuroprotective agents, a group of DA-derived metabolites and natural products were identified as PD-inducing agents with the manifestation of several pathological events both *in vitro* and *in vivo*.

Since impaired DA metabolism leads to the production of many toxic metabolites, these derivatives are under suspicion as potential triggers of PD selective loss of dopaminergic neurons. DA itself is the first mentioned agent that can, under certain circumstances, act as a toxic agent. The first examples of toxic dopaminergic actions can be found during biosynthesis, in which excess of DA is metabolized by MAO-B to form the highly toxic metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) with ammonia and hydrogen peroxide and consequently by the action of aldehyde dehydrogenase or aldehyde/aldose reductase to nontoxic dihydroxyphenylacetic acid (DOPAC) or 3,4-dihydroxyphenylethanol (DOPET).[82] Another example of dysregulation of DA homeostasis was found in connection with α-SYN, a well-known presynaptic protein associated with the pathology of PD called "synucleinopathy".[83] The wild-type protein was found to be essential for the regulation of synaptic transmission and recycling of DA vesicles. It was shown that mutated α-SYN can induce a shortage of monoaminergic vesicles resulting in the accumulation of DA in the cytoplasm.[84] Accumulated DA in

https://doi.org/10.1021/acschemneuro.2c00516
ACS Chem. Neurosci. 2022, 13, 3230−3246

the cytoplasm is prone to autooxidize and produces a high amount of toxic DA quinones.[85]

DOPAL was proposed to be strongly involved in selective loss of dopaminergic neurons as proposed in the "catecholaldehyde hypothesis".[82,86−90] DOPAL is a highly reactive electrophilic molecule reacting with nucleophilic moieties of amino acids, such as primary amines and thiols. Under physiological conditions in dopaminergic neurons the normal concentration of DOPAL is around 2−3 μM, which detoxification enzymes (e.g., aldehyde dehydrogenases or aldehyde/aldose reductase) are able to catabolize sufficiently without any cytotoxicity effect on neurons. On the other hand, when the concentration of DOPAL exceeds 6 μM, toxicity events start to appear. Such cytotoxicity threshold has been observed in many cell lines.[91] *In vivo* studies using intracranial injection of DOPAL, DOPET, and DA to the substantia nigra of animal model showed that the loss of dopaminergic neurons after DOPAL administration was dramatically higher than after DOPET and DA.[86]

More importantly, DOPAL was also responsible for oligomerization of α-SYN.[92−95] Additionally, it was shown that an essential part of the ubiquitin proteasome system, used for protein clearance, was also negatively affected by DOPAL. Another finding indicates the role of DOPAL on α-SYN aggregation through DOPAL-mediated lysine modification of small ubiquitin-like modifier.[94] In the case of cellular PD pathological events, DOPAL was associated with direct generation of ROS, e.g., hydroxyl radical and hydrogen peroxide,[90] as DOPAL can autooxidize to semiquinone and ortho-quinone.[96] DOPAL was also linked with neuroinflammation through cyclooxygenase-2 which is up-regulated during PD, increasing oxidative stress.

DA under neutral pH is highly unstable, creating dopamine quinones (DAQs) by autoxidation. Besides the main DAQs, such as dopamine-*o*-quinone (DA-*o*-quinone), aminochrome, and 5,6-indolequinone previously mentioned, DOPAL can also undergo autoxidation resulting in formation of its own quinone acting in similar toxic ways as DAQs. Since the DA-*o*-quinone is highly unstable, the molecule is subjected to cyclization and oxidation to aminochrome and superoxide radical.[97−100] Finally, aminochrome is rearranged to 5,6-dihydroxyindole which is further oxidized to 5,6-indolequinone.[101]

In the case of biological activity of these DAQs, DA-*o*-quinone as an electrophile usually binds nucleophilic parts of several molecules or proteins, such as glutathione,[102] parkin,[103] tyrosine hydroxylase,[104] mitochondrial glutathione peroxidase 4,[105] dopamine transporter,[106] quinoprotein adducts,[107] mitochondrial complexes I, III, and V, isocitrate dehydrogenase, superoxide dismutase 2, JC-1, UCHL-1,[108,109] and tryptophan hydroxylase.[110] Since the DA-*o*-quinone is highly unstable and is prone to rapid cyclization to aminochrome, some authors indicate that these protein adducts can be accounted to aminochrome.[97,111] In fact, aminochrome was associated with covalent modification of parkin,[103] UCHL-1,[109] and α-SYN[112,113] (similarly as 5,6-indolequinone[114]). Additionally, both aminochrome and 5,6-indolequinone were responsible for formation of toxic α-SYN protofibrils in dopaminergic neurons containing neuromelanin.[115] Interestingly, aminochrome interacts with α- and β-tubulin leading toward cytoskeleton architecture disruption, which impairs mitochondrial motility.[116] Other proteins, such as DAT and tryptophan hydroxylase, were negatively affected by aminochrome as well.[104,106,110] Since several proteins, e.g., parkin and UCHL-1, are negatively influenced by aminochrome, the impairment of proteasome ubiquitin,[117,118] autophagy,[116,119] and lysosomal systems[120] was identified.

Pathological activity of DAQs is not limited to modification of proteins but also one-electron redox reaction cycles resulting in elevation of oxidative stress. Specifically, NADH and NADPH as sources of electrons react with aminochrome (acceptor) forming leukoaminochrome-*o*-semiquinone radical, which is highly unstable. Leukoaminochrome-*o*-semiquinone then reacts with oxygen to form a superoxide radical with further autoxidation to aminochrome.[119,121] Such redox cycle continues until the levels of NADH or NADPH and oxygen are depleted. Consequently, depletion of NADH, NADPH, and oxygen leads to blockade of production of ATP in mitochondria, elevation of oxidative stress, and prevalence of oxidized glutathione.[116,122−129]

6-Hydroxydopamine (6-OHDA), similarly as DAQs, is produced from DA by oxidation, which was shown to be mediated by iron(III) ions.[85,130] Unlike DAQs, 6-OHDA is a minor metabolite but it is associated with higher acute toxicity.[131] 6-OHDA is one the most common PD inducer both in *in vitro* and *in vivo*.[132,133] Similar to previous DA-derived neurotoxic agents, 6-OHDA has high affinity toward dopamine transporter and noradrenaline transporter and induces toxicity via oxidative stress by autoxidation or production of hydrogen peroxide via MAO-B, mitochondrial dysfunction by inhibition of complexes I and IV,[134] ER stress[135,136] causing cell death by apoptosis,[137−139] but also necroptosis[140] or autophagy.[135] In contrast to DAQs, 6-OHDA did not recapitulate α-SYN pathology.[132,133]

4. OVERVIEW OF ACQUIRED DATA

This section summarizes data obtained from the articles (65 in total) included in our systematic review, as described in the Introduction.

4.1. Tetrahydroisoquinoline Determination in Mammalian Bodily Fluids and Tissues. A biomarker is a molecule that indicates a physiological state, such as PD (or specific stage of PD). Biomarkers that are directly linked to the clinical manifestations and outcome of a particular disease are extremely valuable.[141] They are highly important for improving diagnosis in early stages of chronic neurodegenerative diseases, when therapies are likely to be most effective. Currently, clinical diagnostic methods are limited,[5−8] so there are urgent needs for specific biochemical markers that could help diagnosis of presymptomatic and symptomatic stages of neurodegenerative diseases.[141]

Biomarkers can be monitored in any tissue or bodily fluid, but easily accessible biological matrices are required for their routine determination. Thus, blood and urine are widely used for biomarker monitoring.[142] In addition, plasma and urine respectively reflect early stages of metabolism and averaged states of the entire metabolic system within a specific time.[143] Neurological biomarkers that are abundant in CSF can be studied using CSF samples, but this requires invasive, painful collection through lumbar puncture by medical professionals. The analysis of biological fluids such as blood and urine has clear advantages because the sampling is simple. Compared to the collection of CSF, the collection of urine and blood is almost noninvasive. However, blood and urine analyses usually provide only partial information on brain chemistry because the BBB forms a limited permeable barrier between the brain's internal environment and the body's vascular system.[143]

https://doi.org/10.1021/acschemneuro.2c00516
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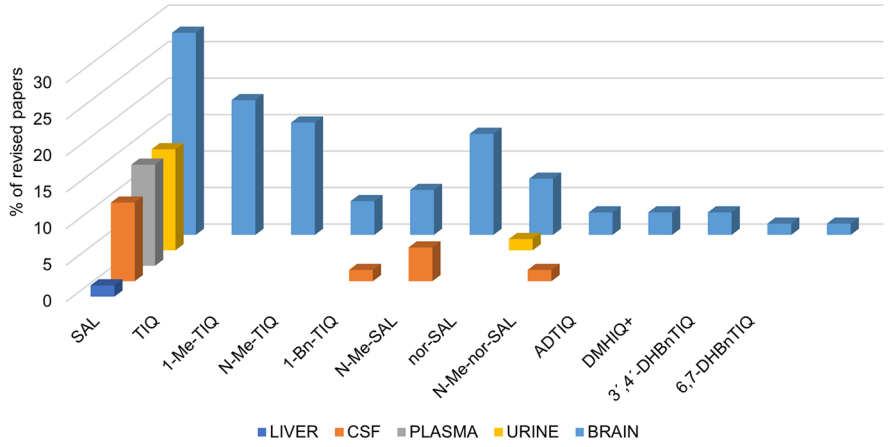

Figure 2. TIQs detected in indicated mammalian fluids and tissues of any species (with no distinction of brain tissues) in reviewed articles. Percentages of studies in which each biomarker was analyzed are shown (abbreviations explained in the text).

To date, the following TIQs have been isolated, detected, and quantified in mammals as potential biomarkers: SAL and nor-SAL in human urine; SAL in human plasma; SAL, 1-Bn-TIQ, *N*-Me-SAL, and *N*-Me-nor-SAL in human CSF; SAL, TIQ, 1-Me-TIQ, *N*-Me-TIQ, 1-Bn-TIQ, *N*-Me-SAL, nor-SAL, *N*-Me-nor-SAL, ADTIQ, and $DMDHIQ^+$ in human brain; SAL, TIQ, 1-Me-TIQ, *N*-Me-TIQ, 1-Bn-TIQ, *N*-Me-SAL, nor-SAL, and *N*-Me-nor-SAL in rat brain; SAL in rat liver; SAL, TIQ, 1-Me-TIQ, *N*-Me-TIQ, 1-Bn-TIQ, *N*-Me-SAL, nor-SAL, *N*-Me-nor-SAL, ADTIQ, 3′,4′-DHBnTIQ, and 6,7-DHBnTIQ in mouse brain; and TIQ, 1-Me-TIQ, and 1-Bn-TIQ in monkey brain (Table S2). The most commonly analyzed biomarker in the reviewed studies was SAL, which was included in more than 60% of both single- and multi-TIQ analyses. Table S2 presents (and Figure 2 summarizes) recorded TIQ levels and their locations in biological fluids and tissues of various mammal species.

Most studies in which TIQs have been detected, qualitatively or quantitatively, in mammalian biological fluids and tissue samples focused on individual compounds with little consideration of regional distributions, enantiomers, or differences among species. However, in 2008 a much more comprehensive regional and quantitative picture of TIQ derivatives in mouse, rat, and human brains was presented.[60] TIQ derivatives were detected and quantified in all brain regions analyzed of all three species. However, (*R*/*S*)-SAL and its methylated derivatives were detected in regions with relatively high DA content in both rodents and humans. Racemic mixtures of (*R*)- and (*S*)-enantiomers were also detected in dopaminergic regions.[60] It has been shown that TIQ derivatives are not short-lived, unstable intermediates and could probably accumulate in catecholaminergic neuronal populations with aging. Interestingly, presented results indicated that TIQ, 1-Me-TIQ, *N*-Me-TIQ, and 1-Bn-TIQ are widely distributed in rat and mice brain with minimal regional variation in concentrations and do not accumulate in tissues expressing catecholaminergic pathways. It was concluded that they might have exogenous sources since all of these compounds readily cross the BBB.[72,73] Alternatively, they may arise from endogenous synthetic pathways that do not involve catecholamines.[60]

Various TIQs have also been detected in urine, CSF, and brains of PD patients. These include SAL, TIQ, 1-Me-TIQ, *N*-methyl-TIQ, nor-SAL, *N*-Me-nor-SAL, *N*-Me-SAL, 1-Bn-TIQ, and ADTIQ (Table S2). In comparison to human controls, higher SAL concentrations have been found in PD patients' urine,[59] CSF,[144] and brain locus coeruleus, frontal cortex, and hippocampus;[60] comparable levels in their brain substantia nigra[60] and CSF;[50,59,145,146] and significantly lower concentrations in their cerebellar cortex and inferior olive.[60] Significant variations in *N*-Me-SAL levels among PD brain regions relative to normal levels have also been recorded. They are reportedly higher in the frontal cortex and hippocampus of PD brains, lower in substantia nigra, caudate, and locus coeruleus but not significantly different in putamen, inferior olive and cerebellar cortex.[60] Higher *N*-Me-SAL levels have been detected in human PD CSF.[41,145,146]

Nor-SAL levels are reportedly significantly lower in PD putamen than in healthy controls but otherwise unaffected.[60] In contrast to undetectable levels in human controls, quantifiable *N*-Me-nor-SAL levels (16−60 pmol/L) have been detected in CSF of PD patients.[50] No significant differences in *N*-Me-nor-SAL levels between normal and PD brains have been detected except higher levels in substantia nigra and lower levels in caudate of PD brains.[147] In addition, higher levels of 1-Bn-TIQ have been detected in human PD CSF,[46] and ADTIQ in caudate and putamen of PD brains.[148] As found in rodents, TIQ, 1-Me-TIQ, *N*-Me-TIQ, and 1-Bn-TIQ are reportedly widely distributed in the human brain, with no significant concentration differences among regions or between normal human and PD brains.[60,148] TIQ levels in normal and PD human brain regions are summarized in Table S2.

4.2. Bioanalytical Method Validation. Rigorous demonstration that detected TIQs that are not analytical artifacts generated during sample workup and/or analysis is essential. The possibility that artificial condensation of catecholamines with contaminated aldehydes may increase TIQ levels should also be considered. Generally, use of validated methods is crucial to establish biomarkers' selectivity for diagnosing particular diseases,[149,150] and full validation is required for any new analytical method applied and any methodology applied to a new analyte.[150,151] Guidelines for bioanalytical method validation issued by the U.S. Food and Drug Administration (2018) and European Medicines Agency (2011) for drug development include key defined parameters for validating bioanalytical methods. Full validation requires aspects such as demonstration of a method's reliability for

https://doi.org/10.1021/acschemneuro.2c00516
ACS Chem. Neurosci. 2022, 13, 3230−3246

determining an analyte's concentration along with limits of detection or quantification values for target biomarkers in specific biological matrices. Therefore, it is essential to use well-characterized bioanalytical platforms to ensure the reliability of analyses of particular biological samples. Matrices can also dramatically affect the reliability of both identifications and quantifications of analytes. In such cases, use of stable isotopic-labeled analogues as internal standards (SIL-ISs) in combination with MS detection markedly enhances the reliability of applied methods.[152] Use of SIL-ISs of analytes, which have the same chemical properties and retention times as the nonlabeled analogues, is highly recommended in method validation. However, it is essential to use SIL-ILs with high isotope purity.[150] In stable isotope dilution methodology,[153] SIL-ISs are usually added at the start of the sample preparation procedure at known concentrations and the resulting analyte:labeled compound response ratios are used to generate standard curves to estimate absolute concentrations of the analytes in samples. This enables correction for any losses or gains from sample preparation or matrix effects caused by coeluting components during ionization in a MS ion source. Fully validated stable isotope dilution methods provide the most reliable quantitative determinations possible for any analytes.[152]

Although validation is mandatory,[150,151] few fully validated methodologies for quantitative determination of TIQs have been published. The following text summarizes validation efforts described in the reviewed articles. Zhang et al. (2004) studied the reproducibility of a method involving liquid chromatography connected to electrochemical detection (LC-ED) with a standard test mixture, providing information on intra- and interday precision data, linearity ranges, and the long-term stability of TIQs.[146] DeCuypere et al. (2008) investigated TIQ derivatives' stability in aqueous solution during thermal and freeze/thaw treatments.[60] Mao et al. (2010) reported the linear range, limit of detection (LOD), and repeatability of an LC-ED-based assay, as well as the recovery of SAL in neonatal rats.[154] Rojkovicova et al. (2008) partially validated a liquid chromatography−mass spectrometry (LC-MS) method for determining SAL in different brain regions of rats genetically predisposed to alcoholism, presenting information including its dynamic range and LOD.[155] Inoue et al. (2008) reported the precision, linearity, and LOD of a liquid chromatography with fluorescence detection (LC-FD) method for determining TIQ, 1-Me-TIQ, and 1-Bn-TIQ using standard solutions.[156] Starkey et al. (2006) developed and fully validated a new analytical approach for simultaneous LC-MS measurements of endogenous SAL and major catecholamines in brain tissue of experimental animals.[157] Further, a method for simultaneous determination of SAL enantiomers in human plasma and CSF by chemical derivatization coupled to chiral LC-MS was developed and fully validated by Lee et al. (2007).[158] Zhang et al. (2012) have described the most comprehensive validation of a LC-MS method for determining SAL and *N*-Me-SAL in rat brain, including assessment of its selectivity, linearity, precision, and accuracy, as well as the analytes' stability.[159] Various chemicals, such as dibenzylamine,[46,55,64,160−162] *N*-Me-TIQ,[78] *N*-Me-nor-SAL,[66,163,164] 3,4-dihydroxynorephedrine,[165] vanillic acid,[50,71,166,167] 3,4-dihydroxybenzylamine,[60,147,155,157,168−171] isoproterenol,[159,172] 6-methylquinoxaline,[173] *N*-ethylbenzylamine,[156] and benzyloxyamine,[174,175] have been employed as internal standards (ISs). Selected ISs have been chosen because they have structural similarities to TIQs of interest and efficiently trapped on solid phase extraction (SPE) columns, thus enabling reliable correction for loss of TIQs during extraction and derivatization steps of the methods. The SIL-ISs used in reviewed papers are summarized in Table S1. Undoubtedly, their use considerably increases bioanalytical results' credibility. However, there are several difficulties in developing and validating stable isotope dilution methods for TIQs based on MS detection. First, no SIL-ISs are commercially available for some target analytes and obtaining them for most metabolites is a complex task. Moreover, they are very expensive when available for purchase. Hence, the combination of stable isotope dilution and MS detection was only used in 58 and 10% of the reviewed TIQs' quantitative analyses by gas chromatography−mass spectrometry (GC-MS) and LC-MS, respectively. For SAL determination, d_2-SAL[176−186] and d_4-SAL[158,187] have been used in the quantitative analysis of human urine, human plasma, peripheral mononuclear cells and lymphocytes, and rat brain. d_3-TIQ and d_4-TIQ have been used as SIL-ISs for quantification of TIQ in human and monkey brain.[188,189] In addition, d_2-nor-SAL has been used for quantitative determination of nor-SAL in human brain tissue.[181−183]

4.3. Sample Preparation. Detected levels of TIQ compounds in mammalian brain tissue and bodily fluids are very low (ng/g and pg-ng/mL levels, respectively; Table S2). Thus, their determination requires more sensitive methodology than measurement of classical neurotransmitters, and sample preparation is as crucial for success as any other part of the analysis. Analytes must be separated from substances in the complex sampled biological matrices. Matrix components, such as nondetected metabolites, salts, or exogenous compounds, may interfere in analyte retention, in addition to reducing purification recovery and method sensitivity. Moreover, matrix effects may either reduce or increase responses (through ion suppression and ion enhancement, respectively) when MS detectors are used.[190] All the papers reviewed described the steps designed to extract analytes and cleanup matrix components. A typical sample-processing protocol commonly comprises an extraction step and a derivatization process, if required, prior to instrumental analysis. The methodologies used for preparing samples and determining TIQs in different biological matrices are summarized in Figure 3 and Table S1.

The main sample preparation procedures described in the reviewed literature are very similar, although SPE replaced liquid−liquid extraction (LLE) as the most common extraction technique during the period covered by the reviewed studies

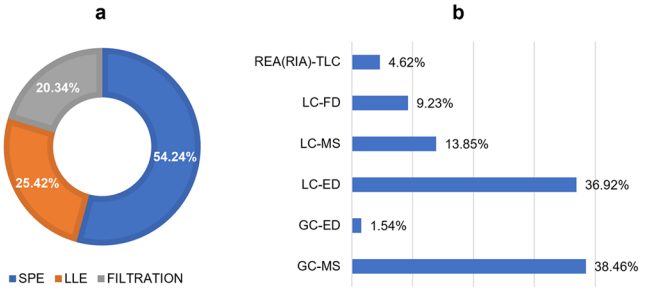

Figure 3. Purification (a) and separation/detection techniques (b) for determining TIQs in mammalian fluids and tissues applied in the reviewed studies. The percentage of reviewed studies in which each technique was used is indicated (abbreviations explained in the text).

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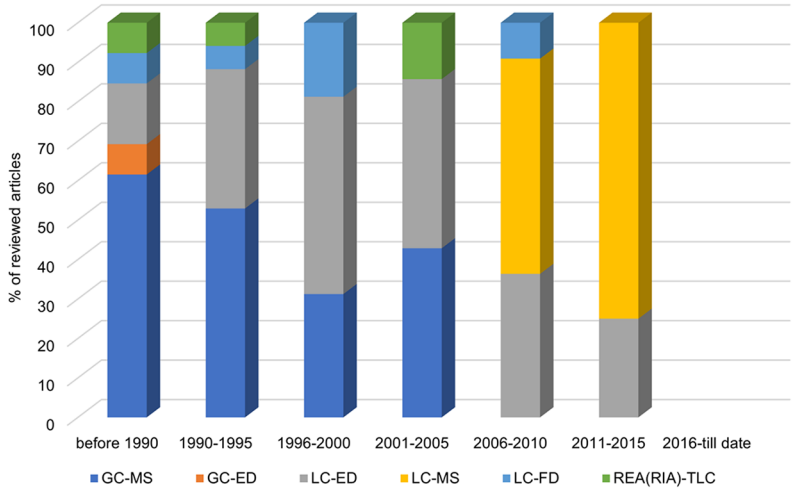

Figure 4. Analytical techniques used to determine TIQs in mammalian urine, plasma, CSF, and brain tissue during the period covered by the reviewed articles. The percentage of studies in which each technique was used is shown (abbreviations explained in the text).

(Figure 3). The methodology sometimes included simple dilution and filtration through a 0.22 or 0.45 μm cellulose membrane or polyvinylidene fluoride (PVDF) filter followed by direct injection.[41,66,144,147,154,159,163,173,191−194] Filtration before analysis was included in 20.34% of the reviewed methods. LLE with different organic solvents was also used for processing samples containing TIQs. Organic solvents found to be suitable for these extraction processes include ethyl acetate/ammonium hydroxide[176] and dichloromethane.[46,55,59,64,78,156,160−162,188,189,195,196] Extractive pentafluorobenzylations have also been employed.[180] LLE was applied in 25.42% of the reviewed studies (Table S1, Figure 3). The most widely used TIQ-related extraction, cleanup, and enrichment procedure was manual SPE,[197] applied in 54.24% of the reviewed methodological studies (Figure 3). None of the published methods involved use of some available online sample extraction and preconcentration devices enabling automated analyses with column-switching and high-throughput SPE with use of a multiwell plate. However, manual SPE has been used to purify and preconcentrate TIQs by retention of TIQs on a stationary phase using phenylboronic acid (PBA) cartridges,[42,50,71,158,166,167,177−179,181−183,185,187,198−202] Al_2O_3 columns,[164,168,171,184] AG SOW-X4 Biorex 70 cation exchange resin cartridges,[165,169,203] Amberlite CG-50 type II,[172,204] primary and secondary amine (PSA) cartridges,[199−201] and two types of reverse phase cartridges Oasis HLB[60] and Varian C18[155,170] (Table S1, Figure 3). Several recently developed procedures and devices for sample preparation, such as Captiva EMR-lipid cartridges[149] and immunoaffinity columns,[205] have not been employed for TIQs' determination. However, their use could potentially reduce matrix effects and markedly improve methods' selectivity and sensitivity

4.4. Analytical Techniques for Tetrahydroisoquinolines. As endogenous levels of TIQs are very low, their determination clearly requires highly sensitive and validated analytical methods. The following approaches have been used to determine TIQ biomarkers in biological matrices: GC-MS, gas chromatography-electrochemical detection (GC-ED), LC-MS, LC-ED, LC-FD, radioenzymatic assay, and radioimmunoassay thin layer chromatography (REA and RIA-TLC) (Figures 3 and 4, Table S1).

4.4.1. Chromatographic Techniques for Analyzing Tetrahydroisoquinolines. Generally, methods involving use of a chromatographic instrument (LC or GC) coupled to a specific detector have been most commonly used for quantifying TIQs in mammals. For analysis of a single TIQ (e.g., SAL) in the investigated biological matrices traditional LC with ED used to be the most widely used method (Figures 3 and 4). However, there are increasing needs to determine multiple analytes, including TIQs. Therefore, LC-MS has become the technique of choice as it enables simultaneous determination of various TIQs of both catechol and non-catechol families.[60] The GC and LC analytical platforms used in the reviewed studies are summarized in Table S1.

Coupling GC and MS detection has enabled measurement of the low levels of various endogenous TIQs in biological samples (Table S1). GC-MS with prederivatization used to be considered a well-established technique with advantages including highly standardized sample preparation procedures, affordability, and readily available libraries that could be conveniently searched for compound identification. Thus, this analytical platform (with MS or ED detection systems) was used in 40% of the retrieved studies. However, since it can only be applied to volatile compounds, a derivatization step is required.[155] Thus, various methods have been applied to convert TIQs to volatile derivatives prior to GC-MS analysis.[177,180] The most popular agents for derivatizing TIQs are pentafluoropropionyl anhydride (PFPA),[78,184,187−189] *p*-tyramine (3,5-bis(trifluoromethyl)-*N*-[2-(4-trimethylsilyloxyphenyl)ethyl]benzamide; DTFMB-TMS),[176] *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) and *R*-(−)-2-phenylbutyrylic acid,[177−179,181−183,185] pentafluorobenzoyl chloride (PFB-Cl),[180] heptafluorobutyric anhydride (HFBA),[42,46,55,64,160−162,188,189,195,196,203] HFBA and perfluoro-2-propoxypropionyl chloride,[59] and *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS).[198] The resulting volatile TIQ forms can be analyzed using routine GC-MS methods, and in principle the GC-MS methods offer the sensitivity and selectivity needed for positive identification and quantification of the TIQs at low levels. However, the complex derivatization steps required prior to GC-MS analysis are time-consuming

https://doi.org/10.1021/acschemneuro.2c00516
ACS Chem. Neurosci. 2022, 13, 3230−3246

and introduce risks of loss and transformation of TIQs, low recovery, and poor reproducibility. Moreover, sample injection reproducibility and analyte instability at the elution temperatures may vary.[206] Therefore, methods that can exploit the selectivity of MS with no requirement or prior sample derivatization have been sought.

High-performance liquid chromatography (HPLC) with reversed-phase or chiral (e.g., β-cyclodextrin) columns has become the main approach for determining TIQs in mammalian fluids and tissues. This analytical platform was used in 60% of all referenced studies. Methods for analyzing TIQ metabolites have typically involved use of reversed-phase columns with C_{18}, C_8, octadecyl-silica, pentafluorophenylpropyl, or porous activated graphite stationary phases. The mobile phases for the ED methods have usually consisted of sodium phosphate buffer modified with citric acid, sulfonates, and EDTA. Commonly used mobile phases in LC-MS methods have contained volatile acids or buffers such as formic acid or ammonium formate. Organic modifiers such as methanol, acetonitrile, and isopropyl alcohol have been commonly used with either isocratic or gradient conditions for analyte elution. HPLC methods with chiral β-cyclodextrin stationary phases (β-cyclodextrin-OH or AD-H columns) or sulfated β-cyclodextrins as chiral mobile phase additives have also been reported (Table S1). The chiral mobile phase additive β-cyclodextrin is a cyclic oligosaccharide that preferentially binds to one face of an enantiomer, thus changing hydrogen-bonding patterns of the enantiomers and enabling their separation using a standard reversed-phase column. Methods involving use of a reversed-phase column and a chiral mobile phase additive (β-cyclodextrin) have been described for determining SAL,[60,66,163] *N*-Me-SAL,[60,66,163] TIQ,[60] 1-Me-TIQ,[60] *N*-Me-TIQ,[60] and 1-Bn-TIQ.[60] Chiral columns have been used for the analysis of SAL,[41,155,170,191,193,199−201] *N*-Me-SAL,[41,191,193] and $DMDHIQ^+$.[41,193]

Compared to traditional HPLC, ultra-high-performance liquid chromatography (UHPLC) involves use of a more uniform column packing material with ≤2 μm particles, smaller diameter columns, and new pumps. It provides higher numbers of theoretical plates and significantly reduces analysis times and solvent consumption while increasing both chromatographic resolution and efficiency. Thus, UHPLC provides sharper peaks while decreasing sample loading relative to conventional HPLC. Such properties may provide high-throughput analysis of trace levels of target analytes in complex biological matrices.[207] Surprisingly, however, there are no commercially available columns with sub-2-μm solid-core particles that enable large increases in efficiency of TIQs analysis as yet.

4.4.2. Detection Methods. High-performance liquid chromatography with electrochemical detection (HPLC-ED) used to be the most commonly and widely applied approach for determining TIQs in mammalian fluids and tissues (Figures 3 and 4). It was used in 36.92% of the review articles, but as shown in Figure 4, its use seems to be declining for the determination of TIQs. It has been used for detecting catecholamines in biological fluids and tissues due to its excellent detection limits, simple operation, low cost, and no requirement for sample derivation.[146,208,209] However, although it provides valuable information, this method has drawbacks, including inability to positively identify and detect metabolites or verify identities of peaks in chromatograms obtained from analysis of complex biological extracts (a serious limitation). In addition, it is not usually selective enough to discriminate between TIQs and various other constituents of physiological samples.[210] This problem is being addressed by advances in LC-ED analysis, such as use of chemically modified electrodes (CMEs). The fabrication and application of a novel ED system with functionalized multiwall carbon CME nanotubes for detecting (*R*)-SAL and (*R*)-*N*-Me-SAL in PD patients' CSF, which conveniently enhance sensitivity and selectivity, has been reported.[146]

In addition to ED, other types of detection systems have also been combined with LC or GC for detecting TIQs in mammals, including MS and FD (Table S1). Along with popularization of modern MS technology, LC-MS has been increasingly used for quantitative analysis of TIQs in bodily fluids and tissue matrices in recent years (Figure 4). It has clearly been the most commonly used technique in recent studies, and it may become a viable alternative to GC-MS for TIQ analysis in the coming years. Chromatographic systems coupled with tandem MS (MS/MS) have become commonly used for this purpose, including in 13.85% of referenced studies (Figure 3). The main types of ionization used in MS detection of TIQs have been electrospray ionization[60,158,159,173] and atmospheric pressure photoionization.[157] The most commonly used mass analyzers have been single quadrupole,[160,177−183] triple quadrupole,[155,158,159,170,173,187] and ion-trap MS[60,157] analyzers. MS detectors can separate the precursor ions formed. Single quadrupole instruments are operated in selected ion monitoring mode, in which only ions with a selected m/z value are detected. In triple quadrupole instruments precursor ions are selected in the first quadrupole and allowed to pass into a collision chamber for collision-induced dissociation fragmentation into product ions. Fragmentation products pass into the third quadrupole for MS/MS detection in multiple reaction monitoring mode. Use of MS/MS detection affords the greatest sensitivity and specificity; thus it is particularly important in investigations of biomarkers present in samples in pg or low ng levels. Increasing numbers of studies have focused on monitoring several TIQs in mammalian fluids and tissues, with a tendency to use LC and MS detection (Figure 4). Derivatization-free LC-MS methods have advantages of simple sample-processing, favorable recoveries, short running times, high selectivity and sensitivity, and possibilities of structural elucidation. However, when using electrospray ionization, matrix effects might pose significant challenges that affect the quantitative accuracy of TIQs' determination in complex biological matrices by liquid chromatography−tandem mass spectrometry (LC-MS/MS).[211] In such cases, SIL-ISs can provide significant benefits for the correction of signal deviations, but as yet stable isotope dilution methodology has only been applied in determinations of single TIQs (Table S1).

Despite significant matrix effects on MS signals, expensive instrumentation, and requirements for high technical personnel training, the use of MS detectors in TIQ determination is a valuable and confirmatory technique. Several applications of LC-MS and LC-MS/MS have been developed for single-TIQ analysis.[154,155,158,170,173] LC-MS methodology also offers possibilities for simultaneously detecting multiple TIQs in a single run.[60,157,159,194] Accordingly, it is being increasingly used for the simultaneous determination of both catechol and non-catechol TIQs. As many as nine TIQs can be detected in brain tissue in a single high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) run,[60] which

https://doi.org/10.1021/acschemneuro.2c00516
ACS Chem. Neurosci. 2022, 13, 3230−3246

would certainly be extremely difficult using LC with FD or ED detection systems.

Further, high-performance liquid chromatography with fluorescence detection (HPLC-FD) methods have been proposed for the single determination, in mammalian bodily fluids and tissues, of SAL,[171,204] TIQ,[156] 1-Me-TIQ,[156] 1-Bn-TIQ,[156] nor-SAL,[164] and DMDHIQ$^+$.[41,193] FD provides high sensitivity with convenience, and it is less expensive than other methods (e.g., MS). However, a derivatization is typically required for determination and sample preparation, so this technique is time-consuming. Generally, the precolumn derivatization process involves a complex and time-consuming concentration procedure that cannot be performed online. Moreover, due to the complex chemical constitutions of bodily fluids and tissues, interference from the biological matrix may change analytes' retention times, resulting in incorrect identification of substances. Therefore, the results require further confirmation using more reliable detectors, such as MS systems. Of all the TIQ analytical platforms, LC-FD was used less frequently, in just 9.23% of the reviewed studies.[41,156,164,171,193,204] It has been used both with underivatized samples and with precolumn derivatization protocols for TIQ determinations. When the samples were derivatized before the detection procedure, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride and $NaIO_4$ were the commonly used derivatization regents.[156,164]

5. CONCLUSIONS

Partly because other neurodegenerative diseases may mimic early stages of idiopathic PD, there are urgent needs for earlier diagnostic markers and techniques for differentiating PD from other syndromes. Reliable biomarkers would also improve understanding of PD pathogenesis, assessment of disease progression, and development of potent therapies. Thus, identifying reliable PD biomarkers is essential.

This critical overview covers available analytical methods for determining TIQs, as potential PD biomarkers that are putatively involved in the multifactorial etiology of the disease. We discuss limitations of current techniques and highlight trends in development of methods for determining TIQs. We also cover traditional and current extraction and derivatization procedures, as well as the benefits and drawbacks of separation techniques (GC, LC) and the most widely used detectors (ED, FD, MS). We also show that ultra-high-performance liquid chromatography−mass spectrometry (UHPLC-MS) with a simplified sample-treatment procedure should be employed as a strong alternative to conventional high-performance liquid chromatography−mass spectrometry (HPLC-MS) or GC-MS with derivatization steps. Generally, chromatographic methods have been commonly used for the analysis of TIQs and their metabolites in diverse mammalian matrices (urine, plasma, CSF, brain tissue). The LODs have been quite diverse due to variations in the efficiency of extraction/preconcentration techniques, recoveries from chromatography columns, and the sensitivity of the detectors used. Notably, in most of the reviewed studies single TIQs were determined, but multiple TIQs are being simultaneously detected in single runs increasingly often to save time and reduce costs. LC coupled with MS detection has proven to be a particularly useful analytical technique for multi-TIQ biomonitoring. Moreover, the small sample requirements enhance the suitability of MS for analyzing TIQs in complex biological matrices. Advances in MS have also greatly reduced the complications associated with selectivity and sensitivity for target analytes. Further, the use of appropriate SIL-ISs in stable isotope dilution assays can greatly enhance the robustness and accuracy of LC-MS analyses of TIQs. Without capacities for rigorous bioanalytical validation of TIQ assays, subsequent costly clinical studies will not have adequate sensitivity and specificity to distinguish different PD states. Therefore, strenuous efforts are required to develop, optimize, and rigorously validate stable isotope dilution methodologies for quantitative determinations of TIQs.

In the future, metabolomic profiling, based mainly on high-resolution MS, for more numerous groups of TIQ derivatives, should be considered. These analytical techniques seem certain to become more widely applied, and potent, in coming years. The determination of unknown TIQ derivatives, and their distributions, is crucial to gain more detailed insights into endogenous TIQ metabolites and their roles in mammals and other organisms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.2c00516.

Table S1 listing overview of analytical platforms for the analysis of TIQs in human urine, plasma, CSF, and brain tissue samples; Table S2 listing control and parkinsonian levels of TIQs in indicated species and localizations (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Michal Kaleta − *Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacký University, 783 71 Olomouc, Czech Republic; Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, 779 00 Olomouc, Czech Republic;* orcid.org/0000-0003-0875-2912; Email: michal.kaleta@upol.cz

Authors

Eva Hényková − *Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacký University, 783 71 Olomouc, Czech Republic; Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, 779 00 Olomouc, Czech Republic;* orcid.org/0000-0003-4033-7786

Kateřina Klíčová − *Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, 779 00 Olomouc, Czech Republic;* orcid.org/0000-0002-7528-4146

Gabriel Gonzalez − *Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, 779 00 Olomouc, Czech Republic; Department of Experimental Biology, Faculty of Science, Palacky University Olomouc, 783 71 Olomouc, Czech Republic*

Ondřej Novák − *Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacký University, 783 71 Olomouc, Czech Republic;* orcid.org/0000-0003-3452-0154

https://doi.org/10.1021/acschemneuro.2c00516
ACS Chem. Neurosci. 2022, 13, 3230−3246

Miroslav Strnad − *Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacký University, 783 71 Olomouc, Czech Republic; Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, 779 00 Olomouc, Czech Republic;* orcid.org/0000-0002-2806-794X

Petr Kaňovský − *Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, 779 00 Olomouc, Czech Republic*

Complete contact information is available at: https://pubs.acs.org/10.1021/acschemneuro.2c00516

Author Contributions

[‖]E.H. and M.K. contributed equally to this paper. E.H., M.K., K.K., and G.G. were involved in writing this manuscript. M.S., P.K., and O.N. reviewed and edited the structure of the article. All authors have read and agreed to the published version of the manuscript.

Funding

This work was financially supported by the ENOCH project "Molecular, Cellular and Clinical Approach to Healthy Aging", with registration number CZ.02.1.01/0.0/0.0/16_019/0000868, by the student project IGA UPOL_PřF_2022_016 of the Palacky University in Olomouc, and by the Czech Science Foundation Grant 20-15621S.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Lucie Tučková, M.D., for reviewing the medical terminology and Ota Blahoušek for graphic editing of the figures.

■ ABBREVIATIONS

(*R*)-*N*-Me-SAL, (1*R*)-1,2-dimethyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol; (*R*)-SAL, (1*R*)-1-methyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol, (*R*)-salsolinol; (*S*)-*N*-Me-SAL, (1*S*)-1,2-dimethyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol; (*S*)-SAL, (1*S*)-1-methyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol, (*S*)-salsolinol; 1-Bn-TIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline; 1-Me-TIQ, 1-methyl-1,2,3,4-tetrahydroisoquinoline; 3′,4′-DHBnTIQ, 1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline; 6,7-DHBnTIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol; 6-OHDA, 6-hydroxydopamine; ADTIQ, 1-acetyl-6,7-dihydroxyl-1,2,3,4-tetrahydroisoquinoline; BBB, blood−brain barrier; Bn-TIQ, benzyl-tetrahydroisoquinoline; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; CME, chemically modified electrode; CSF, cerebrospinal fluid; DA, dopamine; DA-*o*-quinone, dopamine-*o*-quinone; DAQ, dopamine quinone; $DMDHIQ^+$, 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion; DOPAC, dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPET, 3,4-dihydroxyphenylethanol; DTFMB-TMS, *p*-tyramine (3,5-bis(trifluoromethyl)-*N*-[2-(4-trimethylsilyloxyphenyl)ethyl]-benzamide); ED, electrochemical detection; FD, fluorescence detection; GC, gas chromatography; HFBA, heptafluorobutyric anhydride; HPLC, high-performance liquid chromatography; IS, internal standard; LC, liquid chromatography; LLE, liquid−liquid extraction; LOD, limit of detection; MAO-B, monoamine oxidase B; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS(/MS), (tandem) mass spectrometry; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoracetamide; *N*-Me-nor-SAL, 2-methyl-3,4-dihydro-1*H*-isoquinoline-6,7-diol; *N*-Me-TIQ, 2-methyl-1,2,3,4-tetrahydroisoquinoline, 2-methyl-3,4-dihydro-1*H*-isoquinoline; $NMIQ^+$, *N*-methylisoquinolinium ion; nor-SAL, 1,2,3,4-tetrahydroisoquinoline-6,7-diol; PBA, phenylboronic acid; PD, Parkinson's disease; PEA, 2-phenylethylamine; PFB-Cl, pentafluorobenzoyl chloride; PFPA, pentafluoropropionyl anhydride; PSA, primary and secondary amine; PVDF, polyvinylidene fluoride; TLC, thin layer chromatography; REA, radioenzymatic assay; RIA, radioimmunoassay; ROS, reactive oxygen species; SIL-IS, stable isotopic-labeled internal standard; SPE, solid-phase extraction; TIQ, 1,2,3,4-tetrahydroisoquinoline; TIQ, tetrahydroisoquinoline; TMCS, trimethylchlorosilane; UHPLC, ultrahigh-performance liquid chromatography; α-SYN, α-synuclein

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Supporting Information

Quantitative Determination of Endogenous Tetrahydroisoquinolines, Potential Parkinson's Disease Biomarkers, in Mammals

Eva Hényková[ab§], Michal Kaleta[ab§*], Kateřina Klíčová[b], Gabriel Gonzalez[bc], Ondřej Novák[a], Miroslav Strnad[ab], Petr Kaňovský[b]

[a]Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacký University, Šlechtitelů 27, 783 71, Olomouc, Czech Republic

[b]Department of Neurology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, I. P. Pavlova 6, 779 00, Olomouc, Czech Republic

[c]Department of Experimental Biology, Palacky University Olomouc, Faculty of Science, Šlechtitelů 27, 78371 Olomouc, Czech Republic

[§]E.H. and M.K. contributed equally to this paper.

*Email: michal.kaleta@upol.cz

Table S1. Overview of analytical platforms for the analysis of TIQs in human urine, plasma, CSF and brain tissue samples.

Analytical platform	Compound	Internal standard	Extraction method/type	Method comments	LOD	*References*
GC-MS	**SAL**	d4-SAL	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	derivatization with pentafluoropropionyl anhydride (PFPA)	0.55 pmol/mL	[187]
		d2-SAL	liqiuid-liquid extraction (LLE)/ethyl acetate and ammonium hydroxide	derivatization with p-Tyramine (DTFMB-TMS)	5-20 pg/mL	[176]
		d2-SAL	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	derivatization with *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) and *R*-(-)-2-phenylbutyrylic acid	50 pg/mL resp. 30 fmol/mg protein	[177–179,186]
		d2-SAL	liqiuid-liquid extraction (LLE)/extractive pentafluorobenzoylatio n	derivatization with pentafluorobenzoyl chloride (PFB-Cl)	10 fmol/mL	[180]
		d2-SAL	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	chiral separation; derivatization with *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) and *R*-(-)-2-phenylbutyrylic acid (chloride)	0.2-0.5 ng/g wet weight	[181–183]
		d2-SAL	solid phase extraction (SPE)/ Al_2O_3 columns	derivatization with pentafluoropropionic anhydride (PFPA)	0.4 pmol/inj.	[184]
	TIQ	dibenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[160]
		N-Me-TIQ	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with pentafluoropropionic anhydride (PFPA)	not specified	[78]
		d3-TIQ and d4-TIQ	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[188,189]
		dibenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[55,64,161,162]
		d4-2-phenylethylam ine	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[195,196]
	1-Me-TIQ	dibenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[160]
		not specified	liqiuid-liquid extraction (LLE)/dichloromethane	chiral separation; derivatization with heptafluorobutyric anhydride (HFBA) and perfluoro-2-propoxypropionylchloride	5 pg/inj.	[59]

		dibenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[55,64,161,162]
		N-Me-TIQ	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with pentafluoropropionic anhydride (PFPA)	not specified	[78]
	_N_-Me-TIQ	*N*-Me-TIQ	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with pentafluoropropionic anhydride (PFPA)	not specified	[78]
		not specified	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with pentafluoropropionic anhydride (PFPA)	not specified	[188,189]
	3',4'-DHBnTIQ	not specified	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[42]
	6,7-DHBnTIQ	not specified	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[42]
	1-Bn-TIQ	dibenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	derivatization with heptafluorobutyric anhydride (HFBA)	not specified	[46]
	nor-SAL	d2-norSAL	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	chiral separation; derivatization with *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) and *R*-(2)-2-phenylbutyrylic acid (chloride)	0.2-0.5 ng/g wet weight	[181–183]
	***N*-Me-nor-SAL**	not specified	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS)	not specified	[198]
	***N*-Me-SAL**	not specified	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS)	not specified	[198]
GC-ED	**SAL**	not specified	solid phase extraction (SPE)/cation-exchange resin (AG SOW-X4) cartridge	derivatization with heptafluorobutyric anhydride (HFBA)	2.5 ng/brain sample	[203]
LC-ED	**SAL**	not specified	filtration/celluloid 0.2 µm filters	reverse-phase HPLC	10 pmol/mL	[144]
		not specified	solid phase extraction (SPE)/primary and secondary amine (PSA) and a phenylboronic acid (PBA) cartridge	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	0.02 ng/mL	[199–201]

	not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	0.047-0.079 pmol/inj.	[191]
	N-Me-norSAL	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.047-0.073 pmol/inj.	[66,163]
	3,4-dihydroxynorephedrine	solid phase extraction (SPE)/cation-exchange resin (Biorex 70)	reverse-phase HPLC	1 ng/mL	[165]
	not specified	filtration/Millipore HV filter (pore size 0.45 µm)	reverse-phase HPLC	not specified	[192]
	vanillic acid	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	reverse-phase HPLC	5-10 pmol/mL	[50,71,166,167]
	3,4-dihydroxybenzylamin	solid phase extraction (SPE)/Al_2O_3 columns	reverse-phase HPLC	0.2 ng/g wet weight	[168]
	3,4-dihydroxybenzylamin	solid phase xtraction (SPE)/cation-exchange resin (Biorex 70)	ion-pair reverse-phase HPLC	6 nmol/L	[169]
	isoproterenol	solid phase extraction (SPE)/weakly acidic cation exchange resin (Amberlite CG-50 type II)	reverse-phase HPLC	40 fmol/inj.	[172]
	not specified	none/active reverse microdialysis membrane (rat brain)	reverse-phase HPLC	not specified	[210]
	not specified	microdialysis sampling/CMA 12 microdialysis probe	reverse-phase HPLC	0.25 nmol/L	[145,146]
	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.0629 ng/mL (R)-SAL; 0.0634 ng/mL (S)-SAL	[60]
	not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	0.01 nmol/L	[41,193]
nor-SAL	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/Al_2O_3 columns	reverse-phase HPLC	0.2 ng/g wet weight	[168]
	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.0549 ng/mL	[60]

	***N*-Me-nor-SAL**	3,4-dihydroxybenzylamine	filtration/ Millipore polyvinylidene fluoride (PVDF) syringe-driven membrane filters (pore size 0.22 µm)	reverse-phase HPLC; determination of the cellular localization with monoclonal antibody	20 pg/inj. (standard); 28 pg/inj. (tissue)	[147]
		vanillic acid	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	reverse-phase HPLC	10 pmol/mL	[50,71,166,167]
	***N*-Me-SAL**	not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.047-0.079 pmol/inj.	[66,163]
		not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	0.047-0.079 pmol/inj.	[191]
		not specified	microdialysis sampling/CMA 12 microdialysis probe	reverse-phase HPLC	0.25-0.50 nmol/L	[145,146]
		3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.0643 ng/mL (R)-N-MeSAL; 0.0651 ng/mL (S)-N-MeSAL	[60]
		not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	50 fmol/inj.	[41,193]
	TIQ	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.0288 ng/mL	[60]
	1-Me-TIQ	3,4-ihydroxybenzylamine)	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.0254 ng/mL	[60]
	***N*-Me-TIQ**	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.0294 ng/mL	[60]
	1-Bn-TIQ	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	HPLC chiral separation with β-cyclodextrin as a chiral mobile phase additive	0.0219 ng/mL	[60]
	ADTIQ	not specified	centrifugation/22 000 g for 10 min	reverse-phase HPLC	not specified	[148]
	DMDHIQ$^+$	not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	500 fmol/inj.	[41,193]
LC-MS	**SAL**	d4-SAL	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	chiral separation; derivatization with pentafluorobenzyl bromide (PFBBr)	10 pg (LOQ)	[158]
		not specified	filtration/0.22 µm cellulose membranes	reverse-phase HPLC	0.5 µg/L	[154,194]

		3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase 96-well plates (Varian C18)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	2.5 pg/µL	[155]
		3, 4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Varian C18)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	0.05 ng/mL	[170]
		3,4-dihydroxybenzylamine	solid phase extraction (SPE)/reverse phase cartridge (Waters C18)	reverse-phase HPLC	0.03 ng	[157]
		isoproterenol	filtration/Millipore HV filter (pore size 0.22 µm)	reverse-phase HPLC	0.98 nmol/L	[159]
		not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.70 ng/mL (LOQ)	[60]
	***N*-Me-SAL**	isoproterenol	filtration/Millipore HV filter (pore size 0.22 µm)	reverse-phase HPLC	0.98 nmol/L	[159]
		not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.70 ng/mL (LOQ)	[60]
	ADTIQ	6-methylquinoxaline	filtration/not specified filter (pore size 0.22 µm)	reverse-phase HPLC	not specified	[173]
	TIQ	not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.30 ng/mL (LOQ)	[60]
	***N*-Me-TIQ**	not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.30 ng/mL (LOQ)	[60]
	1-Me-TIQ	not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.30 ng/mL (LOQ)	[60]
	1-Bn-TIQ	not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.30 ng/mL (LOQ)	[60]
	nor-SAL	not specified	solid phase extraction (SPE)/reverse phase cartridge (Waters Oasis HLB)	reverse-phase HPLC	0.70 ng/mL (LOQ)	[60]
LC-FD	**SAL**	3,4-dihydroxybenzylamine	solid phase extraction (SPE)/Al_2O_3 sorbent	reverse-phase HPLC; without derivatization	20 pmol/mL	[171]
		not specified	solid phase extraction (SPE)/weakly acidic cation exchange resin (Amberlite CG-50)	reverse-phase HPLC	2 pmol/mL	[204]

TIQ	*N*-ethylbenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	reverse-phase HPLC; photochemical derivatization with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride	8-9 fmol/inj.	[156]
1-Me-TIQ	*N*-ethylbenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	reverse-phase HPLC; photochemical derivatization with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride	8-9 fmol/inj.	[156]
1-Bn-TIQ	*N*-ethylbenzylamine	liqiuid-liquid extraction (LLE)/dichloromethane	reverse-phase HPLC; photochemical derivatization with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride	8-9 fmol/inj.	[156]
nor-SAL	*N*-Me-norSAL	solid phase extraction (SPE)/Al_2O_3 sorbent	weak anion exchange HPLC; derivatization with $NaIO_4$	5 pmol/mL	[164]
DMDHIQ$^+$	not specified	filtration/Millipore HV filter (pore size 0.45 µm)	HPLC chiral separation without derivatization, using β-cyclodextrin phase column	500 fmol/inj.	[41,193]

REA-TLC	**SAL**	benzyloxyamine	catecholamine radioenzymatic kit/CAT-A-KIT	incubation with [methyl-^{3}H]5-adenosyl-L-methion	10-15 pg/g	[174,175]
RIA-TLC	**1-Me-TIQ**	not specified	solid phase extraction (SPE)/ phenylboronic acid (PBA) cartridges	incubation with [^{3}H]1MeTIQ	0.5 pmol	[202]

Table S2. Control and parkinsonian levels of TIQs in indicated species and localizations.

Compound	Racemic form determined	Species/Location	Control levels	Parkinsonian levels (if involved in study)	*References*
salsolinol (SAL)	racemic mix	human/urine	1-6 pmol/mL		[187]
	not specified	human/urine	3043 ± 2068 ng/24hrs (1.89 ± 0.85 ng/mL)		[165]
	racemic mix	human/urine	14.9 ± 17.2 µg/24hrs		[171]
	(*R*) and (*S*)	human/urine	detected, not quantified		[177]
	not specified	human/urine	detected, not quantified		[180]
	(*R*) and (*S*)	human/urine	(*R*)-SAL 0.41-3.38 µg/12hrs, (*S*)-SAL 0.43-3.98 µg/12hrs		[178]
	not specified	human/urine	0.028 nmol/mL	0.303-0.848 nmol/mL	[167]
	not specified	human/urine	464 nmol/L		[169]
	not specified	human/urine	< LOD-30 pmol/mL		[204]
	not specified	human/plasma	185 ± 78 pg/mL		[176]
	(*R*) and (*S*)	human/plasma	< LOD-0.95 ng/mL		[199]
	not specified	human/plasma	SAL-sulphate 0-232 pg/mL; free SAL < LOD		[174]
	(*R*) and (*S*)	human/plasma	detected, not quantified		[177]
	(*R*) and (*S*)	human/plasma	(*R*)-SAL 0.06-0.75 ng/mL, (*S*)-SAL 0.05-0.55 ng/mL		[178]
	(*R*) and (*S*)	human/plasma	(*R*)-SAL 173 ± 220 pg/mL; (*S*)-SAL 136 ± 167 pg/mL		[158]
	(*R*) and (*S*)	human/plasma	(*R*)-SAL 0.24 ± 0.07 ng/mL, (*S*)-SAL 0.08 ng/mL (only in 1 of 20 samples)		[200]
	racemic mix	human/plasma, peripheral mononuclear cells	1.25 ± 0.32 ng/1.10^6 cells; plasma 2.6 nmol/L		[185]
	racemic mix	human/lymphocytes	0.58-1.00 ng/1.10^6 cells		[179]
	not specified	human/CSF	118 ± 10 ng/mL	215 ± 26 ng/mL (early PD), 175 ± 55 ng/mL (advanced PD)	[144]
	not specified	human/CSF	SAL-sulphate 405 ± 477 pg/mL; free SAL 43 ± 29 pg/mL		[175]
	(*R*) and (*S*)	human/CSF	(*R*)-SAL 115 ± 128 pg/mL; (*S*)-SAL 92 ± 109 pg/mL		[158]
	not specified	human/CSF	< LOD	< LOD - 28 pmol/mL	[50]
	(*R*) and (*S*)	human/CSF	(*R*)-SAL 166.8 ± 149.4 pg/mL; (*S*)-SAL 177.5 ± 118.4 pg/mL	(*R*)-SAL 150.0 ± 92.8 pg/mL; (*S*)-SAL 207.6 ± 157.6 pg/mL	[201]
	(*R*)	human/CSF	4.91 ± 1.57 nmol/L	4.52 ± 1.61 nmol/L	[145]
	(*R*)	human/CSF	4.83 ± 1.53 nmol/L	4.49 ± 1.57nmol/L	[146]
	(*R*) and (*S*)	human/brain, substantia nigra	(*R*)-SAL 204.79 ± 21.91; (*S*)-SAL 213.19 ± 25.83 ng/g wet tissue	(*R*)-SAL 221.32 ± 15.90; (*S*)-SAL 210.88 ± 17.73 ng/g wet tissue	[60]
	(*R*) and (*S*)	human/brain, substantia nigra	(*R*)-SAL 94.5 ± 78.7 pmol/g; (*S*)-SAL < LOD		[193]
	racemic mix	human/brain, substantia nigra	5.1 ± 3.7 ng/g		[168]
	(*R*) and (*S*)	human/brain, substantia nigra	(*R*)-SAL 28.6 ±18.3; (*S*)-SAL 18.5 ± 14.1 ng/g		[181]
	(*R*)	human/brain, substantia nigra	94.5 ± 78.8 pmol/g		[41]
	(*R*) and (*S*)	human/brain, nucleus caudatus	(*R*)-SAL 393.40 ± 10.95; (*S*)-SAL 394.38 ± 15.51 ng/g wet tissue	(*R*)-SAL 255.79 ± 18.66; (*S*)-SAL 249.69 ± 17.34 ng/g wet tissue	[60]
	(*R*) and (*S*)	human/brain, nucleus caudatus	(*R*)-SAL 73.3 ± 79.9 pmol/g; (*S*)-SAL < LOD		[193]
	racemic mix	human/brain, nucleus caudatus	38.5 ± 35.3 ng/g		[168]

(*R*) and (*S*)	human/brain, nucleus caudatus	(*R*)-SAL 12.5 ± 8.9; (*S*)-SAL 7.9 ± 6.3 ng/g		181
(*R*) and (*S*)	human/brain, nucleus caudatus	(*R*)-SAL 19.8 ± 17.6; (*S*)-SAL 5.5 ± 4.1 ng/g		182
(*R*) and (*S*)	human/brain, nucleus caudatus	(*R*)-SAL 21.2 ± 20.7; (*S*)-SAL 5.2 ± 3.9 ng/g		183
(*R*)	human/brain, nucleus caudatus	73.3 ± 79.9 pmol/g		41
not specified	human/brain, nucleus caudatus	218 ± 95 pmol/g wet tissue		172
(*R*) and (*S*)	human/brain, putamen	(*R*)-SAL 593.68 ± 32.38; (*S*)-SAL 591.87 ± 32.04 ng/g wet tissue	(*R*)-SAL 379.44 ± 23.74; (*S*)-SAL 347.65 ± 36.91 ng/g wet tissue	60
(*R*) and (*S*)	human/brain, putamen	(*R*)-SAL 37.8 ± 23.0 pmol/g; (*S*)-SAL < LOD		193
racemic mix	human/brain, putamen	35.1 ± 30.7 ng/g		168
(*R*) and (*S*)	human/brain, putamen	(*R*)-SAL 24.1 ± 20.9; (*S*)-SAL 14.2 ± 12.0 ng/g		181
(*R*) and (*S*)	human/brain, putamen	(*R*)-SAL 21.0 ± 20.8; (*S*)-SAL 5.1 ± 5.0 ng/g		182
(*R*) and (*S*)	human/brain, putamen	(*R*)-SAL 24.8 ± 24.9; (*S*)-SAL 5.1 ± 5.7 ng/g		183
(*R*)	human/brain, putamen	37.8 ± 23.0 pmol/g		41
not specified	human/brain, putamen	207 ± 86 pmol/g		172
(*R*) and (*S*)	human/brain, cerebral gray matter	detected, not quantified		191
(*R*) and (*S*)	human/brain, cerebral gray matter	detected, not quantified		163
(*R*) and (*S*)	human/brain, cerebral gray matter	(*R*)-SAL 71.4 ± 28.1 pmol/g; (*S*)-SAL < LOD		66
(*R*) and (*S*)	human/brain, cerebellar cortex	(*R*)-SAL 252.46 ± 12.51; (*S*)-SAL 184.88 ± 0.90 ng/g wet tissue	(*R*)-SAL 157.36 ± 19.19; (*S*)-SAL 99.68 ± 17.39 ng/g wet tissue	60
(*R*) and (*S*)	human/brain, locus coeruleus	(*R*)-SAL 168.49 ± 31.91; (*S*)-SAL 177.29 ± 34.83 ng/g wet tissue	(*R*)-SAL 340.12 ± 25.70; (*S*)-SAL 319.78 ± 27.25 ng/g wet tissue	60
(*R*) and (*S*)	human/brain, frontal cortex	(*R*)-SAL 52.63 ± 1.28; (*S*)-SAL 10.84 ± 6.41 ng/g wet tissue	(*R*)-SAL 60.36 ± 3.07; (*S*)-SAL 14.98 ± 6.54 ng/g wet tissue	60
(*R*) and (*S*)	human/brain, frontal cortex	(*R*)-SAL 134 ± 125 pmol/g; (*S*)-SAL < LOD		193
(*R*)	human/brain, frontal lobes	13.4 ± 12.5 pmol/g		41
(*R*) and (*S*)	human/brain, hippocampus	(*R*)-SAL 52.54 ± 12.54; (*S*)-SAL 20.19 ± 7.92 ng/g wet tissue	(*R*)-SAL 182.73 ± 12.73; (*S*)-SAL 194.86 ± 19.49 ng/g wet tissue	60
(*R*) and (*S*)	human/brain, inferior olive	(*R*)-SAL 23.78 ± 2.10; (*S*)-SAL 24.05 ± 4.58 ng/g wet tissue	(*R*)-SAL 14.61 ± 5.64; (*S*)-SAL 8.27 ± 3.37 ng/g wet tissue	60
racemic mix	human/brain, nucleus accumbens	43.1 ± 33.7 ng/g		168
(*R*) and (*S*)	human/brain, nucleus accumbens	(*R*)-SAL 16.5 ± 6.3; (*S*)-SAL 7.8 ± 6.1 ng/g		181
(*R*) and (*S*)	human/brain, nucleus accumbens	(*R*)-SAL 13.2 ± 14.9; (*S*)-SAL 3.6 ± 4.7 ng/g		182
(*R*) and (*S*)	human/brain, nucleus accumbens	(*R*)-SAL 15.1 ± 16.6; (*S*)-SAL 3.3 ± 3.6 ng/g		183
(*R*) and (*S*)	human/brain, hypothalamus	(*R*)-SAL 11.9 ± 9.8; (*S*)-SAL 12.8 ± 10.8 ng/g		181
(*R*) and (*S*)	human/brain, hypothalamus	(*R*)-SAL 1.3 ± 2.3; (*S*)-SAL 3.0 ± 4.2 ng/g		182
(*R*) and (*S*)	human/brain, hypothalamus	(*R*)-SAL 1.6 ± 2.7; (*S*)-SAL 2.9 ± 3.9 ng/g		183
racemic mix	human/brain, hypothalamus	0.5 ± 1.1 ng/g		168
(*R*) and (*S*)	human/brain, ventral tegmental area	(*R*)-SAL 3.8 ± 5.0; (*S*)-SAL 1.3 ± 2.1 ng/g		182
(*R*) and (*S*)	human/brain, ventral tegmental area	(*R*)-SAL 3.7 ± 5.0; (*S*)-SAL 0.9 ± 1.6 ng/g		183

	not specified	beef-cattle/posterior pituitary (PP) gland	20 ng/PP extract		[192]
	(*R*) and (*S*)	rat/whole brain	(*R*)-SAL 1.2-4.2; (*S*)-SAL 0.2-1.1 pg/mg tissue		[155]
	not specified	rat/whole brain	0.85-1.03 nmol/g		[184]
	not specified	rat/whole brain	0.06 ± 0.03 ng/mg		[157]
	not specified	rat/whole brain	6.94-8.33 ng/g of protein		[159]
	(*R*) and (*S*)	rat/brain, frontal cortex	(*R*)-SAL 15.08 ± 4.55; (*S*)-SAL 4.18 ± 1.29 ng/g wet tissue		[60]
	(*R*) and (*S*)	rat/brain, striatum	(*R*)-SAL 28.00 ± 3.27; (*S*)-SAL 24.50 ± 1.79 ng/g wet tissue		[60]
	(*R*) and (*S*)	rat/brain, ventral midbrain	(*R*)-SAL 211.02 ± 13.76; (*S*)-SAL 170.89 ± 18.89 ng/g wet tissue		[60]
	(*R*) and (*S*)	rat/brain, hippocampus	(*R*)-SAL 12.10 ± 2.50; (*S*)-SAL 6.38 ± 1.70 ng/g wet tissue		[60]
	(*R*) and (*S*)	rat/brain, cerebellum	(*R*)-SAL 12.26 ± 2.53; (*S*)-SAL 7.28 ± 2.07 ng/g wet tissue		[60]
	not specified	rat/whole neonatal brain	1.68 ng/g		[154]
	not specified	rat/whole neonatal brain	4.02 ± 0.85 ng/g		[194]
	not specified	rat/liver	1.96 ± 0.92 nmol/g		[184]
	(*R*) and (*S*)	mouse/brain, striatum	(*R*)-SAL 122.50 ± 30.33; (*S*)-SAL 106.45 ± 22.95 ng/g wet tissue		[60]
	(*R*) and (*S*)	mouse/brain, ventral midbrain	(*R*)-SAL 148.82 ± 11.73; (*S*)-SAL 80.25 ± 22.27 ng/g wet tissue		[60]
	(*R*) and (*S*)	mouse/brain, frontal cortex	(*R*)-SAL 23.48 ± 1.40; (*S*)-SAL 17.58 ± 6.22 ng/g wet tissue		[60]
	(*R*) and (*S*)	mouse/brain, hippocampus	(*R*)-SAL 34.20 ± 1.55; (*S*)-SAL 19.30 ± 2.51 ng/g wet tissue		[60]
	(*R*) and (*S*)	mouse/brain, cerebellum	(*R*)-SAL 16.84 ± 1.20; (*S*)-SAL 9.65 ± 1.85 ng/g wet tissue		[60]
TIQ		human/brain, substantia nigra	4.48 ± 0.91 ng/g wet tissue	3.97 ± 0.92 ng/g wet tissue	[60]
		human/brain, nucleus caudatus	2.84 ± 0.82 ng/g wet tissue	3.81 ± 1.10 ng/g wet tissue	[60]
		human/brain, nucleus caudatus	0.64 ± 0.24 ng/g	0.25 ± 0.08 ng/g	[161]
		human/brain, putamen	3.49 ± 0.97 ng/g wet tissue	4.45 ± 1.31 ng/g wet tissue	[60]
		human/brain, locus coeruleus	5.61 ± 1.81 ng/g wet tissue	4.92 ± 1.05 ng/g wet tissue	[60]
		human/brain, frontal cortex	4.61 ± 1.03 ng/g wet tissue	5.22 ± 1.37 ng/g wet tissue	[60]
		human/brain, frontal cortex	1 ng/g		[188]
		human/brain, frontal lobe	0.86 ± 0.23 ng/g	0.58 ± 0.20 ng/g	[161]
		human/brain, hippocampus	2.84 ± 0.53 ng/g wet tissue	3.19 ± 1.05 ng/g wet tissue	[60]
		human/brain, inferior olive	1.95 ± 0.26 ng/g wet tissue	2.31 ± 0.80 ng/g wet tissue	[60]
		human/brain, cerebellar cortex	3.28 ± 0.94 ng/g wet tissue	4.17 ± 0.94 ng/g wet tissue	[60]
		human/brain, occipital cortex		7ng/g	[195]
		monkey/substantia nigra	140 pmol/g		[162]
		monkey/striatum	20 pmol/g		[162]
		monkey/cerebrum	80 pmol/g		[162]
		monkey/cerebellum	10 pmol/g		[162]
		monkey/medulla	30 pmol/g		[162]

	monkey/thalamus	25 pmol/g		162
	monkey/whole brain	0.149 -0 .193 µg/g wet tissue		189
	squirrel monkey/whole brain	0.153 ± 0.041 µg/g		196
	rat/brain, substantia nigra	~ 1.7 (young rats), ~ 1.3 (old rats) ng/mg of tissue		160
	rat/brain, striatum	~ 0.6 (young rats), ~ 1.0 (old rats) ng/mg of tissue		160
	rat/brain, striatum	4.64 ± 1.19 ng/g wet tissue		60
	rat/brain, ventral midbrain	5.43 ± 1.14 ng/g wet tissue		60
	rat/brain, frontal cortex	7.32 ± 1.91 ng/g wet tissue		60
	rat/brain, hippocampus	6.75 ± 1.40 ng/g wet tissue		60
	rat/brain, cerebellum	3.03 ± 0.90 ng/g wet tissue		60
	rat/whole brain	0.7 ± 0.3 pmol/g		156
	rat/whole brain	5-7 ng/g		78
	rat/whole brain	4.2 ng/g		64
	mouse/brain, striatum	5.58 ± 1.2 ng/g wet tissue		60
	mouse/brain, ventral midbrain	7.18 ± 1.22 ng/g wet tissue		60
	mouse/brain, frontal cortex	10.19 ± 1.57 ng/g wet tissue		60
	mouse/brain, hippocampus	6.04 ± 1.89 ng/g wet tissue		60
	mouse/brain, cerebellum	4.12 ± 0.32 ng/g wet tissue		60
	mouse/whole brain	1.1 ng/g		64
	mouse/whole brain	approx. 1 ng/g		55
1-Me-TIQ	human/brain, substantia nigra	1.63 ± 0.17 ng/g wet tissue	2.05 ± 0.88 ng/g wet tissue	60
	human/brain, nucleus caudatus	1.33 ± 0.77 ng/g wet tissue	2.00 ± 0.21 ng/g wet tissue	60
	human/brain, nucleus caudatus	0.52 ± 0.15 ng/g	0.12 ± 0.03 ng/g	161
	human/brain, putamen	2.12 ± 0.68 ng/g wet tissue	1.62 ± 0.81 ng/g wet tissue	60
	human/brain, locus coeruleus	2.03 ± 0.25 ng/g wet tissue	1.86 ± 0.61 ng/g wet tissue	60
	human/brain, frontal cortex	1.24 ± 0.64 ng/g wet tissue	0.77 ± 0.24 ng/g wet tissue	60
	human/brain, frontal lobe	0.75 ± 0.25 ng/g	0.05 ± 0.02 ng/g	161
	human/brain, hippocampus	0.44 ± 0.06 ng/g wet tissue	0.72 ± 0.20 ng/g wet tissue	60
	human/brain, inferior olive	0.53 ± 0.18 ng/g wet tissue	0.79 ± 0.21 ng/g wet tissue	60
	human/brain, cerebellar cortex	1.76 ± 0.59 ng/g wet tissue	1.47 ± 0.24 ng/g wet tissue	60
	monkey/substantia nigra	475 pmol/g		162
	monkey/striatum	300 pmol/g		162
	monkey/cerebrum	160 pmol/g		162
	monkey/cerebellum	90 pmol/g		162
	monkey/medulla	70 pmol/g		162
	monkey/thalamus	65 pmol/g		162
	rat/brain, substantia nigra	~ 1.3 (young rats), ~ 0.6 (old rats) ng/mg of tissue		160
	rat/brain, striatum	~ 0.4(young rats), ~ 0.5 (old rats) ng/mg of tissue		160
	rat/brain, striatum	1.06 ± 0.30 ng/g wet tissue		60

	rat/brain, ventral midbrain	1.13 ± 0.57 ng/g wet tissue		60
	rat/brain, frontal cortex	3.20 ± 0.93 ng/g wet tissue		60
	rat/brain, hippocampus	2.32 ± 0.90 ng/g wet tissue		60
	rat/brain, cerebellum	0.79 ± 0.21 ng/g wet tissue		60
	rat/whole brain	3.4 ± 1.5 pmol/g		156
	rat/whole brain	15.95 + 6.35 ng/g brain		202
	rat/whole brain	1.6-2.6 ng/g		78
	rat/whole brain	1.7 ng/g		64
	mouse/brain, striatum	2.00 ± 0.76 ng/g wet tissue		60
	mouse/brain, ventral midbrain	2.52 ± 0.43 ng/g wet tissue		60
	mouse/brain, frontal cortex	1.89 ± 0.33 ng/g wet tissue		60
	mouse/brain, hippocampus	5.88 ± 1.55 ng/g wet tissue		60
	mouse/brain, cerebellum	1.24 ± 0.21 ng/g wet tissue		60
racemic mix?	mouse/whole brain	9.8 ± 1.8 ng/g wet tissue		59
	mouse/brain	~ 10ng/g		55
***N*-Me-TIQ**	human/brain, substantia nigra	2.16 ± 0.30 ng/g wet tissue	1.83 ± 0.67 ng/g wet tissue	60
	human/brain, nucleus caudatus	0.93 ± 0.25 ng/g wet tissue	1.40 ± 0.36 ng/g wet tissue	60
	human/brain, putamen	1.90 ± 0.42 ng/g wet tissue	1.34 ± 0.24 ng/g wet tissue	60
	human/brain, locus ceruleus	1.85 ± 0.40 ng/g wet tissue	1.74 ± 0.55 ng/g wet tissue	60
	human/brain, frontal cortex	1.03 ± 0.33 ng/g wet tissue	0.61 ± 0.13 ng/g wet tissue	60
	human/brain, frontal cortex	3 ng/g		188
	human/brain, hippocampus	0.69 ± 0.10 ng/g wet tissue	1.04 ± 0.15 ng/g wet tissue	60
	human/brain, inferior olive	0.60 ± 0.07 ng/g wet tissue	1.18 ± 0.44 ng/g wet tissue	60
	human/brain, cerebellar cortex	1.34 ± 0.23 ng/g wet tissue	1.63 ± 0.36 ng/g wet tissue	60
	rat/brain, striatum	0.88 ± 0.24 ng/g wet tissue		60
	rat/brain, ventral midbrain	0.82 ± 0.27ng/g wet tissue		60
	rat/brain, frontal cortex	1.70 ± 0.63 ng/g wet tissue		60
	rat/brain, hippocampus	3.46 ± 1.04 ng/g wet tissue		60
	rat/brain, cerebellum	1.92 ± 0.46 ng/g wet tissue		60
	rat/whole brain	1 - 3ng/g ng/g		78
	mouse/brain, striatum	1.22 ± 0.27 ng/g wet tissue		60
	mouse/brain, ventral midbrain	1.73 ± 0.21 ng/g wet tissue		60
	mouse/brain, frontal cortex	2.03 ± 0.71 ng/g wet tissue		60
	mouse/brain, hippocampus	1.31 ± 0.81ng/g wet tissue		60
	mouse/brain, cerebellum	0.69 ± 0.13 ng/g wet tissue		60

1-Bn-TIQ		human/CSF	0.40 - 0.10 ng/mL	1 .17 ± 0.35 ng/mL	[46]
		human/brain, substantia nigra	1.49 ± 0.42 ng/g wet tissue	0.90 ± 0.19 ng/g wet tissue	[60]
		human/brain, nucleus caudatus	1.37 ± 0.41 ng/g wet tissue	1.79 ± 0.27 ng/g wet tissue	[60]
		human/brain, putamen	1.42 ± 0.36 ng/g wet tissue	0.73 ± 0.16 ng/g wet tissue	[60]
		human/brain, locus coeruleus	2.19 ± 0.52 ng/g wet tissue	1.87 ± 0.33 ng/g wet tissue	[60]
		human/brain, frontal cortex	0.89 ± 0.24 ng/g wet tissue	0.80 ± 0.19 ng/g wet tissue	[60]
		human/brain, hippocampus	1.30 ± 0.27 ng/g wet tissue	0.70 ± 0.08 ng/g wet tissue	[60]
		human/brain, inferior olive	1.52 ± 0.44ng/g wet tissue	0.91 ± 0.31 ng/g wet tissue	[60]
		human/brain, cerebellar cortex	2.02 ± 0.63 ng/g wet tissue	1.19 ± 0.14 ng/g wet tissue	[60]
		monkey/substantia nigra	110 pmol/g		[162]
		monkey/striatum	30 pmol/g		[162]
		monkey/cerebrum	55 pmol/g		[162]
		monkey/cerebellum	15 pmol/g		[162]
		monkey/medulla	10 pmol/g		[162]
		monkey/thalamus	20 pmol/g		[162]
		rat/brain, striatum	2.19 ± 0.92 ng/g wet tissue		[60]
		rat/brain, ventral midbrain	3.39 ± 1.20 ng/g wet tissue		[60]
		rat/brain, frontal cortex	0.93 ± 0.36 ng/g wet tissue		[60]
		rat/brain, hippocampus	5.29 ± 1.44 ng/g wet tissue		[60]
		rat/brain, cerebellum	0.88 ± 0.16 ng/g wet tissue		[60]
		rat/whole brain	1.3 ± 1.8 pmol/g		[156]
		mouse/brain, striatum	4.12 ± 1.11 ng/g wet tissue		[60]
		mouse/brain, ventral midbrain	2.14 ± 0.31 ng/g wet tissue		[60]
		mouse/brain, frontal cortex	2.52 ± 0.63 ng/g wet tissue		[60]
		mouse/brain, hippocampus	4.55 ± 1.28 ng/g wet tissue		[60]
		mouse/brain, cerebellum	1.24 ± 0.27 ng/g wet tissue		[60]
		mouse/whole brain	7.67 ± 1 .60 ng/g of wet tissue		[46]

***N*-Me-SAL**	(*R*)	human/CSF	4.53 ± 2.08 nmol/L	8.32 ± 2.89 nmol/L	[41]
	(*R*)	human/CSF	5.42 ± 2.06 nmol/L	8.39 ± 1.92 nmol/L	[145]
	(*R*)	human/CSF	5.45 ± 2.13 nmol/L	8.34 ± 1.85 nmol/L	[146]
	(*R*) and (*S*)	human/brain, substantia nigra	*N*-Me-(*R*)-SAL 174.92 ± 19.42; *N*-Me-(*S*)-SAL 187.22 ± 24.54 ng/g wet tissue	*N*-Me-(*R*)-SAL 61.44 ± 6.26; *N*-Me-(*S*)-SAL 56.25 ± 5.67 ng/g wet tissue	[60]
	(*R*)	human/brain, substantia nigra	76.6 ± 23.0 pmol/g wet weight		[193]
	(*R*)	human/brain, substantia nigra	76.6 ± 23.0 pmol/g wet weight		[41]
	(*R*) and (*S*)	human/brain, nucleus caudatus	*N*-Me-(*R*)-SAL 272.06 ± 20.58; *N*-Me-(*S*)-SAL 281.92 ± 13.91 ng/g wet tissue	*N*-Me-(*R*)-SAL 90.30 ± 13.98; *N*-Me-(*S*)-SAL 83.57 ± 14.06 ng/g wet tissue	[60]
	(*R*)	human/brain, nucleus caudatus	65.7 ± 88.3 pmol/g wet weight		[193]
	(*R*)	human/brain, nucleus caudatus	66.7 ± 88.3 pmol/g wet weight		[41]
	(*R*) and (*S*)	human/brain, putamen	*N*-Me-(*R*)-SAL 383.03 ± 31.31; *N*-Me-(*S*)-SAL 376.81 ± 17.68 ng/g wet tissue	*N*-Me-(*R*)-SAL 341.20 ± 27.87; *N*-Me-(*S*)-SAL 358.01 ± 43.85 ng/g wet tissue	[60]

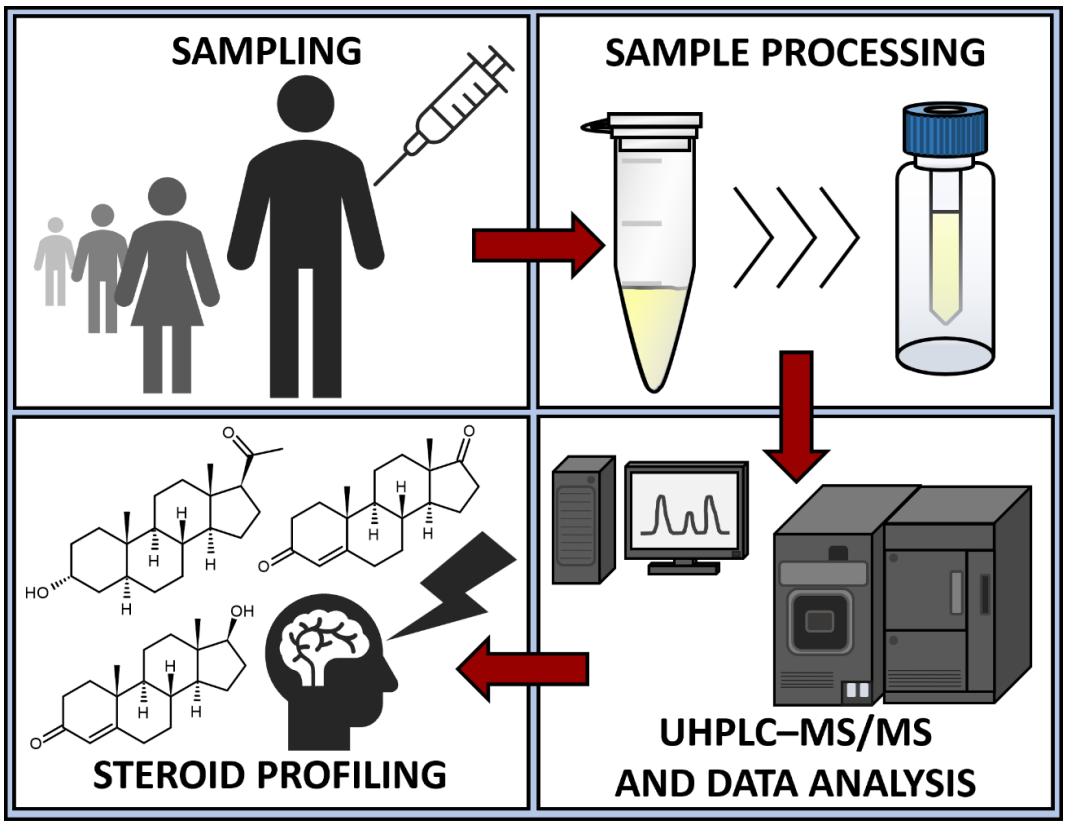
(*R*)	human/brain, putamen	110 ± 126 pmol/g wet weight		[193]
(*R*)	human/brain, putamen	110 ± 126 pmol/g wet weight		[41]
(*R*) and (*S*)	human/brain, locus coeruleus	*N*-Me-(*R*)-SAL 193.02 ± 21.32; *N*-Me-(*S*)-SAL 181.43 ± 32.54 ng/g wet tissue	*N*-Me-(*R*)-SAL 82.36 ± 13.54; *N*-Me-(*S*)-SAL 102.54 ± 18.70 ng/g wet tissue	[60]
(*R*) and (*S*)	human/brain, frontal cortex	*N*-Me-(*R*)-SAL 71.30 ± 2.36; *N*-Me-(*S*)-SAL 66.75 ± 4.77 ng/g wet tissue	*N*-Me-(*R*)-SAL 168.80 ± 15.82; *N*-Me-(*S*)-SAL 135.43 ± 22.05 ng/g wet tissue	[60]
(*R*)	human/brain, frontal cortex	< LOD		[193]
(*R*) and (*S*)	human/brain, frontal cortex	1 ng/g		[198]
(*R*)	human/brain, frontal lobes	< LOD		[41]
(*R*) and (*S*)	human/brain, hippocampus	*N*-Me-(*R*)-SAL 125.90 ± 20.50; *N*-Me-(*S*)-SAL 35.26 ± 8.39 ng/g wet tissue	*N*-Me-(*R*)-SAL 612.65 ± 27.23; *N*-Me-(*S*)-SAL 445.45 ± 40.55 ng/g wet tissue	[60]
(*R*) and (*S*)	human/brain, inferior olive	*N*-Me-(*R*)-SAL 46.36 ± 8.99; *N*-Me-(*S*)-SAL 16.77 ± 4.21 ng/g wet tissue	*N*-Me-(*R*)-SAL 56.33 ± 14.60; *N*-Me-(*S*)-SAL 31.75 ± 8.12 ng/g wet tissue	[60]
(*R*) and (*S*)	human/brain, cerebellar cortex	*N*-Me-(*R*)-SAL 60.85 ± 6.63; *N*-Me-(*S*)-SAL 11.97 ± 1.62 ng/g wet tissue	*N*-Me-(*R*)-SAL 33.45 ± 6.01; *N*-Me-(*S*)-SAL 9.11 ± 2.35 ng/g wet tissue	[60]
(*R*) and (*S*)	human/brain, cerebral gray matter	detected, not quantified		[191]
(*R*) and (*S*)	human/brain, cerebral gray matter	detected, not quantified		[163]
(*R*) and (*S*)	human/brain, cerebral gray matter	*N*-Me-(*R*)-SAL 71.4 ± 28.1 pmol/g; *N*-Me-(*S*)-SAL < LOD		[66]
(*R*) and (*S*)	rat/brain, striatum	*N*-Me-(*R*)-SAL 19.98 ± 2.60; *N*-Me-(*S*)-SAL 17.38 ± 1.45ng/g wet tissue		[60]
(*R*) and (*S*)	rat/brain, ventral midbrain	*N*-Me-(*R*)-SAL 240.90 ± 15.75; *N*-Me-(*S*)-SAL 233.50 ± 4.49 ng/g wet tissue		[60]
(*R*) and (*S*)	rat/brain, frontal cortex	*N*-Me-(*R*)-SAL 45.64 ± 6.08; *N*-Me-(*S*)-SAL 25.19 ± 3.34 ng/g wet tissue		[60]
(*R*) and (*S*)	rat/brain, hippocampus	*N*-Me-(*R*)-SAL 7.07 ± 1.10; *N*-Me-(*S*)-SAL 5.50 ± 0.74 ng/g wet tissue		[60]
(*R*) and (*S*)	rat/brain, cerebellum	*N*-Me-(*R*)-SAL 15.80 ± 2.85; *N*-Me-(*S*)-SAL 8.93 ± 2.65 ng/g wet tissue		[60]
not specified	rat/whole neonatal brain	2.63 ± 0.65 ng/g		[194]
(*R*)	rat/whole brain	61.9 - 93.1 ng/g protein		[159]
(*R*) and (*S*)	mouse/brain, striatum	*N*-Me-(*R*)-SAL 100.40 ± 8.13; *N*-Me-(*S*)-SAL 92.07 ± 6.55 ng/g wet tissue		[60]
(*R*) and (*S*)	mouse/brain, ventral midbrain	*N*-Me-(*R*)-SAL 215.58 ± 8.55; *N*-Me-(*S*)-SAL 194.84 ± 3.71 ng/g wet tissue		[60]
(*R*) and (*S*)	mouse/brain, frontal cortex	*N*-Me-(*R*)-SAL 12.66 ± 6.98; *N*-Me-(*S*)-SAL 8.32 ± 1.14 ng/g wet tissue		[60]
(*R*) and (*S*)	mouse/brain, hippocampus	*N*-Me-(*R*)-SAL 8.75 ± 1.09; *N*-Me-(*S*)-SAL 3.19 ± 0.31 ng/g wet tissue		[60]
(*R*) and (*S*)	mouse/brain, cerebellum	*N*-Me-(*R*)-SAL 17.10 ± 3.86; *N*-Me-(*S*)-SAL 3.52 ± 0.43 ng/g wet tissue		[60]
nor-SAL	human/urine	2.8 ± 1.7 nmol/g creatinine		[164]
	human/brain, substantia nigra	62.24 ± 7.55 ng/g wet tissue	54.60 ± 9.54 ng/g wet tissue	[60]
	human/brain, substantia nigra	8.1 ± 7.7 ng/g		[168]
	human/brain, substantia nigra	12.3 ± 9.2 ng/g		[181]
	human/brain, nucleus caudatus	65.97 ± 5.96 ng/g wet tissue	50.17 ± 3.01 ng/g wet tissue	[60]
	human/brain, nucleus caudatus	50.4 ± 43.7 ng/g		[168]

	human/brain, nucleus caudatus	42.1 ± 37.8 ng/g		[181]
	human/brain, nucleus caudatus	26.5 ± 50.1 ng/g		[182]
	human/brain, nucleus caudatus	40.7 ± 68.2 ng/g		[183]
	human/brain, putamen	109.59 ± 15.04 ng/g wet tissue	50.21 ± 4.09 ng/g wet tissue	[60]
	human/brain, putamen	25.1 ± 30.7 ng/g		[168]
	human/brain, putamen	22.1 ± 52.7 ng/g		[182]
	human/brain, putamen	30.1 ± 66.5 ng/g		[183]
	human/brain, putamen	40.0 ± 20.3 ng/g		[181]
	human/brain, locus coeruleus	42.24 ± 12.55 ng/g wet tissue	44.54 ± 10.74 ng/g wet tissue	[60]
	human/brain, frontal cortex	50.40 ± 16.02 ng/g wet tissue	44.07 ± 10.92 ng/g wet tissue	[60]
	human/brain, hippocampus	70.94 ± 4.21 ng/g wet tissue	68.79 ± 3.79 ng/g wet tissue	[60]
	human/brain, inferior olive	65.47 ± 7.18 ng/g wet tissue	87.45 ± 11.96 ng/g wet tissue	[60]
	human/brain, cerebellar cortex	112.62 ± 9.47 ng/g wet tissue	78.12 ± 10.11 ng/g wet tissue	[60]
	human/brain, nucleus accumbens	45.1 ± 43.9 ng/g		[168]
	human/brain, nucleus accumbens	55.5 ± 28.9 ng/g		[181]
	human/brain, nucleus accumbens	13.7 ± 38.7 ng/g		[182]
	human/brain, nucleus accumbens	20.1 ± 55.2 ng/g		[183]
	human/brain, hypothalamus	0.6 ± 0.8 ng/g		[168]
	human/brain, hypothalamus	4.5 ± 2.8 ng/g		[181]
	human/brain, hypothalamus	4.2 ± 22.9 ng/g		[182]
	human/brain, hypothalamus	6.5 ± 31.2 ng/g		[183]
	human/brain, ventral tegmental area	0.6 ± 1.7 ng/g		[182]
	human/brain, ventral tegmental area	0.2 ± 0.3 ng/g		[183]
	rat/brain, striatum	5.13 ± 1.34 ng/g wet tissue		[60]
	rat/brain, ventral midbrain	6.40 ± 1.04 ng/g wet tissue		[60]
	rat/brain, frontal cortex	5.22 ± 0.71 ng/g wet tissue		[60]
	rat/brain, hippocampus	8.38 ± 1.52 ng/g wet tissue		[60]
	rat/brain, cerebellum	5.40 ± 1.65 ng/g wet tissue		[60]
	mouse/brain, striatum	5.19 ± 1.74 ng/g wet tissue		[60]
	mouse/brain, ventral midbrain	24.04 ± 2.03 ng/g wet tissue		[60]
	mouse/brain, frontal cortex	4.22 ± 0.93 ng/g wet tissue		[60]
	mouse/brain, hippocampus	6.57 ± 2.06 ng/g wet tissue		[60]
	mouse/brain, cerebellum	8.49 ± 1.32 ng/g wet tissue		[60]

***N*-Me-nor-SAL**		human/CSF	< LOD	16-60 pmol/mL	[50]
		human/brain, substantia nigra	191.17 ± 12.91 ng/g wet tissue	65.75 ± 6.27 ng/g wet tissue	[147]
		human/brain, nucleus caudatus	570.03 ± 43.44 ng/g wet tissue	105.63 ± 23.55 ng/g wet tissue	[147]
		human/brain, putamen	314.87 ± 28.73 ng/g wet tissue	265.47 ± 39.03 ng/g wet tissue	[147]
		human/brain, locus coeruleus	18.44 ± 3.66 ng/g wet tissue	21.53 ± 5.67 ng/g wet tissue	[147]
		human/brain, frontal cortex	62.32 ± 14.76 ng/g wet tissue	35.77 ± 16.28 ng/g wet tissue	[147]
		human/brain, frontal cortex	1 ng/g		[189]
		human/brain, hippocampus	39.02 ± 7.82 ng/g wet tissue	46.78 ± 5.95 ng/g wet tissue	[147]
		human/brain, inferior olive	31.54 ± 9.04 ng/g wet tissue	29.77 ± 7.75 ng/g wet tissue	[147]
		human/brain, cerebellar cortex	28.15 ± 7.35 ng/g wet tissue	23.76 ± 4.31 ng/g wet tissue	[147]
		rat/brain, striatum	10.52 ± 1.95 ng/g wet tissue		[147]
		rat/brain, ventral midbrain	12.36 ± 2.43 ng/g wet tissue		[147]
		rat/brain, frontal cortex	9.84 ± 3.16 ng/g wet tissue		[147]
		rat/brain, hippocampus	14.23 ± 2.35 ng/g wet tissue		[147]
		rat/brain, cerebellum	9.54 ± 2.82 ng/g wet tissue		[147]
		mouse/brain, striatum	14.53 ± 4.54 ng/g wet tissue		[147]
		mouse/brain, ventral midbrain	10.42 ± 3.34 ng/g wet tissue		[147]
		mouse/brain, frontal cortex	12.88 ± 1.51 ng/g wet tissue		[147]
		mouse/brain, hippocampus	18.08 ± 7.87 ng/g wet tissue		[147]
		mouse/brain, cerebellum	12.75 ± 3.16 ng/g wet tissue		[147]
ADTIQ		human/brain, nucleus caudatus	0.06 ± 0.01 nmol/g wet tissue	0.43 ± 0.07 nmol/g wet tissue	[148]
		human/brain, putamen	0.10 ± 0.01 nmol/g wet tissue	0.76 ± 0.27 nmol/g wet tissue	[148]
		mouse/whole brain	~ 2.5fM/mg protein		[173]
DMDHIQ$^+$		human/brain, substantia nigra	254 ± 59.0 pmol/g wet weight		[193]
		human/brain, substantia nigra	254 ± 59.0 pmol/g wet weight		[41]
		human/brain, nucleus caudatus	< LOD		[193]
		human/brain, caudate nuclei	< LOD		[41]
		human/brain, putamen	< LOD		[193]
		human/brain, putamen	< LOD		[41]
		human/brain, frontal cortex	< LOD		[193]
		human/brain, frontal lobes	< LOD		[41]
3´,4´-DHBnTIQ		mouse/whole brain	detected, not quantified		[42]
6,7-DHBnTIQ		mouse/whole brain	detected, not quantified		[42]

Supplement III

Simultaneous Determination of Selected Steroids with Neuroactive Effects in Human Serum by Ultra-High Performance Liquid Chromatography–Tandem Mass Spectrometry



Simultaneous Determination of Selected Steroids with Neuroactive Effects in Human Serum by Ultra-high Performance Liquid Chromatography–Tandem Mass Spectrometry

Michal Kaleta [1,3] · Jana Oklestkova [1] · Miroslav Kvasnica [2] · Petr Kaňovský [3,4] · Miroslav Strnad [1] · Ondřej Novák [1]

[1]Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Faculty of Science, Palacký University, Šlechtitelů 27, 783 71, Olomouc, Czech Republic

[2]Department of Experimental Biology, Faculty of Science, Palacky University Olomouc, Šlechtitelů 27, 783 71 Olomouc, Czech Republic.

[3]Department of Neurology, University Hospital Olomouc, 779 00 Olomouc, Czech Republic

[4]Department of Neurology, Faculty of Medicine and Dentistry, Palacky University, 779 00 Olomouc, Czech Republic

Correspondence

Ondřej Novák
E-mail: *novako@ueb.cas.cz*

Michal Kaleta
E-mail: *michal.kaleta@upol.cz*

Author information

Michal Kaleta; ORCID: 0000-0003-0875-2912; *E-mail: michal.kaleta@upol.cz*

Jana Oklestkova; ORCID: 0000-0002-7423-5537; *E-mail: jana.oklestkova@upol.cz*

Miroslav Kvasnica; ORCID: 0000-0002-0597-3261; *E-mail: miroslav.kvasnica@upol.cz*

Petr Kaňovský; ORCID: 0000-0002-9009-3241; *E-mail: petr.kanovsky@fnol.cz*

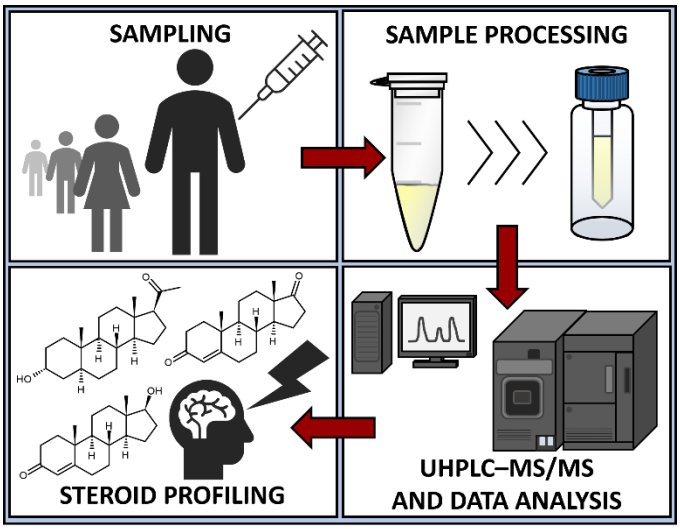
Miroslav Strnad; ORCID: 0000-0002-2806-794X; *E-mail: miroslav.strnad@upol.cz*

Ondřej Novák; ORCID: 0000-0003-3452-0154; *E-mail: novako@ueb.cas.cz*

Abstract

Neuroactive steroids are a group of steroid molecules that are involved in the regulation of functions of the nervous system. The nervous system is not only the site of their action, but their biosynthesis can also occur there. Neuroactive steroid levels depend not only on the physiological state of an individual (person's sex, age, diurnal variation, etc.) but they are also affected by various pathological processes in the nervous system (some neurological and psychiatric diseases or injuries) and new knowledge can be gained by monitoring these processes. The aim of our research was to develop and validate a comprehensive method for

the simultaneous determination of selected steroids with neuroactive effects in human serum. The developed method enables high-throughput and a sensitive quantitative analysis of nine neuroactive steroid substances (pregnenolone, progesterone, 5α-dihydroprogesterone, allopregnanolone, testosterone, 5α-dihydrotestosterone, androstenedione, dehydroepiandrosterone, and epiandrosterone) in 150 µL human plasma by ultra-high performance liquid chromatography with tandem mass spectrometry. The correlation coefficients above 0.999 indicated that the developed analytical procedure was linear in the range of 1.8 fmol to 57 pmol/inj. in human serum. The precision and accuracy of the method for all analytes ranged from 83 to 118% and from 0.9 to 14.1%, respectively. This described method could contribute to a deeper understanding of the pathophysiology of various diseases. Similarly, it can also be helpful in the search for new biomarkers and diagnostic options or new therapeutic approaches.



Keywords

Neurosteroids · Ultra-high-performance liquid chromatography–tandem mass spectrometry · Serum

Abbreviations: DHP, 5α-dihydroprogesterone; DHT, 5α-dihydrotestosterone; ACN, acetonitrile; ALLO, allopregnanolone; ANDRO, androstenedione; CV, coefficient of variation; d_3-T, testosterone-d_3; d_4-ALLO, allopregnanolone-d_4; d_4-PREG, pregnenolone-d_4; d_6-DHEA, dehydroepiandrosterone-d_6; d_9-PROG, progesterone-d_9; DHEA, dehydroepiandrosterone; EMA, European Medicine Agency; EPIA, epiandrosterone; ESI, electrospray ionization; FDA, Food and Drug Administration; GC, gas chromatography; HQ, high quality control; IS, internal standard; LC, liquid chromatography; LLOQ, lower limit of quantification; LOD, limit of detection; LQ, low quality control; ME, matrix effect; MeOH, methanol; MQ, medium quality

control; MRM, multiple-reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, Molecular weight; NAS, neuroactive steroid; PBS, phosphate-buffered saline; PREG, pregnenolone; PROG, progesterone; QC, quality control; RE, recovery; RIA, radioimmunoassay; RT, retention time; SD, standard deviation; T, testosterone; UHPLC–ESI–MS/MS, ultra-high-performance liquid chromatography with electrospray tandem mass spectrometry; UHQ, ultra-high quality control; ULOQ, upper limit of quantification.

Introduction

The human nervous system is the source and the target tissue for the action of many neuroactive substances. This group undoubtedly includes compounds derived from cholesterol – steroid hormones. All steroids, whether of natural or synthetic origin, that modulate the development and activity of the nervous system and thus the whole organism are referred to as neuroactive steroids (NASs) [1]. They have been observed to be involved in the regulation of neurogenesis, neuritogenesis, synaptogenesis, neuronal survival, myelin formation, synaptic plasticity, and many other processes. Based on these mechanisms, they are involved, for example, in the regulation of mood or behavior. In addition, some of them have a neuroprotective activity that may be sexually dimorphic [2]. Furthermore, they are involved in learning processes, general activity, memory, and excitatory or inhibitory effects of various neurotransmitter systems [3]. This large group of steroid substances includes hormonal steroids produced by "typical" steroidogenic peripheral tissues (mainly gonads and adrenal glands) and a specified subgroup of steroids biosynthesised by neurons and glial cells in the central and peripheral nervous system (so-called neurosteroids) [1, 2]. The NASs group also includes synthetic steroid substances capable of regulating neural activity. The central and peripheral nervous system has molecular mechanisms involved in the biosynthesis and metabolism of some NASs. Interestingly, NASs act not only via "classical" intracellular steroid receptors that modulate gene transcription (relatively slow genomic effects), but also through ion channels and membrane receptors (rapid non-genomic effects) such as γ-aminobutyric acid receptors [3, 4]. The genomic mechanism of action is typical for NASs and leads to the modulation of neuronal excitability. In addition, interactions of NASs with *N*-methyl-D-aspartate receptors, voltage-gated calcium channels, serotonin receptors, voltage-dependent anion channels, microtubule-associated protein 2, etc. are also described.

In addition to brain imaging methods, various biological markers, especially those of a protein origin (beta-amyloid protein, alpha-synuclein, etc.), are used to predict, prognose, diagnose or track the progression of neurodegenerative diseases [5]. Currently, attention is also

focused on the possibility of using low molecular weight substances [6]. The levels of NASs can also be changed by various pathological events and processes in the nervous system [1]. For instance, alterations in the levels of some NASs have been observed in psychiatric and neurological diseases such as multiple sclerosis [7, 8], Parkinson's disease [9], Alzheimer's disease or non-Alzheimer's dementia [10, 11], Huntington's disease [12], and in some injuries such as traumatic brain injury [13, 14]. In addition to health status, steroidome depends also physiologically on a person's sex, age, time of sampling (e.g., diurnal variation, menstruation cycle), medication (e.g. oral contraceptive pills in females), dietary patterns, and other factors [15–19].

The study of steroid substances was initially limited to various arduous colorimetry-based methods or bioassays [20–22]. The introduction of immunoassays, specifically the radioimmunoassay (RIA), and subsequent modifications of this method marked a major revolution in endocrinology and the analysis of not only steroid hormones [23, 24]. Several platforms based on the RIA and the enzyme immunoassay (especially the enzyme-linked immunosorbent assay – know as ELISA) are available for the determination of steroid analytes [25–28]. However, immunoassays can have a number of limitations, such as lack of specificity and associated cross-reactivity with structurally related compounds, limited dynamic range, interference with the matrix, or the ability to analyze only one analyte on a single platform [29]. Continuous advances have enabled a wider spread of mass spectrometry (MS) in the bioanalysis of steroid hormones. In recent years, it has become the method of first choice for the determination of steroids [24]. The use of MS allows highly selective, sensitive, accurate, and precise determination of a large number of steroid analytes in a single analytical run [30]. For more than half a century, gas chromatography combined with mass spectrometry (GC–MS) has been used for the analysis of steroid substances [31]. GC-based techniques using single MS or tandem mass spectrometry (MS/MS) are available [7, 32–35]. Approaches to the steroid analysis have been enriched in recent years in particular by the use of liquid chromatography (LC) [8, 17, 43–52, 29, 36–42]. The LC–MS/MS techniques offer several advantages over GC–MS, such as the high-throughput analysis (more suitable for a large set of samples), less time-consuming and generally easier sample preparation (usually no derivatization is required), or the ability to quantify intact steroid conjugates (sulfates or glucuronides) [53]. These properties make LC–MS/MS more suitable for routine use in clinical laboratories. Reversed-phase chromatographic separation is widely applied in the analysis of steroid substances. These are stationary phases based on hydrocarbon chains of various lengths (mainly C18) [17, 36, 45–52, 37–44], phenyl-hexyl [39] or, for example, pentafluorophenyl [29]. Detection is most

commonly provided by triple quadrupole MS [17, 29, 47–51, 36, 38, 40–45], but various hybrid approaches are also available [37, 39, 46, 52]. NASs are studied by LC–MS/MS in various types of mammalian biological matrices such as plasma or serum [8, 17, 29, 36, 38, 42–44], cerebrospinal fluid [8, 26, 38], and brain tissue [42, 45, 46]. But others can also be used: urine [47, 48], saliva [37, 49, 50], hair [41, 51], or nails [52]. LC–MS/MS-based methods have been successfully used to determine selected representatives of androgens, estrogens, progestins, and corticosteroids [54].

Even when the best possible endpoint analytical approach is available, sample processing is still a critical step in the analysis. Sample preparation for the LC–MS/MS analysis of low abundance endogenous compounds in complex biological matrices usually involves protein precipitation (e.g. with acetonitrile, methanol/water containing zinc sulphate) [29, 36, 39, 49], solid-phase extraction (online or offline) [8, 17, 49, 51, 52, 37, 39–41, 43, 46–48], or liquid-liquid extraction [29, 38, 41, 42, 44, 48, 50, 52] in various combinations and modifications. In addition, various derivatization procedures can be used to improve detection capabilities (e.g. with dansyl chloride, hydroxylamine hydrochloride, or picolinic acid) [29, 38, 44–46, 51]. Enzymatic hydrolysis can also be part of the sample preparation for urinary steroid analysis [48]. However, it is important to note that the design of the purification process depends on the specific analytical requirements (e.g. the type and quantity of the sample, the purpose of the analysis).

The aim of this work was to develop and validate a comprehensive isotope dilution method for a simultaneous analysis of selected NASs, such as the progestins pregnenolone (PREG), progesterone (PROG), 5α-dihydroprogesterone (DHP), allopregnanolone (ALLO) and the androgens dehydroepiandrosterone (DHEA), androstenedione (ANDRO), testosterone (T), 5α-dihydrotestosterone (DHT), and epiandrosterone (EPIA) in human blood serum. The chemical structures of the individual analytes and their physico-chemical properties are shown in Fig. 1 and Table 1, respectively. These analytes are very important representatives of female and male steroid hormones and have been also studied in other NAS-focused research [7–9, 26, 42]. The developed method combines a very simple and rapid extraction process with sensitive detection and quantification by ultra-high-performance liquid chromatography with electrospray tandem mass spectrometry (UHPLC–ESI–MS/MS). To the best of our knowledge, no rapid and high-throughput comprehensive method is currently available for the simultaneously targeted profiling of a selected set of steroids with neuroactive effects in human serum.

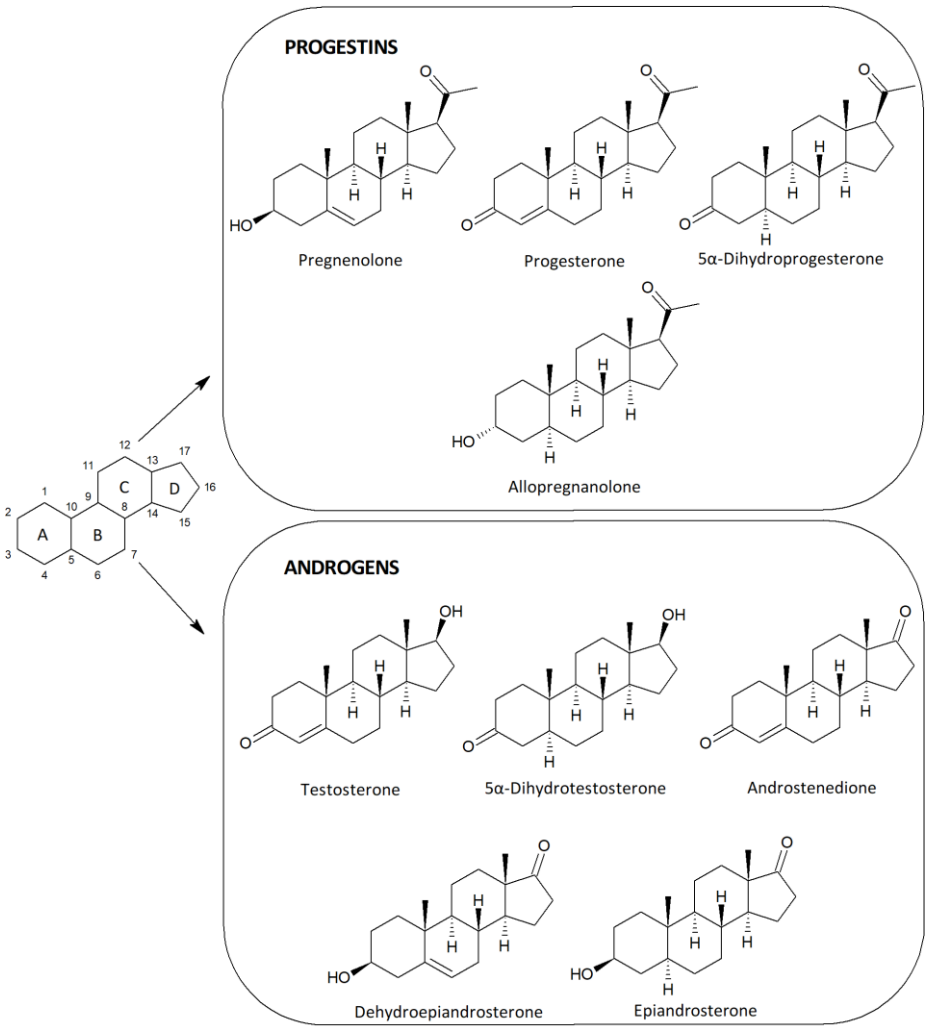


Fig. 1 Structures of the analysed steroid hormones

Table 1 Selected physico-chemical properties of the analytes (data source used HMBD: Human Metabolome Database [55]).

Analyte	**Log P**	**pKa_1** (strongest acidic)	**pKa_2** (strongest basic)	**Hydrogen acceptor count**	**Hydrogen donor count**
DHEA	3.36	18.20	-1.40	2	1
T	3.37	19.09	-0.88	2	1
EPIA	3.77	18.30	-1.40	2	1
DHT	3.41	19.38	-0.88	2	1
ANDRO	3.93	19.03	-4.80	2	0
PREG	3.58	18.20	-1.40	2	1
ALLO	3.99	18.30	-1.40	2	1
PROG	4.15	18.92	-4.80	2	0
DHP	4.19	19.34	-7.10	2	0

ALLO: Allopregnanolone, ANDRO: Androstenedione, DHEA: Dehydroepiandrosterone, DHP: α-Dihydroprogesterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, PREG: Pregnenolone, PROG: Progesterone, T: Testosterone.

Materials and methods

Human and Animal Rights Statement

The use of human serum samples was approved by the institutional ethics committee of the Faculty of Medicine and Dentistry, Palacky University in Olomouc and University Hospital Olomouc. Written informed consent form was obtained from all participants.

Chemicals and materials

Unlabeled standards PREG, ALLO, PROG, ANDRO, and DHP were purchased from Sigma–Aldrich (Germany). The T standard was obtained from Fluka (Netherlands), DHEA and DHT from the National Measurement Institute (Australia). EPIA was prepared in the Laboratory of Growth Regulators. Internal standards (ISs) labeled with deuterium pregnenolone-d_4 (d_4-PREG), allopregnanolone-d_4 (d_4-ALLO), and progesterone-d_9 (d_9-PROG) were obtained from Cambridge Isotope Laboratories, Inc. (USA). Testosterone-d_3 (d_3-T) and dehydroepiandrosterone-d_6 (d_6-DHEA) were purchased from Sigma–Aldrich (USA). All stocks and working solutions of standards (ISs, unlabeled standards) were dissolved in 100% methanol (MeOH) and stored in the dark at -70 °C until analysis.

The solvents MeOH gradient grade for LC, MeOH hypergrade for LC–MS, and acetonitrile (ACN) hypergrade for LC–MS were purchased from Merck Millipore (Germany). Pure water was prepared using the Direct-Q® 3 UV Water Purification System (Merck Millipore, Germany). Formic acid was obtained from Fluka (USA), butylated hydroxytoluene and bovine serum albumin was obtained from Sigma–Aldrich (USA). All other chemicals used were purchased from Lach-ner (Czech Republic).

Method calibration was performed using artificial serum prepared by dissolution of 4% bovine serum albumine in 10 $mmol.L^{-1}$ phosphate-buffered saline (PBS), pH 7.4. The PBS buffer was composed of 136.9 $mmol.L^{-1}$ sodium chloride, 2.7 $mmol.L^{-1}$ potassium chloride, 10.1 $mmol.L^{-1}$ disodium phosphate dodecahydrate, and 1.8 $mmol.L^{-1}$ monopotassium phosphate. The artificial serum was aliquoted and stored in the dark at -70 °C. A new aliquot of artificial serum was used for each experiment.

Sample collection, pre-treatment, and storage

Human serum samples required for the development and validation of the method were obtained from the Department of Neurology of the University Hospital Olomouc, Czech Republic. Ethics approval was granted according to University Hospital Olomouc standard SM-

L031, and ethics committee reference numbers: 139/10 and 76/15. The collection of blood samples from participants and their pre-treatment, transport, and storage were performed according to the established methodologies.

Blood was collected into collection tubes (VACUETTE® 9 mL Z Serum Separator Clot Activator) and centrifuged at 4.000 rpm and 4 °C for 5 min (Universal 320R, Hettich, Germany). The obtained sera were transferred into dark amber glass vials and treated in an ultrasonic bath for 5 min (Elmasonic S 10 H, Elma, Germany). Serum samples in vials were then bubbled with a stream of argon for 2 min to create an inert atmosphere. Argon provides prevention against unwanted oxidation of analytes. Finally, these samples were stored in the dark at -70 °C until analysis.

Serum sample processing

First, 5 µL µl of a stock solution containing a mixture of stable isotopically labeled ISs (addition of IS mixture A) was added to 150 µL of cooled human serum. A list of ISs and their additions are listed in Table 2. The modified sample preparation is based on previously published protocol [56]. Briefly, serum proteins were completely precipitated by adding 595 µL of ice-cold ACN (-20 °C) containing 0.05% (v/v) butylated hydroxytoluene (prevention of autoxidation [57]). The addition of ACN ensures both the precipitation of serum proteins and the release of steroid substances from their carrier proteins. Finally, 50 µL of MeOH is also added, corresponding to the addition of steroid standards in the calibration curve samples. Serum samples were kept cold during all pipetting steps (CoolBox™, Biocision, USA). The resulting precipitate was vortexed for 30 s (Wizard Advanced IR Vortex Mixer, VELP Scientifica, Italy). The samples were placed on a rotator (SB3, Stuart, UK), and incubated for 60 min and 19 rpm at -20 °C to ensure protein precipitation. After further vortexing (30 s), the samples were centrifuged at 10.000 rpm for 10 min at 4 °C (Heraeus™ Multifuge™ X1R, Thermo Scientific, USA). The supernatant obtained was transferred to a mini-spin centrifuge filter tube with a nylon membrane and a pore size of 0.20 µm (Mini Spin Columns + 0.2 NY). The samples were filtered at 10.000 rpm for 5 min and 4 °C. The filtrate was then evaporated to dryness under a gentle stream of nitrogen at 37 °C for as long as necessary (TurboVap® Classic LV, Biotage, Sweden). The dry residues were then dissolved in 50 µL of 100% ice-cold MeOH, vortexed to rinse the microtube walls (30 s), and placed in an ultrasonic bath (3 min) (Sonorex RK 510S, Bandelin, Germany). The dissolved samples were then transferred to a mini-spin centrifuge filter tube, centrifuged at 10.000 rpm for 3 min at 4 °C and pipetted into vial inserts for LC–MS measurements. The processed serum samples in triplicates were then immediately placed in the

cooled autosampler (4 °C) of the UHPLC–MS/MS instrument and analysed. A schematic overview of the main steps in the analysis of NASs of interest is shown in Fig. 2.

Table 2 An overview of individual analytes, the corresponding deuterated internal standards, and their additions

		Calibration range (pmol/inj.)	
		0.00018–0.18	0.5692–56.9210
Analyte	**IS**	**Addition IS mix A** (pmol/inj.)	**Addition IS mix B** (pmol/inj.)
DHEA	d_6-DHEA	0.20	2.00
T	d_3-T	0.02	0.20
EPIA	d_3-T	0.02	0.20
DHT	d_3-T	0.02	0.20
ANDRO	d_3-T	0.02	0.20
PREG	d_4-PREG	1.00	10.00
ALLO	d_4-ALLO	2.00	20.00
PROG	d_9-PROG	0.02	0.20
DHP	d_9-PROG	0.02	0.20

ALLO: Allopregnanolone, ANDRO: Androstenedione, d_3-T: d_3-Testosterone, d_4-ALLO: d_4-Allopregnanolone, d_4-PREG: d_4-Pregnenolone, d_6-DHEA: d_6-Dehydroepiandrosterone, d_9-PROG: d_9-Progesterone, DHEA: Dehydroepiandrosterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, PREG: Pregnenolone, IS: Internal standard, T: Testosterone.

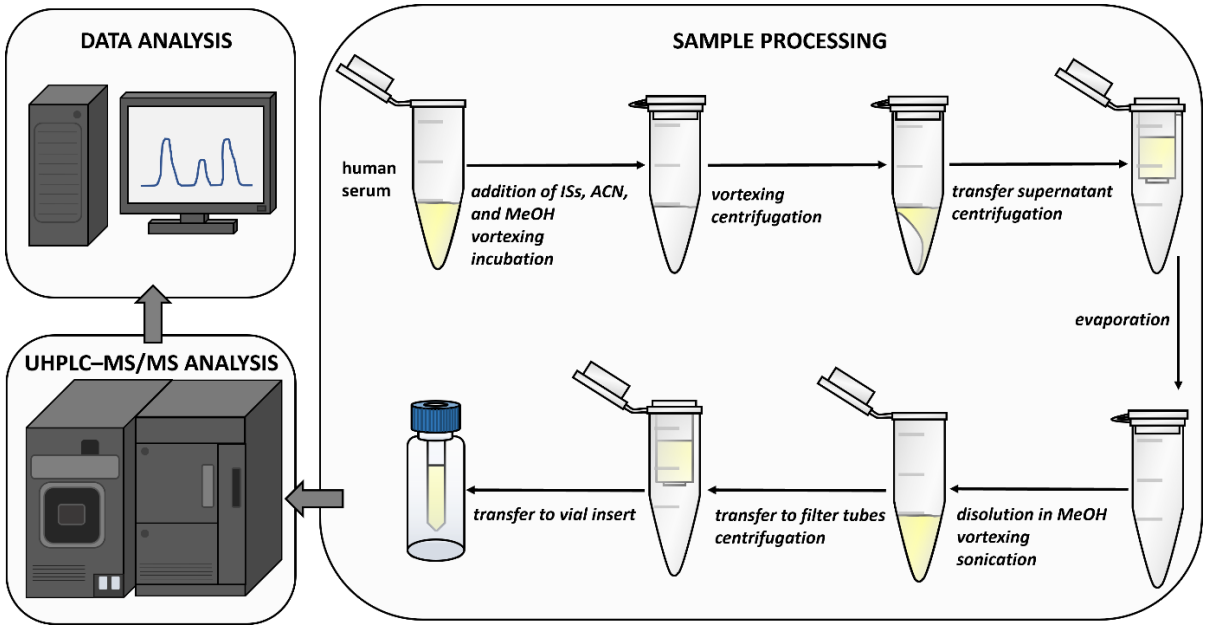


Fig. 2 Main steps of the developed method for the profiling of selected neuroactive steroids in serum: Sample processing, UHPLC–MS/MS analysis, and Data analysis. UHPLC–MS/MS: ultra-high-performance liquid chromatography–tandem mass spectrometry, ISs: internal standards, ACN: acetonitrile, MeOH: methanol

Chromatographic separation and mass spectrometric detection

The UHPLC–MS/MS analysis of targeted steroid compounds was performed using an ACQUITY^{TM} UPLC^{TM} H-Class PLUS System (Waters, USA) connected to a triple quadrupole MS Xevo® TQ-S micro (Waters, UK) with electrospray ionization (ESI) source. Data acquisition and processing were performed using the MassLynx^{TM} 4.1 (Waters) and Microsoft Office (Microsoft) software packages.

A Kinetex® Biphenyl column (100 × 2.1 mm, 1.7 μm, 100 Å; Phenonemex, USA) equipped with an ACQUITY Column In-Line Filter kit (Waters) was used for chromatographic separation of steroid compounds. The column was maintained at 40 °C with a flow rate 0.5 mL.min^{-1} of the mobile phase containing of 100% MeOH (A) and 7.5 mmol.L^{-1} aqueous solution of formic acid (B). The gradient was as follows: 0 min, 60:40 (A:B); 10 min, 75:25 (A:B); 12 min, 85:15 (A:B); 12.25–12.75 min, 99:1 (A:B); 13–15 min, 60:40 (A:B). A wash and equilibration steps were included at the end of the gradient used. The column was washed with 99% MeOH for 0.50 min. After the washing step, initial separation conditions were achieved using a 2-min equilibration. The course of the gradient is shown in Fig. 3. The mobile phase was directed into the MS from the 3.51 min of the gradient. Thus, only at the moment of the expected elution of the first analytes. This prevented unnecessary fouling of the MS instrument components. Similarly, the mobile phase flow was directed to the waste at the end of the gradient (11.49 min). During the UHPLC–MS/MS analysis, samples were placed in an autosampler maintained at 4 °C in the dark. Samples dissolved in 100% MeOH were injected (constant injection volume 2 μL) onto a reverse phase column. The representative chromatographic separation of the steroid standards in 100% MeOH and their retention times (RTs) are shown in Fig 3. The chromatographic column was washed at the end of the analysis and stored long-term in 65% ACN in water. The total run time was 15 min per sample.

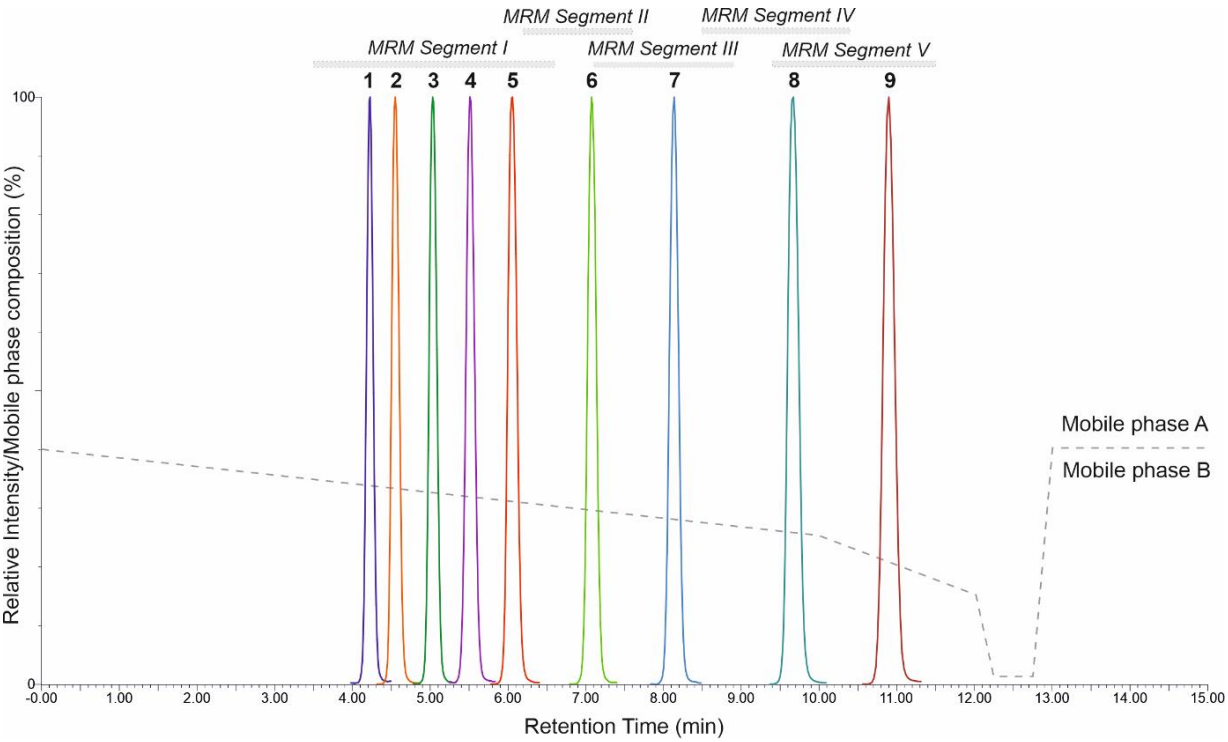


Fig. 3 Reveversed-phase chromatographic separation of nine steroid standards dissolved in 100% methanol (1.8 pmol/inj.): Dehydroepiandrosterone (1), Testosterone (2), Epiandrosterone (3), 5α-Dihydrotestosterone (4), Androstenedione (5), Pregnenolone (6), Allopregnanolone (7), Progesterone (8), 5α-Dihydroprogesterone (9). The chromatographic run is divided into five multiple-reaction monitoring (MRM) scan segments.

The triple quadrupole MS instrument was operated in positive ESI mode using multiple-reaction monitoring (MRM) transitions. The optimised conditions for the MS analysis were the following: source temperature 150 °C, desolvation temperature 600 °C, desolvation nitrogen gas flow rate 1000 $L.h^{-1}$, capillary voltage 2.5 kV, cone voltage 19–45 V, and collision energy 11–30 eV. A specific quantification and confirmation MRM transitions were selected for each steroid analyte. An MRM transition was also selected for each IS. In addition, based on the RT knowledge of the individual analytes, the MS/MS measurement was divided into five separate MRM scan segments. In these short RT window, only the mass transitions of the expected analytes were measured. The dwell times were determined based on the width of the chromatographic peak. The dwell time value was set on the MS so that the obtained chromatographic peaks were covered by at least 15 scan points. The MRM transitions and other selected MS parameters for individual analytes and the corresponding ISs are listed in Table 3.

Table 3 Summary of multiple-reaction monitoring transitions, optimised instrument settings, and retention times (min; mean ± SD; n = 60) for individual analytes and internal standards.

Analyte/ Internal standard	RT ± SD (min)	Precursor ion	Molecular weight ($g.mol^{-1}$)	Quantif. MRM transition	Confirm. MRM transition	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
MRM scan segment I (3.5–6.7 min)								
DHEA	4.16±0.02	$[M\text{-}H_2O+H]^+$	288	271 > 253	271 > 213	20/27	13/16	0.050
d_6-DHEA	4.10±0.02	$[M\text{-}H_2O+H]^+$	294	277 > 259	-	29	13	0.050
T	4.49±0.02	$[M+H]^+$	288	289 > 97	289 > 109	25/22	19/22	0.050
d_3-T	4.45±0.03	$[M+H]^+$	291	292 > 97	-	25	19	0.050
EPIA	4.97±0.02	$[M\text{-}H_2O+H]^+$	290	273 > 255	273 > 161	30/30	11/18	0.050
DHT	5.45±0.03	$[M+H]^+$	290	291 >255	291 > 159	30/30	13/21	0.050
ANDRO	5.98±0.03	$[M+H]^+$	286	288 > 97	288 > 109	20/20	18/21	0.050
MRM scan segment II (6.2–7.6 min)								
PREG	7.00±0.03	$[M\text{-}H_2O+H]^+$	316	317 > 159	317 > 81	25/25	22/25	0.095
d_4-PREG	6.91±0.03	$[M\text{-}H_2O+H]^+$	320	321 > 159	-	25	22	0.095
MRM scan segment III (7.1–8.9 min)								
ALLO	8.06±0.03	$[M\text{-}H_2O+H]^+$	318	301 > 81	301 > 95	45/45	30/25	0.225
d_4-ALLO	7.97±0.03	$[M\text{-}H_2O+H]^+$	322	305 > 81	-	45	30	0.225
MRM scan segment IV (8.5–10.4 min)								
PROG	9.58±0.03	$[M+H]^+$	314	315 > 97	315 > 109	23/25	19/22	0.250
d_9-PROG	9.40±0.03	$[M+H]^+$	323	324 > 100	-	19	21	0.250
MRM scan segment V (9.9–11.5 min)								
DHP	10.83±0.03	$[M+H]^+$	316	317 > 85	317 > 281	32/30	13/13	0.240

ALLO: Allopregnanolone, ANDRO: Androstenedione, d_3-T: d_3-Testosterone, d_4-ALLO: d_4-Allopregnanolone, d_4-PREG: d_4-Pregnenolone, d_6-DHEA: d_6-Dehydroepiandrosterone, d_9-PROG: d_9-Progesterone, DHEA: Dehydroepiandrosterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, MRM: Multiple-reaction monitoring, PREG: Pregnenolone, RT: Retention time, SD: Standard deviation, T: Testosterone.

Bioanalytical method validation

Validation is a tool that can be used to assess whether a bioanalytical method is suitable for its intended purpose. The parameters that should be verified during validation and the criteria that the method should meet are described in detail in the European Medicine Agency (EMA) [58] and Food and Drug Administration (FDA) [59] guidelines. Four quality control (QC) levels of

analyte concentration were used for the following series of validation experiments, namely low QC (LQ), medium QC (MQ), high QC (HQ), and ultra-high QC (UHQ) level. The QC levels were chosen to cover the linear calibration range of each analyte with respect to endogenous serum steroid levels. The LQ, MQ, HQ, and UHQ levels correspond to 0.0569, 0.18, 1.8, and 5.6921 pmol/inj., respectively.

Method calibration

When analyzing metabolites in biological matrices, it is recommended to prepare calibration points in the same, usually artificial matrix [58]. Matrix-matched calibration curves prepared from 4% bovine serum albumine solution in 10 $mmol.L^{-1}$ PBS buffer were used the for quantification of steroid analytes [56]. This artificial matrix replaces real human serum. Each calibration point (prepared in triplicate) contained artificial serum (150 µL), a mixture of unlabeled standards (45 µL), a defined addition of stable isotopically labeled ISs (5 µL, addition of IS mixture A or B) (Table 2), and 100% ice-cold ACN (550 µL, -20 °C). The calibration range was divided into two parts to obtain the optimal matrix-matched calibration curves. Each part of the calibration points contains a mixture of ISs with different concentrations (addition of IS mixtures A or B). Table 2 shows both parts of the calibration range and the optimised IS additions. The same extraction protocol was applied to the calibration samples in artificial serum as well as to the real blood serum samples (based on the procedure described in the "Serum sample processing" section).

Finally, the defined addition of ISs allows the quantification of endogenous analytes in unknown samples using the isotope dilution method [60]. This method is based on knowing the ratio between the area of the analyte and the labeled standard in the sample (so-called response), which is then plotted on a calibration curve. The result of this interpolation is the absolute quantification of the analyte in the sample.

Method precision and accuracy

Within-run precision and accuracy were determined using four sets of neat solution samples (100% MeOH) spiked with a constant amount of labeled ISs (addition IS mixture A or B) and unlabeled standards at LQ, MQ, HQ, or UHQ levels. The samples were analysed in five replicates for each QC level. The UHPLC–MS/MS analysis of the prepared samples was performed within one run of the instrument. The same sample sets of neat solutions were used to determine the between-run precision and accuracy of the method. These were analysed for each concentration level in five replicates on three different runs on two different days.

Within-run precision and accuracy were also determined using donor pool serum. The serum was divided into four sets based on the addition of unlabeled standards. All sets were spiked with a constant addition of ISs (addition IS mixture A or B) and unlabeled analytes at the LQ, MQ, HQ, or UHQ level. The samples were analysed within one run of the instrument.

Method recovery and matrix effect

Four individual human serum donors were selected to determine the analytical recovery (RE) and the matrix effect (ME). These validation parameters were determined for each analyte at four concentration levels (LQ, MQ, HQ, or UHQ). To establish appropriate QC concentration levels, the endogenous levels of the analytes of interest in the human serum were preliminarily measured. The first set of serum samples was spiked with a mixture of unlabeled standards to four concentration levels and a constant amount of ISs (addition IS mixture A or B) at the beginning of the extraction protocol. Individual QC levels were represented by samples in triplicate. The same extraction protocol described in the "Serum sample processing" section was applied to the prepared samples. At the same time, the other corresponding set of samples was prepared, spiked with standards after extraction prior to the UHPLC–MS/MS analysis. Adequate blanks were also prepared for both sets of samples to subtract endogenous analyte levels (spiked with ISs only).

The RE (1) of the analytical method was calculated from the mean peak area of the analyte in the matrix spiked with standards before extraction (member C) to the mean peak area of the analyte in the matrix spiked after extraction (member B). The RE values were calculated based on the following previously published equations [61].

$$\text{RE (\%)} = \frac{C}{B} \cdot 100 \quad (1)$$

The ME (2) was calculated by knowing the ratio of the mean peak area of the analyte in the matrix spiked standards after extraction (member B) to the mean peak area in the neat solution of the analyte without the presence of matrix (member A) [61, 62]. The resulting ME is reported as a percentage. A ME value greater than 100% reflects an enhancement of ionization and a value less than 100% indicates a suppression of ionization. It was determined based on the following calculation:

$$\text{ME (\%)} = \frac{B}{A} \cdot 100 \quad (2)$$

Furthermore, the so-called IS-normalised ME (3) was also determined [63]. As in the previous case, this is also a post-extraction addition technique for ME evaluation. Its calculation

is based on the ratio of the response in the matrix (member D; spiked after extraction) to the response in the neat solution (member E). The response is determined as the ratio between the peak area of the analyte and the IS. The IS-normalised ME was expressed by the equation:

$$\text{IS-normalised ME (\%)} = \frac{D}{E} \cdot 100 \quad (3)$$

Results and discussion

Chromatographic separation and mass spectrometric detection

A high percentage of published methods rely on reversed-phase chromatographic columns [17, 37, 65, 66, 39–41, 43, 44, 50, 51, 64] and therefore this strategy was chosen in this case. As is well known, retention during reversed-phase separation is usually based mainly on hydrophobic and van der Waals interactions [67]. Therefore, we used a biphenyl LC column to improve the chromatographic selectivity for steroid compounds. Nine target steroids were successfully separated at baseline using the Kinetex® Biphenyl column (100 × 2.1 mm, 1.7 μm, 100 Å; Phenonemex, USA) equipped with an ACQUITY Column In-Line Filter kit (Waters) (Fig. 4). RTs of the analytes studied ranged from 4.16 (DHEA) to 10.83 min (DHP). A high degree of RT stability was observed for all compounds during the UHPLC–MS/MS analysis. The maximum standard deviation (SD) of RTs between injections (n = 60) was 0.03 min (Table 3). The CV values ranged from 0.29 to 0.57% for all substances tested.

Analyte profiling was performed using a triple quadrupole with ESI^+ in MRM acquisition mode. The MRM mode is characterised by exceptional selectivity and sensitivity in ion recording methods and is therefore widely used for the quantification of low abundance target analytes [68]. Increased reliability in the quantification of analytes was achieved by combining the UHPLC–MS/MS approach with the stable isotope dilution method [60]. Two MRM transitions were monitored for each analyte. The more intense mass transition was used as a quantification transition, the other was used as a confirmation transition (Table 3). Importantly, the selection of MRM transitions of individual analytes and labeled standards were verified on the basis of existing publications using the LC–MS/MS system for the analysis of steroid compounds [64–66, 69–73]. Precursor ions were either protonated $[M+H]^+$ molecules or $[M-H_2O+H]^+$ molecules formed by loss of water molecules due to instability in the ESI source (see Table 3). In addition, the cone voltage but especially the collision energy for each MRM transition have been also optimised to achieve the highest possible sensitivity. The values that showed the largest peak area in the UHPLC–MS/MS measurements were selected and are

reported in Table 3. The optimised cone voltage and collision energy values ranged from 20 to 45 V and 11 to 30 eV, respectively. To achieve optimal sensitivity, the chromatographic window was divided into five MRM scan segments based on the expected RTs of the analytes. Dwell time values were set between 0.050 and 0.250 ms to achieve at least 15 scan points per chromatographic peak.

Method calibration

Quantification of the analytes was performed using matrix-matched calibration curves (4% bovine albumine in 10 mmol.L^{-1} PBS buffer). An important step in their construction was the determination of the parameters that characterize them, namely the linear range of the calibration curve, the limit of detection (LOD), the lower limit of quantification (LLOQ), and the upper limit of quantification (ULOQ). The lower limit of quantification (LLOQ) is defined as a signal-to-noise ratio (S/N) ≥5 and is represented by the lowest concentration point in the calibration range [58]. The LOD values (S/N = 3) were estimated by knowledge of the signal-to-noise ratio of the LLOQ points.

Overall, calibration parameters for all target analytes are listed in Table 4. The LLOQ values of selected steroids in the artificial matrix range from 0.0018 to 0.0569 pmol/inj. The lowest LLOQ values in the blank matrix were obtained for T, ANDRO, and PROG (0.0018 pmol/inj), while the highest was obtained for DHP (0.0569 pmol/inj.). In addition, some analytes (T, ANDRO, and PROG) can be detected at concentrations lower than 1 fmol/inj. L. Such low values allowed the profiling of analytes at the trace level in serum samples (see below). Based on the available data, the LLOQ values achieved for the vast majority of analytes were at or close to the expected endogenous levels of the target analytes [55]. It should be noted that lower LLOQ and LOD values can of course be achieved using the calibration curves prepared in pure solvent. However, it is recommended to use the matrix-matched calibration curve for the analysis of metabolites in biological samples [58]. Compared to many available studies, LLOQ values for T [29, 34, 42, 69, 70, 73], DHEA [30, 34, 36, 40, 42, 69, 70, 73], PROG [30, 34, 36, 39, 42], PREG [34, 42], ANDRO [34, 36, 69, 70, 73], ALLO [74, 75], and DHT [34] were better or at least within one order of magnitude. Lower quantification limits for some substances in other methods may be due to the use of different ionisation techniques [36]. For example, APPI or APCI techniques seem to be more suitable for steroid analysis. However, none of these techniques were available in our laboratory, and therefore the ESI source was used. Yuan et al. (2020) achieved better LLOQ values for many analytes (PREG, DHEA, ANDRO, and PROG),

but their sample preparation involves derivatization steps, specifically acylation with isonicotinoyl chloride [29].

A minimum seven-point matrix-matched calibration curve was obtained for all analytes tested. To assess linearity, the analyte concentration was back-calculated for each point on the calibration curve and related to the nominal concentration of that point. The difference between the calculated value and the nominal value did not exceed ±15 %, in the case of LLOQs ±20 % [58]. In constructing the calibration curves, each calibration point was interleaved with a linear regression line. Individual calibration curves are defined by the line equation (slope and intercept) and by the coefficient of determination (r^2). Linearity was excellent with r^2 varying between 0.9989 and 0.9998 for all analytes tested (Table 4).

The analysis of a solvent sample (100% MeOH) beyond the most concentrated point (ULOQ) of the calibration curve confirmed no significant carry-over between samples. This confirms that there is no interference between samples (even between the samples with high concentrations of analytes) that would interfere with the analysis.

Table 4 Summary of linear ranges, detection and quantification limits, and regression parameters of serum matrix-matched calibration curves

				Regression parameters		
Analyte	**Linear range**[a]	**LOD**[a]	**LLOQ**[a]	**Slope**	**Intercept**	**r^2**
DHEA	0.0180–56.9210	0.0082	0.0180	0.9991	0.3067	0.9996
T	0.0018–56.9210	0.0003	0.0018	0.9836	0.1294	0.9996
EPIA	0.0180–56.9210	0.0044	0.0180	1.0355	-0.7937	0.9998
DHT	0.0057–56.9210	0.0021	0.0057	1.0247	-0.6112	0.9996
ANDRO	0.0018–56.9210	0.0006	0.0018	0.9979	-0.1018	0.9996
PREG	0.0057–56.9210	0.0025	0.0057	1.0072	-0.1642	0.9997
ALLO	0.0180–56.9210	0.0063	0.0180	0.9993	0.2419	0.9995
PROG	0.0018–18.0000	0.0007	0.0018	0.9973	0.3037	0.9996
DHP	0.0569–56.9210	0.0238	0.0569	0.9639	-1.2306	0.9989

ALLO: Allopregnanolone, ANDRO: Androstenedione, DHEA: Dehydroepiandrosterone, DHP: α-Dihydroprogesterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, LLOQ: Lower limit of quantification (S/N ≥5), LOD: Limit of detection (S/N = 3), PREG: Pregnenolone, PROG: Progesterone, T: Testosterone.

[a] pmol/inj. (2 μL) for all analytes

Method precision and accuracy

The within-run and between-run precision and accuracy of the analytical method was determined using four sets of neat solution samples, each set spiked to one QC concentration

level (LQ, MQ, HQ, and UHQ). Each QC level was represented by five samples. The accuracy is expressed as a percentage and represents the closeness of the measured concentration to the reference value [58]. In contrast, the precision of the method is expressed as a coefficient of variation (CV). To determine the between-run parameters, the same set of samples was analysed in three different analytical runs on two different days. The precision and accuracy values for selected steroid analytes in solvent are shown in Table 5.

Both the within-run and the between-run accuracy fell within ±15% for all the steroids. This is consistent with the EMA requirements [58]. The lowest within-run and between-run accuracy was determined for EPIA (91%) and the highest for DHEA and DHP (114%). The SD were below 10% for all steroid analytes. The requirements were also met in the case of method precision. Specifically, CVs ranged between 0.2% for the PROG and 6.8% for the EPIA (both at the UHQ level).

Table 5 Within-run and between-run precision and accuracy at low (LQ), medium (MQ), high (HQ), and ultra-high (UHQ) levels of steroid analytes in neat solutions

	Accuracy mean (%) (SD)								**Precision** CV (%)							
	Within-run				Between-run				Within-run				Between-run			
QC levels[a]	LQ	MQ	HQ	UHQ	LQ	MQ	HQ	UHQ	LQ	MQ	HQ	UHQ	LQ	MQ	HQ	UHQ
DHEA	100 (4.3)	102 (4.3)	114 (2.1)	102 (1.8)	100 (3.7)	101 (3.2)	114 (1.5)	102 (1.7)	4.4	4.2	1.8	1.8	3.7	3.2	1.3	1.7
T	97 (1.1)	97 (1.1)	110 (1.1)	96 (0.9)	97 (1.3)	98 (1.1)	110 (0.9)	95 (1.1)	1.1	1.1	1.0	0.9	1.3	1.2	0.8	1.1
EPIA	94 (4.7)	91 (4.9)	103 (5.8)	94 (5.7)	95 (3.3)	93 (4.5)	105 (3.8)	95 (6.4)	5.0	5.4	5.6	6.0	3.5	4.8	3.6	6.8
DHT	98 (4.4)	95 (2.9)	105 (3.3)	96 (4.0)	97 (3.2)	95 (2.8)	106 (2.1)	95 (4.6)	4.5	3.1	3.1	4.2	3.3	2.9	2.0	4.8
ANDRO	101 (1.0)	98 (1.4)	110 (0.7)	99 (1.2)	100 (1.2)	97 (1.4)	110 (0.8)	98 (1.5)	0.9	1.4	0.6	1.2	1.2	1.4	0.8	1.5
PREG	97 (5.7)	97 (1.6)	111 (0.6)	98 (0.4)	97 (3.7)	98 (1.7)	112 (1.0)	98 (0.5)	5.9	1.7	0.6	0.4	3.8	1.7	0.9	0.5
ALLO	93 (1.7)	93 (0.9)	112 (0.7)	100 (0.6)	94 (2.4)	93 (0.9)	112 (0.8)	100 (0.6)	1.8	0.9	0.6	0.6	2.5	0.9	0.7	0.6
PROG	95 (0.4)	98 (1.0)	112 (1.1)	95 (0.2)	95 (0.8)	98 (0.9)	112 (0.7)	94 (0.5)	0.4	1.0	1.0	0.2	0.8	1.0	0.7	0.6
DHP	96 (2.7)	98 (1.5)	113 (1.2)	102 (0.8)	95 (2.9)	98 (1.9)	114 (2.2)	102 (2.1)	2.8	1.5	1.1	0.8	3.0	2.0	2.0	2.1

ALLO: Allopregnanolone, ANDRO: Androstenedione, DHEA: Dehydroepiandrosterone, DHP: α-Dihydroprogesterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, PREG: Pregnenolone, PROG: Progesterone, T: Testosterone.

Accuracy was expressed as a percentage of the nominal concentration; $n = 5$.

Precision was expressed as a coefficient of variation (CV); $n = 5$.

[a] The low (LQ), medium (MQ), high (HQ), and ultra-high (UHQ) levels correspond to to 0.0569, 0.18, 1.8, and 5.6921 pmol/inj., respectively.

Furthermore, the accuracy and precision of the analytical method were evaluated using the pooled spiked serum (see Table 6). Each QC level (LQ, MQ, HQ, and UHQ) was represented by five samples. To determine the accuracy of the method, mean analyte concentrations were compared to nominal values. For all analytes, 83 to 118% was achieved, indicating the reliability and accuracy of the method. The measured mean concentrations did not deviate from the reference values by more than ± 15% (20%). The accuracy of the developed method is therefore in accordance with the requirements set by the EMA [58]. The CV values also reach the required values (from 0.9 to 14.1%). Based on these results, it can be concluded that the laboratory and systematic error of the method is not significant.

Table 6 Within-run precision and accuracy at low (LQ), medium (MQ), high (HQ), and ultra-high (UHQ) levels of steroid analytes in human serum

	Accuracy mean (%) (SD)				**Precision** CV (%)			
QC levels[A]	LQ	MQ	HQ	UHQ	LQ	MQ	HQ	UHQ
DHEA	87 (4.7)	94 (1.2)	105 (1.4)	106 (4.0)	5.4	1.2	1.3	3.8
T	87 (2.1)	90 (2.3)	100 (0.9)	100 (3.3)	2.4	2.6	0.9	3.2
EPIA	117 (3.0)	106 (12.1)	115 (1.1)	102 (14.4)	2.6	11.3	1.0	14.1
DHT	86 (2.6)	85 (1.5)	92 (0.9)	86 (9.2)	3.0	1.8	0.9	10.7
ANDRO	83 (1.7)	84 (1.5)	93 (0.9)	93 (5.9)	2.1	1.8	1.0	6.3
PREG	96 (2.3)	99 (3.3)	108 (1.8)	108 (4.3)	2.4	3.3	1.7	4.0
ALLO	110 (12.0)	109 (4.9)	115 (2.6)	115 (5.7)	10.9	4.5	2.3	4.9
PROG	100 (3.7)	105 (3.0)	114 (2.3)	114 (2.8)	3.7	2.9	2.0	2.4
DHP	110 (7.3)	102 (2.7)	115 (1.0)	118 (4.2)	6.6	2.7	0.9	3.6

ALLO: Allopregnanolone, ANDRO: Androstenedione, DHEA: Dehydroepiandrosterone, DHP: α-Dihydroprogesterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, PREG: Pregnenolone, PROG: Progesterone, T: Testosterone.

Accuracy was expressed as a percentage of the nominal concentration; $n = 5$.

Precision was expressed as a coefficient of variation (CV %); $n = 5$.

[A]The low (LQ), medium (MQ), high (HQ), and ultra-high (UHQ) levels correspond to to 0.0569, 0.18, 1.8, and 5.6921 pmol/inj., respectively.

Method recovery and matrix effect

The RE of the method was tested at four concentration levels (corresponding to LQ, MQ, HQ, and UHQ) using blood serum from several donors. For the RE determination, the spiked serum samples before and after extraction were compared; the calculation was based on Matuszewski et al. 2003 [61]. The analytical method REs range from 66 to 102% (see Fig. 4). The greatest losses during the purification and extraction process occur with DHEA at the LQ level. However, these results generally indicate efficient extraction of target analytes from serum samples. The higher SDs (from 1 to 50%) can be explained by the use of four lots of blood serum from different donors. Thus, it can be concluded that the RE in this case is highly dependent on the individual characteristics of the samples. Interestingly, hemolysis, icterus, paraproteinemia, and lipemia, for example, can interfere with biochemical tests [76]. We hypothesize that a similar effect, i.e. different extraction efficiency of analytes due to differences in the matrix (e.g. increased hemoglobin, lipid content, etc.), is also possible in this case. The sample preparation of the developed method is relatively simple and rapid, practically it only involves precipitation of serum proteins, filtration, and concentration. Despite this

simplicity, none of the analytes showed a decrease in method RE below 66%. Compensation for these sample processing losses is provided by the use of a defined addition of stable isotopically labeled ISs [77]. In fact, sample processing for many steroid analysis methods usually includes additional steps using, for example, solid-phase extraction [8, 17, 51, 37, 40, 41, 43, 46–49] or even derivatization [29, 38, 44–46, 51]. The elimination of other usually time-consuming steps makes this method high-throughput and relatively cost-effective.

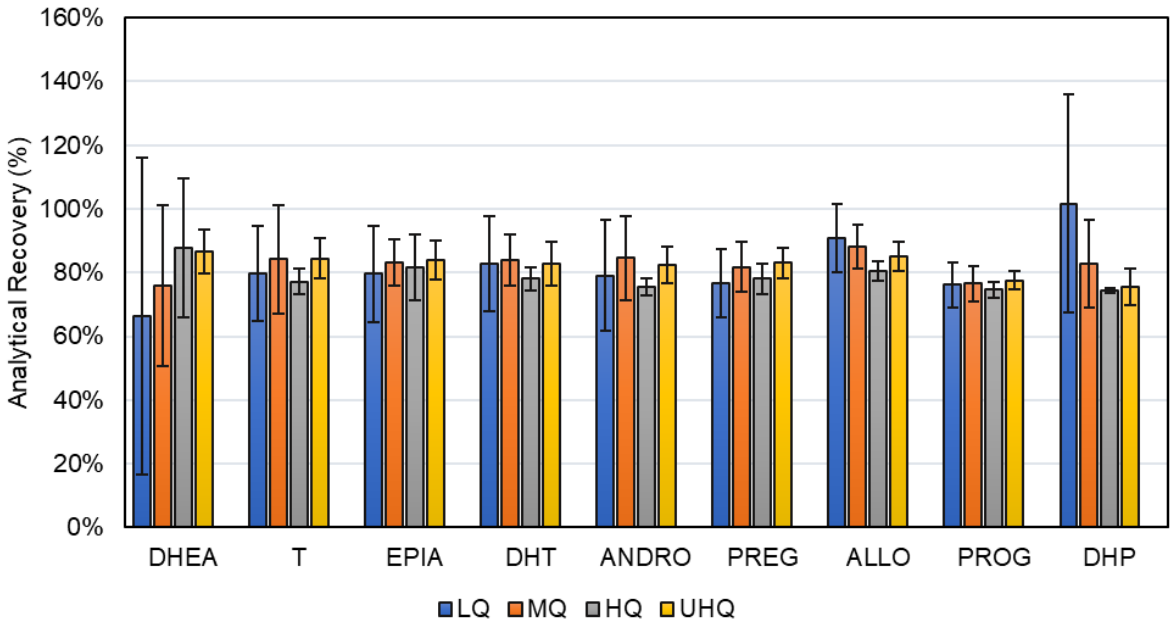


Fig. 4 Analytical recovery of selected steroid analytes in four lots of serum at low (LQ), medium (MQ), high (HQ), and ultra-high (UHQ) quality control levels (n = 12). The abbreviations of the analytes are given in the list of abbreviations.

Another important validation parameter is ME, which can negatively affect the accuracy, precision, or sensitivity of the analytical method [61, 78]. A set of blood serum samples spiked after extraction to four QC levels were used for its determination. In this study, the values of absolute ME of the analysed steroids at all QC levels ranged between 19% (for DHP) and 117% (for DHEA) (Table 7). In addition, the IS-normalised ME was also determined [63], with a maximum CV of 14.4%, which is in accordance with EMA guidelines [58]. For most steroid analytes, the ME is effectively compensated by the ISs used. These results confirm that in addition to matrix-matched calibration curves and optimization of sample preparation, chromatography and mass spectrometry, ISs (structural analogues or stable isotope labeled compounds) can be used to remove or at least reduce ME [78]. The use of ISs increases the robustness of the developed method. The strongest absolute and IS-normalised ME in terms of ion suppression was observed for DHP, specifically from 19 to 24% and from 27 to 33%,

respectively. DHP has the highest Log P value of all the analytes tested, i.e. it is the least polar analyte and has the highest retention on the biphenyl stationary phase of the LC column (Table 1, Fig. 2). Its elution is due to the increasing concentration of organic solvent in the mobile phase (i.e. decreasing polarity). We assume that such a strong ME is due to the elution of DHP at the end of the gradient together with a high proportion of contaminants. Matrix components such as peptides, lipids, salts or urea that elute together with the analyte can interfere with the efficiency of its ionization, either by ion enhancement or, in this case, ion suppression [61, 78]. The reason for the lack of compensation of the ME by the IS may be due to the fact that d_9-PROG was used, which elutes at a different RT than DHP and therefore in an environment containing different interfering substances. Unfortunately, a stable isotopically labeled analogue of DHP was not available in our laboratory. Nevertheless, the developed method allows reliable quantification of DHP, which was confirmed in accuracy and precision testing on blood serum samples. This correction is provided by a matrix-matched calibration curve that is subjected to the same purification and extraction protocol as real samples.

Table 7 Evaluation of matrix effect in human serum

	Absolute ME (SD)[A]				**IS-normalised ME** (CV)[A]			
Analyte	**LQ**	**MQ**	**HQ**	**UHQ**	**LQ**	**MQ**	**HQ**	**UHQ**
DHEA	117 (13.9)	99 (10.9)	100 (15.8)	103 (7.3)	99 (4.5)	100 (5.0)	91 (4.1)	99 (3.1)
T	93 (9.7)	98 (1.8)	98 (7.0)	97 (2.7)	89 (6.9)	90 (4.4)	81 (4.9)	88 (3.8)
EPIA	109 (3.5)	109 (7.7)	109 (9.0)	111 (4.2)	101 (11.3)	101 (3.6)	91 (14.4)	100 (6.6)
DHT	93 (2.4)	90 (4.2)	91 (5.3)	91 (4.5)	86 (10.5)	83 (1.6)	75 (13.6)	82 (7.4)
ANDRO	93 (4.9)	98 (4.8)	102 (1.0)	101 (5.2)	87 (10.1)	90 (3.4)	85 (9.6)	91 (5.7)
PREG	113 (2.4)	113 (5.9)	115 (3.2)	116 (3.4)	96 (2.3)	99 (3.5)	91 (4.7)	99 (3.8)
ALLO	71 (2.4)	71 (3.9)	70 (0.5)	71 (1.6)	113 (4.7)	115 (3.6)	100 (2.8)	107 (2.3)
PROG	74 (2.8)	78 (3.6)	76 (1.9)	79 (2.8)	101 (3.2)	106 (3.2)	97 (3.8)	109 (3.2)
DHP	19 (2.7)	22 (1.8)	24 (1.3)	24 (1.5)	27 (13.3)	30 (4.9)	30 (7.5)	33 (4.8)

ALLO: Allopregnanolone, ANDRO: Androstenedione, DHEA: Dehydroepiandrosterone, DHP: α-Dihydroprogesterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, PREG: Pregnenolone, PROG: Progesterone, T: Testosterone.

[A] The matrix effect (ME), standard deviation (SD), and coefficient of variation (CV) are expressed in percentage. These values were calculated from 4 lots of matrix tested in triplicates (n = 12).

The low (LQ), medium (MQ), high (HQ), and ultra-high (UHQ) levels correspond to to 0.0569, 0.18, 1.8, and 5.6921 pmol/inj., respectively.

Profiling of steroid analytes in serum

Finally, the validated UHPLC–ESI–MS/MS method was applied to the steroid analysis in a selected group of participants. A total of 16 donors with different types of nervous system pathologies were included in this study. Each sample was represented by a triplicate. The participant group consisted of 8 males aged 41–67 years (median 57.5 years) and 8 females aged 21–51 (median 35.5 years). The median and range of the determined endogenous levels of individual analytes are listed in Table 8.

Table 8 Endogenous levels of target steroids in donor serum (n = 16)

Analyte	Median (Range)[a]
DHEA	9.26 (4.16–29.23)
T	4.05 (0.57–30.30)
EPIA	2.00 (2.00–6.01)
DHT	0.99 (0.63–3.38)
ANDRO	2.91 (2.04–5.28)
PREG	2.41 (0.63–6.64)
ALLO	2.00 (2.00–2.00)
PROG	0.32 (0.20–34.82)
DHP	6.33 (6.33–10.52)

ALLO: Allopregnanolone, ANDRO: Androstenedione, DHEA: Dehydroepiandrosterone, DHT: α-Dihydrotestosterone, EPIA: Epiandrosterone, PREG: Pregnenolone, T: Testosterone.

[a] Endogenous levels are given in nmol/L.

The measured concentrations of the analytes in the 16 volunteers corresponded to the expected endogenous levels [55, 79]. Missing analyte levels were replaced by two-thirds of the respective LOQ values [80]. It is important to note that the samples were concentrated several times during the purification and extraction process. Nevertheless, the determination of the lowest endogenous levels in human serum was difficult. However, the developed method can be reliably applied to some physiological conditions in which natural levels increase several-fold. For example, the level of ALLO fluctuates in women of reproductive age from less than 1 to 5 nmol/L (depending on the phase of the menstrual cycle), at the end of the third trimester of pregnancy its level can even reach almost 160 nmol/L [74, 81].

Other studies also used the LC–MS/MS method for the determination of steroids. Zhang et al. (2019) developed and validated a method based on UHPLC–MS/MS for the analysis of selected endogenous and synthetic estrogens and progestins in serum [44]. Unlike the method described here, in this case, more than three times the volume of human serum is used. Even in other cases the sample consumption is several times higher [17, 38, 40, 42]. When small sample volumes (100 μL) are used, the sample preparation for the analysis is usually more time-consuming and involves, for example, protein precipitation, liquid-liquid extraction and

derivatisation steps [29]. Compared to other methods used for the steroid analysis, the described method works with a very small sample volume (150 μL) and does not require any specific purification techniques or chemical derivatisation. Other published methods also use such small volumes of blood serum (100 μL) for the steroid analysis [36]. Yesildal et al. (2019) developed a method based on isotope dilution UHPLC–MS/MS to profile a panel of steroids most commonly analysed in clinical laboratories (aldosterone, corticosterone, cortisol, cortisone, 11-deoxycortisol, ANDRO, DHEA, dehydroepiandrosterone sulfate, DHT, estradiol, 17α-hydroxy progesterone, PROG, and T). Sample preparation also involves only a precipitation step by precipitant solution (ISs, zinc sulfate solution, MeOH), but the sample injection for the analysis is 25 μL. In the case of our method, a small injection volume (only 2 μL) allows a reanalysis of the sample. Our method allows for simultaneous profiling of endogenous levels of progestin and androgen representatives in human blood serum, which was confirmed on a set of volunteer samples (different age and sex). Due to its reliability and simplicity, this method could be used in epidemiological studies.

Conclusion

Our research presents a novel complex method for the determination of selected NASs, including four progestins (PREG, PROG, ALLO, and DHP) and five androgens (DHEA, T, DHT, ANDRO, and EPIA) in human serum within one analytical run. Unlike the collection of cerebrospinal fluid, obtaining blood serum is relatively easy and less invasive and stressful. Therefore, the discovery of new biomarkers of neurodegenerative diseases in this type of sample would bring considerable advantages. Our developed and validated method using very small sample and injection volumes has many potential applications. To illustrate, it can serve as a tool for monitoring the differences between the levels of steroid hormones in different physiological or pathological conditions. The demonstrated method can be an ideal instrument for finding new biomarkers useful in the prevention, diagnosis, or monitoring of conditions associated with changes in NAS levels, for a better understanding of the pathophysiology of certain diseases, as well as for discovering new drugs or developing new therapeutic approaches.

Declarations

Authors' contributions

Michal Kaleta performed the measurements, processed the experimental data, and wrote the original draft of the manuscript. Miroslav Kvasnica synthesized some steroid standards. Jana Oklestkova, Petr Kaňovský, Ondřej Novák, and Miroslav Strnad reviewed and edited the structure of the article. All authors have read and agreed to the published version of the manuscript.

Founding

This work was financially supported by the student project IGA_PrF_2023_031 of the Palacký University Olomouc.

Conflicts of Interest

The authors have no conflict of interest to declare.

Ethics approval

Ethics approval for this study was granted according to University Hospital Olomouc standard SM-L031, and ethics committee reference numbers: 139/10 and 76/15.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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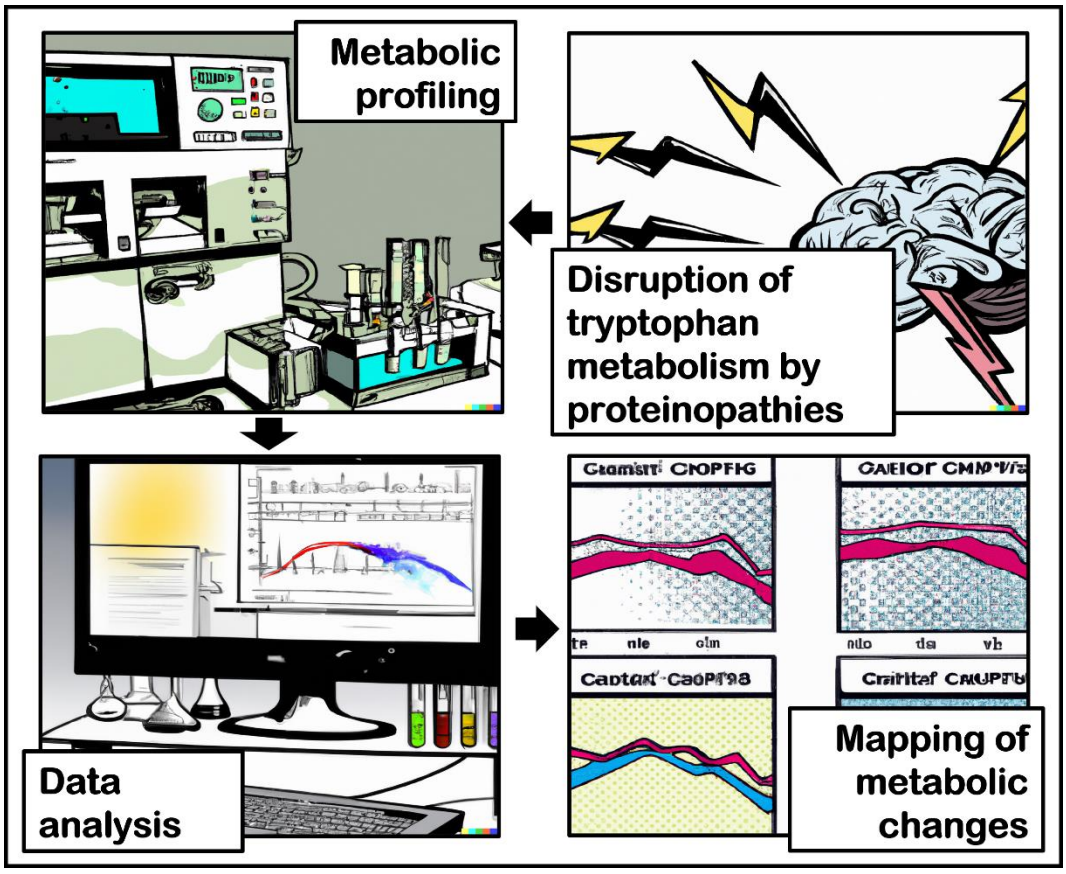
Supplement IV

Patients with Neurodegenerative Proteinopathies Exhibit Altered Tryptophan Metabolism in the Serum and Cerebrospinal Fluid



Patients with Neurodegenerative Proteinopathies Exhibit Altered Tryptophan Metabolism in the Serum and Cerebrospinal Fluid

Michal Kaleta[a,b,c*], Eva Hényková[a,b,c], Kateřina Menšíková[b,c], David Friedecký[d], Aleš Kvasnička[d], Kateřina Klíčová[b,c], Dorota Koníčková[b,c], Miroslav Strnad[a,b,c], Petr Kaňovský[b,c], Ondřej Novák[a*]

[a]Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacky University, Šlechtitelů 27, 783 71, Olomouc, Czech Republic

[b]Department of Neurology, University Hospital Olomouc, 779 00 Olomouc, Czech Republic

[c]Department of Neurology, Faculty of Medicine and Dentistry, Palacky University, 779 00 Olomouc, Czech Republic

[d]Laboratory for Inherited Metabolic Disorders, Department of Clinical Biochemistry, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Zdravotníků 248/7, 779 00 Olomouc, Czech Republic

Correspondence*

Michal Kaleta
E-mail: michal.kaleta@upol.cz

Ondřej Novák
E-mail: novako@ueb.cas.cz

Abstract

Some pathological conditions affecting the human body can also disrupt metabolic pathways and thus alter the overall metabolic profile. Knowledge of metabolic disturbances in specific diseases could thus enable differential diagnosis of otherwise similar conditions. This work therefore aimed to comprehensively characterize changes in tryptophan metabolism in selected neurodegenerative diseases. Levels of 18 tryptophan-related neuroactive substances were determined by high throughput and sensitive ultra-high-performance liquid chromatography–tandem mass spectrometry in time-linked blood serum and cerebrospinal fluid samples from 100 age-matched participants belonging to five cohorts: healthy volunteers (n = 21) and patients with Lewy body disease (Parkinson's disease and dementia with Lewy bodies; n = 31), four-

repeat tauopathy (progressive supranuclear palsy and corticobasal syndrome; *n* = 10), multiple system atrophy (*n* = 13), and Alzheimer's disease (*n* = 25). Although these conditions have different pathologies and clinical symptoms, the discovery of new biomarkers is still important. The most statistically significant differences (with p-values of ≤ 0.05 to ≤ 0.0001) between the study cohorts were observed for three tryptophan metabolites: L-kynurenine in cerebrospinal fluid and 3-hydroxy-L-kynurenine and 5-hydroxy-L-tryptophan in blood serum. This led to the discovery of distinctive correlation patterns between the profiled cerebrospinal fluid and serum metabolites that could provide a basis for differential diagnosis of neurodegenerative tauopathies and synucleopathies. However, further large-scale studies will be needed to determine the direct involvement of these metabolites in the studied neuropathologies, their response to medication, and their potential therapeutic relevance.

Keywords: Tryptophan metabolic pathway, Neurodegenerative disease, Parkinson's disease, Alzheimer's disease, Serum, Cerebrospinal fluid

1. Introduction

The kynurenine pathway plays a key role in L-tryptophan (TRP) metabolism and is the source of many substances essential for the human body [1, 2]. In mammals, the majority (~95%) of ingested TRP is metabolized via this route [3, 4]. The products of this pathway, the so-called kynurenines, include both neurotoxic substances such as 3-hydroxykynurenine (3-OH-KYN) and neuroprotective substances such as kynurenic acid (KA). The remaining minor fraction of ingested TRP is metabolized via the methoxyindole, kynuramine, and intestinal bacterial indole pathways [2, 5-7]. Kynurenine metabolites have been linked to a number of important physiological processes including inflammation, immune responses, and neurotransmission [3]. It has also been suggested that disruption of the kynurenine metabolic pathway contributes significantly to the development of metabolic syndrome, Parkinson’s disease (PD), and Alzheimer’s disease (AD) [8]. Moreover, there is evidence that the initial enzymes of this pathway (hepatic tryptophan 2,3-dioxygenase, EC 1.13.11.11; extrahepatic indoleamine 2,3-dioxygenase, EC 1.13.11.52) are stimulated by glucocorticoids and pro-inflammatory cytokines, prompting suggestions that it is activated preferentially during chronic stress and infection [9]. Under normal conditions, these enzymes are expressed weakly and only in certain areas of the brain [10]. The activity of the kynurenine pathway in the brain therefore depends mainly on the transport of L-kynurenine (KYN) and 3-OH-KYN from peripheral sources across the blood-brain barrier. However, not

all kynurenine pathway metabolites are equally able to cross the blood-brain barrier, so dysregulation of kynurenine metabolism in the periphery and the central compartment can have different functional consequences [8].

Unsurprisingly, most research in this area has focused on the two most common neurodegenerative diseases: PD and AD [11]. Changes in kynurenine metabolism have been characterized in some detail in both PD [8, 10-20], and AD [1, 8, 10, 21-23], revealing some notable characteristic trends. First, PD patients exhibit reduced plasma [16, 20] and serum [8, 10, 12, 17, 19] concentrations of TRP relative to controls. Reduced serum TRP levels may be associated with the psychiatric problems that occur in PD patients [19]. Increased degradation of TRP in peripheral blood leading to reduced serum TRP levels has also been observed in AD [21]. Additionally, some observations indicate that PD patients have reduced KYN levels in both plasma [16] and serum [11, 17] together with elevated KYN levels in the cerebrospinal fluid (CSF) [15]. Moreover, several authors have reported elevated levels of 3-OH-KYN in diverse biological matrices of PD patients, including serum [11, 17], plasma [14], and CSF [13, 15]. However, Oxenkrug et al. (2017) reported that serum KYN concentrations in PD patients were higher than in a control group [8]. These authors were unable to determine levels of 3-OH-KYN because of the low sensitivity of their chosen analytical method. Other metabolic changes observed are described in the discussion section.

Conventional methods for diagnosing neurodegenerative diseases are mainly based on brain imaging but have been enriched in recent years by the possibility of monitoring various predictive, prognostic, or diagnostic biomarkers, especially high molecular weight protein biomarkers [24, 25]. There are several established biomarkers for neuropathologies and new ones have been proposed [24, 26, 27]. Changes in the levels of low molecular weight neurotransmitter metabolites in the serum [8, 10-12, 17, 19, 21, 22], plasma [1, 14, 16, 18, 20] and CSF [1, 10, 12-15, 18, 20, 23] of AD and PD patients have also been studied in detail. However, metabolic dysregulation of low molecular weight metabolites is comparatively under-studied, particularly in less common neuropathologies, and therefore warrants further investigation.

In this study, we used a highly efficient and high-throughput ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for metabolic profiling of 18 TRP-related substances [2], including metabolites of the kynurenine, methoxyindole, and tryptamine and indoles pathways. We analysed these substances in the serum and CSF of a healthy control (HC) group and patient cohorts with PD, dementia with Lewy bodies (DLB),

progressive supranuclear palsy (PSP), corticobasal syndrome (CBS), multiple system atrophy (MSA), and AD. Our basic hypothesis was that the levels of these metabolites may be altered by certain pathological processes affecting the nervous system. The aim of our study was therefore to comprehensively quantitate a wide set of TRP metabolites spanning several metabolic pathways in parallel in two compartmentally separated biological fluids. We also analysed biological samples representing several pathological conditions of the nervous system. This is notable because most previously reported studies have had a much narrower focus, examining only a few analytes and often only a single sample type. Additionally, the available literature data on different metabolites and biological matrices are derived from a wide range of analytical methods, which can result in inconsistent outputs that make it difficult to draw meaningful conclusions when comparing different conditions. We therefore aimed to comprehensively map changes in every TRP metabolic pathway in multiple neuropathologies using a single highly selective and robust analytical method whose high sensitivity enables the mapping of analytes at femtomolar levels.

2. Results

Eighteen TRP metabolites were analysed in blood serum and CSF samples representing selected neurodegenerative proteinopathies using a UHPLC–MS/MS-based method. The concentrations of eight analytes (*N*-methylserotonin, *N*-Me-S; tryptamine, TA; *N*-methyltryptamine, *N*-Me-TA; 5-methoxytryptamine, 5-MeO-TA; *N*-acetylserotonin, *N*-Ac-S; 6-hydroxymelatonin, 6-OH-M; melatonin, M; N^1-acetyl-N^2-formyl-5-methoxykynuramine, AFMK) were below the limit of detection or quantification in all or most participants and were therefore excluded from the statistical evaluation. The remaining ten analytes (TRP; 3-OH-KYN; serotonin, S; KYN; 5-hydroxy-L-tryptophan, 5-OH-TRP; 3-hydroxy-anthranilic acid, 3-OH-AA; 5-hydroxyindole-3-acetic acid, 5-OH-IAA; KA; anthranilic acid, AA; indole-3-acetic acid, IAA) could be quantified and were included. Missing values were imputed using the k-nearest neighbours algorithm. The original data were tested for normality, which was achieved after log-transformation.

Parametric ANOVA with post hoc testing by the Holm-Shidak multiple comparison test was used to compare the study groups. The serum 5-OH-TRP (Figure 1A) concentration in the HC group differed significantly from those in the Lewy body disease (LBD) ($p = 0.00012$) and MSA groups ($p = 0.00722$), while that of the AD group differed significantly from those in the LBD ($p < 0.00001$), four-repeat tauopathy (4R-Tau) ($p = 0.02149$), and MSA ($p = 6 \cdot 10^{-5}$)

groups. The serum 3-OH-KYN (Figure 1B) concentrations of the LBD and AD groups also differed significantly (p = 0.00407), as did the CSF KYN (Figure 1C) concentrations of the LBD and HC groups (p = 0.01917). Here it should be noted that the statistical significance of observed differences depends heavily on the number of samples in each group being compared. The effects of treatments on TRP metabolite concentrations were also evaluated using the Mann-Whitney U test in the LBD (Figure 1D, E) and MSA (Figure 1F, G) patient groups. Treatment significantly increased the concentrations of 5-OH-TRP (p = 0.0037) and 3-OH-KYN (p = 0.0373) in LBD patients. Similar trends existed in the MSA group, but statistical significance was not achieved in this case due to limited number of samples.

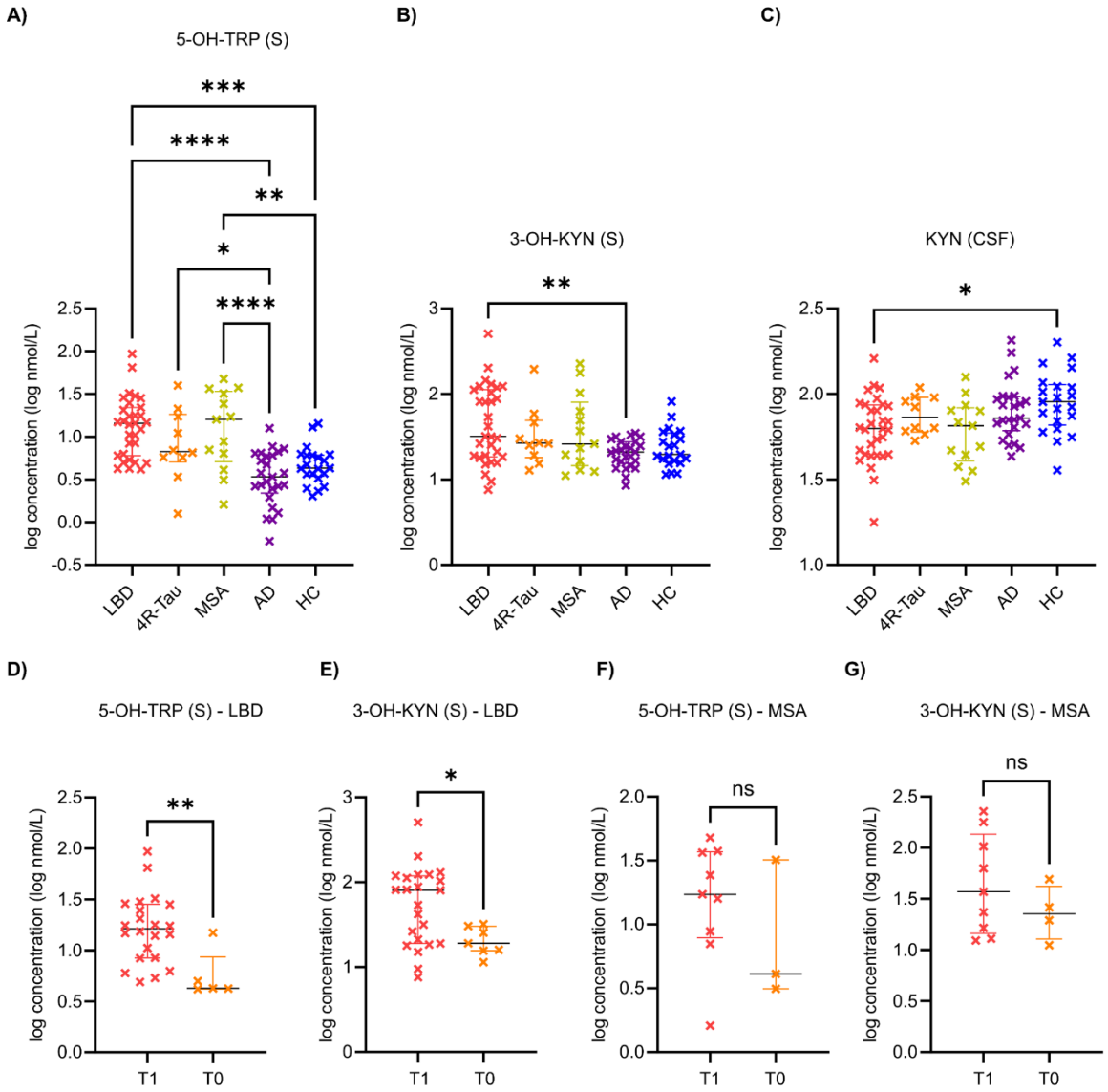


Figure 1. Serum 5-hydroxy-L-tryptophan (5-OH-TRP; A), serum 3-hydroxy-L-kynurenine (3-OH-KYN; B), and CSF kynurenine (KYN; C) concentrations in the Lewy body disease (LBD;

n = 31), four-repeat tauopathy (4R-Tau; *n* = 10), Multiple system atrophy (MSA; *n* = 13), Alzheimer's disease (AD; *n* = 25), and healthy control (HC; *n* = 21) groups. Serum levels of 5-OH-TRP (D, F) and 3-OH-KYN (E, G) in treated (T1) and untreated (T0) LBD (D, E) and MSA (F, G) patients. Data are expressed as log-transformed concentrations (log nmol/L). Asterisks (*, **, ***, and ****) denote p-values ≤ 0.05, ≤ 0.01, ≤ 0.001, and ≤ 0.0001, respectively.

The relationships between the concentrations of ten analytes in the serum and the CSF of each patient group were evaluated based on Pearson correlations. Heat maps of these correlations are presented in Figure 2, where red and blue fields correspond to positive and negative correlations, respectively, and the strengths of the correlations are indicated by the intensity of the coloration and shown explicitly using numbers. Several statistically significant strong, moderate, and weak correlations were found, and there were some clearly different trends within the studied groups. The HC group exhibited only one strong correlation: serum KYN concentrations correlated positively with those in the CSF (r = 0.80). The AD, LBD, and MSA groups had a wider range of positive correlations. In AD patients, there were strong positive correlations between the concentrations of KYN in the serum and CSF (r = 0.74), TRP and 5-OH-TRP in CSF (r = 0.74), KYN and KA in CSF (r = 0.79), and 5-OH-IAA and IAA in CSF (r = 0.77). The LBD group exhibited strong positive correlations between the serum and CSF concentrations of 3-OH-KYN (r = 0.89) and IAA (r = 0.75). In addition, there were strong positive correlations between the concentrations of several metabolites within the CSF, including TRP and KYN (r = 0.75), TRP and 5-OH-TRP (r = 0.76), 3-OH-KYN and 3-OH-AA (r = 0.78), and KYN and 3-OH-AA (r = 0.75). MSA samples exhibited strong positive correlations between serum 3-OH-KYN and 5-OH-TRP (r = 0.74), serum and CSF 3-OH-KYN (r = 0.78), serum and CSF IAA (r = 0.89), CSF 3-OH-KYN and 3-OH-AA (r = 0.72), and CSF 3-OH-AA and KA (r = 0.83). Interestingly, the 4R-Tau group differed significantly from the others in that it had many negative correlations (Figure 2), including strong negative correlations between serum TRP and CSF KYN (r = -0.70), serum 3-OH-KYN and IAA (r = -0.73), serum 3-OH-KYN and CSF 3-OH-AA (r = -0.70), serum 3-OH-KYN and CSF AA (r = -0.81), serum 3-OH-KYN and CSF IAA (r = -0.74), serum and CSF S (r = -0.88), serum 5-OH-TRP and CSF 3-OH-AA (r = -0.73), and serum 5-OH-TRP and 5-OH-IAA (r = -0.82).

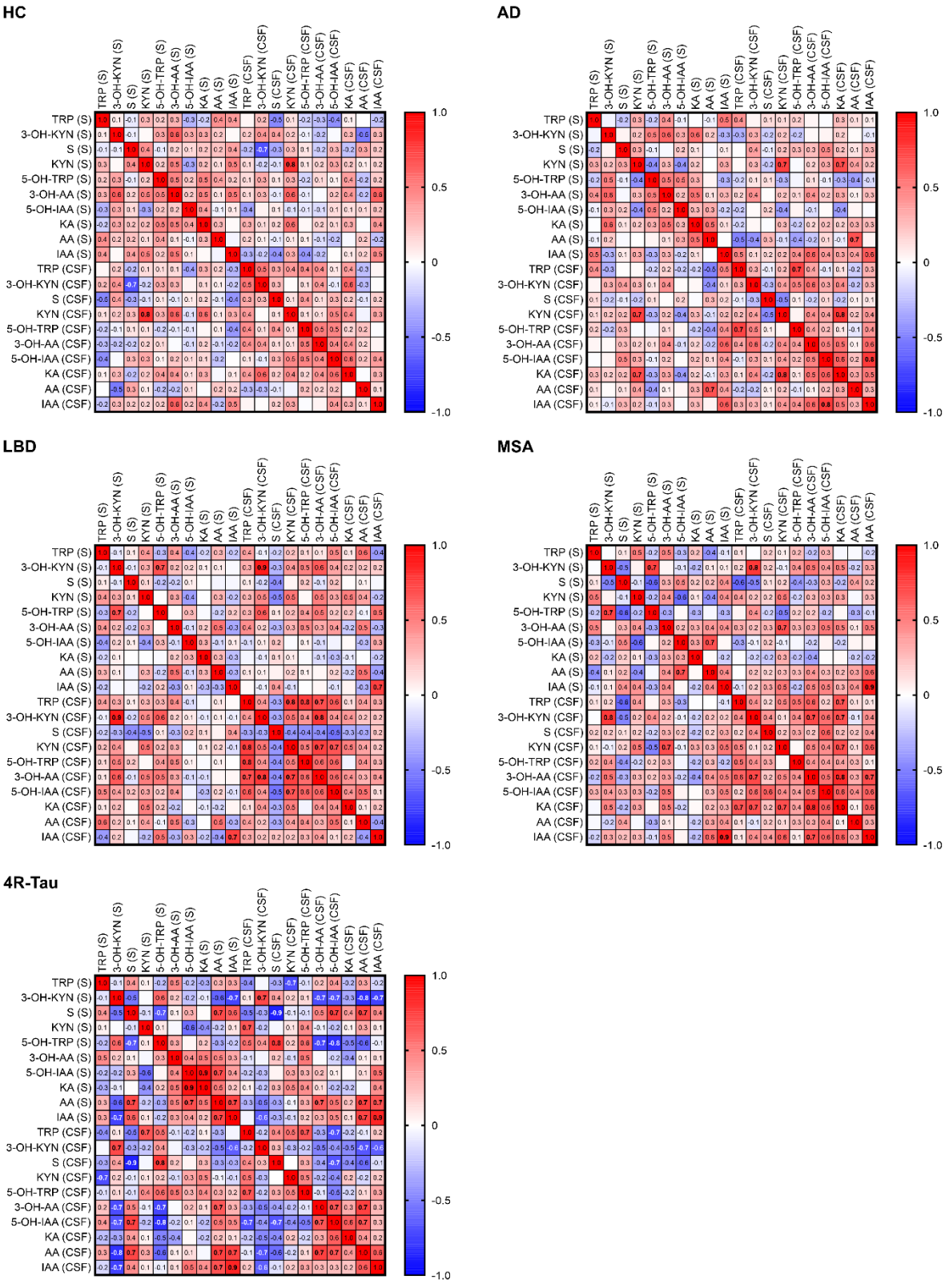


Figure 2. Pearson correlation heatmaps of selected tryptophan metabolites in the healthy control (HC; *n* = 21), Alzheimer's disease (AD; *n* = 25), Lewy body disease (LBD; *n* = 31), Multiple system atrophy (MSA; *n* = 13), and four-repeat tautopathy (4R-Tau; *n* = 10) groups.

Strong significant positive and negative correlations ($r \geq 0.7$ or $r \leq -0.7$) are marked in bold. Red and blue fields correspond to positive and negative correlations, respectively. The metabolites are denoted in accordance with the list of abbreviations.

3. Discussion

This study comprehensively mapped changes in TRP metabolism via the kynurenine, methoxyindole, kynuramine, and intestinal bacterial indole pathways in time-matched CSF and serum samples from patient groups representing four degenerative neuropathologies: AD, LBD, MSA, and 4R-Tau. Such comprehensive mappings are valuable because monitoring of specific metabolic changes (i.e., changes in the levels of selected biomarkers) could facilitate differential diagnosis of these disease states. We found no statistically significant between-cohort differences in the concentrations of TRP, (S, 3-OH-AA, 5-OH-IAA, KA, AA and IAA in either the serum or the CSF. However, significant differences were observed for the serum concentrations of 5-OH-TRP and 3-OH-KYN as well as the CSF concentration of KYN.

Preliminary data indicate that 5-OH-TRP improves global sleep quality in patients with PD and REM sleep behaviour disorder, which are often associated with each other [28]. Moreover, 5-OH-TRP supplementation reportedly reduced depressive symptoms in PD [29] and significantly reduced L-DOPA-induced dyskinesia in PD [30]. Earlier studies examined the use of this aromatic amino acid in the treatment of depression [31, 32] and showed that levels of 5-OH-TRP in Alzheimer-type dementia CSF samples were lower than in matched controls [23]. Additionally, Havelund et al. (2017) reported that 5-OH-TRP levels in blood plasma from PD patients receiving L-DOPA (dyskinetic, $n = 10$; non-dyskinetic, $n = 8$) were roughly twice those in PD patients not receiving L-DOPA ($n = 8$) and controls ($n = 14$) [18]. The authors attributed this to the fact that PD patients are treated with peripheral decarboxylase inhibitors and L-DOPA, which is a substrate of aromatic amino acid decarboxylase (EC 4.1.1.28; DOPA decarboxylase; AADC) – the enzyme that catalyses the metabolic conversion of 5-OH-TRP into S. Substrate competition between L-DOPA and 5-OH-TRP at AADC could thus reduce the rate of 5-OH-TRP conversion and increase its concentration in the body. We found that serum 5-OH-TRP levels were significantly higher in the LBD group (i.e., patients with PD and DLB) than in the HC and AD groups (Figure 1A). Most LBD patients (24 out of 31) were also taking some form of L-DOPA and peripheral decarboxylase inhibitors at the time of blood and CSF sampling. Dividing the LBD group into medicated and unmedicated patients revealed that serum 5-OH-TRP concentrations were significantly higher in patients receiving antiparkinsonian drugs than

in those not receiving such treatment (Figure 1D). Our findings thus agree with those of Havelund et al. (2017).

A similar increase in 5-OH-TRP levels was observed in the 4R-Tau and MSA groups (Figure 1A). The limited number of 4R-Tau patients meant that this group could not be further divided to evaluate the effect of treatment, and no statistically significant treatment effect was observed for the MSA group, although it should be noted that this group had a very small number of untreated patients (Figure 1F). Despite the apparently similar trends in these groups, the origin of the elevated 5-OH-TRP levels in MSA may differ from that in LBD patients. There have been comparatively few studies on metabolic changes in 4R-Tau or MSA but some works have measured concentrations of polyamines (e.g. putrescine, cadaverine, spermidine) [33], catechols (dopamine, norepinephrine, L-DOPA, dihydroxyphenylacetic acid, and dihydroxyphenylglycol) [34], selected amino acids (L-glutamate, L-arginine, and L-citrulline levels) [35], polyunsaturated fatty acids (e.g. arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid) [36], nitrate [37], coenzyme Q10 [38], and glutathione [39] in MSA patients. Additionally, Kaiserova et al. (2021) analysed 5-OH-IAA in the CSF of patients with PD, MSA, PSP and CBS [40], revealing that levels of this metabolite did not differ significantly from controls in the tauopathies PSP and CBS but were significantly reduced in the synucleopathies PD and MSA. The authors suggested that this may be because synucleopathies cause more severe damage to the serotogenic system. While some larger metabolic studies [41, 42] with broader scopes have been reported, we are not aware of any earlier studies that have comprehensively mapped TRP metabolism or any of its individual pathways in MSA and 4R-Tau.

Drugs are not the only factors that may affect TRP metabolite levels in the studied pathologies. For example, the elevated 5-OH-TRP concentrations in PD patients may result from other metabolic changes such as reduced metabolic conversion of 5-OH-TRP. This possibility is supported by the results of Nagatsu and Sawada (2007), who found that the activities and/or mRNA and protein levels of AADC and other enzymes are reduced in the brains of human PD patients [43]. Similarly, Tehranian et al. (2006) observed inhibition of AAAD enzyme activity in dopaminergic cells overexpressing alpha-synuclein [44]. The authors attributed this effect to interactions between AADC and alpha-synuclein, which forms in Lewy bodies and Lewy neurites during PD. Dietary factors may also influence TRP metabolite levels because TRP is an essential amino acid that humans cannot biosynthesize [45]. The relationship between dietary TRP consumption and its levels in the body will thus affect the levels of its derived metabolites. Further work is needed to determine whether the increased 5-OH-TRP levels observed in

various proteinopathies are mainly due to medication (e.g., use of L-DOPA), the pathological process itself, or a combination of these factors.

Our results also suggest that serum 3-OH-KYN could be a target for the treatment of the neurodegenerative diseases examined in this study. This metabolite is known to be neurotoxic and to induce mitochondrial dysfunction and cell death via free radical generation and oxidative stress, possibly in synergy with the excitotoxin quinolinic acid [14]. Free radical generation and increased oxidative activity both cause neuronal damage [11], suggesting that 3-OH-KYN may be involved in the pathogenesis of PD. This suggestion is supported by clinical observations and multiple genome-wide association studies that have revealed an association between neurodegeneration and changes in the kynurenine pathway [14]. Our results showed that LBD (PD and DLB) patients had significantly higher serum levels of 3-OH-KYN than AD patients (Figure 1B) and exhibited a similar but non-significant increase relative to the HC cohort. This finding is consistent with previous studies reporting dysregulation of the kynurenine pathway in PD patients. For example, Heilman et al. (2020) found that plasma levels of 3-OH-KYN were significantly elevated in PD patients, most of which did not exhibit dyskinesia [14]. The authors attributed this to reduced activity of the enzyme kynureninase (EC 3.7.1.3), which catalyses the conversion of 3-OH-KYN into 3-OH-AA. This hypothesis is consistent with the reduced plasma levels of 3-OH-AA observed in their study and, together with the other findings mentioned above, suggests that 3-OH-KYN could serve as a plasma biomarker of PD severity and/or progression. The development of such a biomarker could obviate the need for CSF sampling, which would greatly benefit patients because obtaining blood samples is easier and also less risky and invasive than collecting CSF by lumbar puncture. Elevated plasma levels of 3-OH-KYN have also been observed in PD patients with dyskinesia who were being treated with L-DOPA [18], in accordance with other reports [11, 17]. Similarly increased levels of this strong excitotoxin have also been observed in the CSF of PD patients [13, 15] and in certain brain regions in PD, namely the *putamen*, *prefrontal cortex*, and *pars compacta* of the *substantia nigra* [46]. However, we observed no significant changes in the CSF concentration of 3-OH-KYN. This is consistent with an earlier study [14] in which it was suggested that the differing reported trends in the serum and CSF concentrations of 3-OH-KYN may be due to differences in its production or metabolism in the peripheral and central compartments. The ratio of 3-OH-KYN and KA was also significantly increased in the CSF of PD patients, which supports a proposed therapeutic strategy based on blocking the production of excitotoxic 3-OH-KYN and promoting the synthesis of neuroprotective KA [13]. Disruption of the kynurenine pathway could contribute

to the clinical progression of PD because some of its metabolites increase oxidative stress and cytokine-mediated neuroinflammation in the CNS [15], which again suggests that 3-OH-KYN could be a good therapeutic target for PD treatment. However, it is still important to consider whether elevated 3-OH-KYN levels are a cause or a consequence of PD.

We also considered the possibility that serum 3-OH-KYN levels could be affected by specific therapies because previous studies have demonstrated that certain medications (e.g., antidepressants and L-DOPA) can alter the concentrations of kynurenine pathway metabolites [10, 18]. The published data on this issue are somewhat contradictory, however, because Sorgdrager et al. (2019) found that medication did not affect the levels of six kynurenine pathway metabolites (TRP, KYN, 3-OH-KYN, KA, xanthurenic acid, and quinolinic acid) in PD and AD [10]. Similarly, Oxenkrug et al. (2017) found no differences between untreated and L-DOPA treated PD patients with respect to the plasma levels of TRP, KYN, AA, KA, and 3-OH-KYN, and therefore did not further stratify these patient groups [8]. Nevertheless, we found that therapy influenced 3-OH-KYN levels in LBD and MSA patients (Figure 1E, G), although a statistically significant effect was only observed for the LBD group; the failure to reach significance for the MSA group may be due to its low number of samples. The evidence that established anti-PD treatments may increase levels of neurotoxic 3-OH-KYN suggests that complementary treatments targeting this metabolite could be valuable in the management of PD.

Another interesting metabolite is KYN, whose concentration in the CSF of untreated patients with PD (n = 16) was significantly lower than in controls (n = 16) [47]. A similar reduction was observed previously in PD patients treated with L-DOPA/carbidopa [48], although Iwaoka et al. (2020) reported that KYN levels in the CSF of PD patients (n = 20; 18 without antiparkinsonian medication, 2 treated with L-DOPA) were significantly higher than in controls (n = 13) [15]. We found that the KYN concentrations in the CSF of LBD patients (n = 31) were significantly lower than in the HC group (n = 21) (Figure 1C). However, our patient cohorts were larger than those examined by Iwaoka et al. (2020), and we used mass spectrometric rather than electrochemical detection. A similar trend of slightly reduced KYN and KA concentrations was observed in human postmortem samples of the *prefrontal cortex, putamen,* and *substantia nigra* of PD patients [4, 46], and low KYN concentrations have been found in the plasma of PD patients [16] and the serum of PD patients [11, 12, 17]. However, Oxenkrug et al., (2017) reported elevated serum KYN levels in PD patients [8]. The inconsistencies between these findings may be partly due to the use of different analytical approaches and differences in the studied cohorts' clinical

characteristics (e.g., patient age, disease duration, severity, and gender representation). It should also be noted that TRP metabolism via the kynurenine pathway may be partly regulated by the gut microbiota, which has important implications for CNS functionality [49].

Other studies have also looked for differences between several neuropathological cohorts, but most of these studies have focused on the analysis of protein markers. There are studies investigating different patterns of CSF glial markers in DLB and AD patients [50] or plasma protein biomarkers of neurodegeneration in DLB, AD, frontotemporal dementia, and PSP [51]. A similar experimental design to our work is described in the work of Lourenco et al. (2021) [52]. They focused on the analysis of a panel of 50 analytes including neurotransmitters, cytokines, chemokines, and hormones in the CSF of control participants without dementia and patients with DLB, mild cognitive impairment, and AD. The shared analyte was S, for which, as in our case, no statistically significant changes were observed between patient groups.

Our findings and those reported previously suggest that a testing panel of neuroactive TRP metabolites could have significant diagnostic benefits. For example, the high proportion of negative correlations observed in 4R-Tau samples (see Figure 2) could facilitate the development of a tool for differential diagnosis of neurodegenerative parkinsonism that would distinguish tauopathies (PSP and CBS) from synucleopathies (DLB, PD, and MSA) [53]. Moreover, it may be possible to link observed differences in the concentrations of the panel metabolites to the differing pathophysiologies of these conditions. Changes in different phases of TRP metabolism have been shown to have differing effects on the potential neurotoxicity of protein aggregates including beta amyloid and pathological alpha-synuclein or tau protein aggregates [54-57]. Therefore, alterations in specific stages of the kynurenine metabolic pathway could contribute to the development of different neurodegenerative proteinopathies while also altering the spectrum of TRP metabolites present in CSF or serum. We believe that our discovery of negative correlations between TRP metabolite levels in 4R-Tau patients could be a first step towards the development of tools enabling differential diagnosis of synucleinopathies and tauopathies, but the realisation of such a tool will require further elucidation of the influence of biochemical changes in both groups. To this end, it would be very desirable to measure TRP metabolite concentrations in a larger sample set; the resulting data could reveal differences in the concentrations of these metabolites across the spectrum of neurodegenerative proteinopathies and thus provide a robust basis for their differentiation.

It should be emphasized that the result of our work is not the discovery of specific biomarkers, but the mapping of trends that occur in the studied neurodegenerative entities. As we suggest, finding effective tools for differential diagnosis will not be possible without a deeper understanding of the close relationship between the pharmacological treatment of given conditions and the metabolic changes they cause. This issue should definitely be considered in further research. Based on our observations, we believe that a panel of several relevant biomarkers, preferably both low and high molecular weight, will need to be designed to provide a reliable diagnostic tool. In addition, parallel analysis of multiple biofluids may also be very beneficial.

4. Conclusion

We have successfully quantified the concentrations of 10 TRP-related metabolites in time-linked blood serum and CSF samples in target cohorts representing multiple neurodegenerative proteinopathies and healthy controls. Significant differences between the cohorts were identified, particularly with respect to CSF concentrations of KYN and serum concentrations of 3-OH-KYN and 5-OH-TRP. No significant differences were observed for the CSF and serum concentrations of TRP, 3-OH-AA, 5-OH-IAA, KA, AA, S, and IAA, however. We also observed significant effects of medication on TRP metabolite concentrations in the studied pathologies – in particular, L-DOPA treatment had a notable effect on serum 5-OH-TRP concentrations. A major finding of this study is the discovery of condition-specific patterns of correlations between the serum and CSF concentrations of the studied metabolites, which may eventually enable easy discrimination between tauopathies (PSP and CBS) and synucleopathies (DLB, PD, and MSA). Further testing of larger patient cohorts and longitudinal studies will be needed to identify and validate reliable biomarkers for this purpose. The statistical significance of observed differences depends heavily on the number of samples in each group being compared, so future studies with a larger number of participants will be definitely needed to confirm our results. We are also aware of the fact, that the disease progression together with the continuous pharmacological treatment should certainly jeopardize the results of performed CSF examinations; this would deserve future large, double-blind, and long-term studies targeting the candidate metabolite biomarkers.

Notable strengths of this study include the simultaneous metabolic profiling of a relatively high number of analytes (18 metabolites) in time-linked human serum and CSF samples from all participants and the inclusion of multiple nervous system pathologies: LBD, 4R-Tau, MSA,

and AD. Previous studies in this area have typically focused on fluctuations in a smaller range of TRP metabolites and only examined PD or AD; to our knowledge, this is the first comprehensive study on the metabolic dysregulation of TRP metabolism in proteinopathies. As this study was only observational and thus provides no basis for inference of direct causal relationships, further research is needed to clarify the relationships described herein. Metabolite levels were not corrected for body mass index, which is a limitation of the study and could be investigated in more detail on larger cohorts of participants in the future. Overall, however, the results obtained show that TRP metabolism is impaired in different ways by various pathological conditions affecting the nervous system and by the pharmacological interventions used to treat these conditions, leading to distinct effects on the concentrations of TRP metabolites in the blood and CSF.

5. Material and methods

5.1 Chemicals and reagents

The deuterated internal standards D_4-S, D_3-5-OH-TRP, D_4-TA, D_5-TRP, D_4-5-MeO-TA, D_5-5-OH-IAA, D_5-KA, D_4-AA, D_4-M, and D_4-6-OH-M were purchased from C/D/N Isotopes (Canada). D_5-IAA was obtained from Olchemim Ltd. (Czech Republic), D_6-KYN from Cambridge Isotope Laboratories (USA), and [$^{13}C_2{}^{15}N_1$]-3-OH-KYN and D_3-3-OH-AA from Toronto Research Chemicals (Canada). The internal standards D_3-*N*-Me-S, D_3-*N*-Me-TA, D_3-*N*-Ac-S, and D_3-AFMK were synthesized using published procedures [58, 59]. Corresponding unlabelled standards, bovine serum albumin, and formic acid were purchased from Sigma Aldrich (USA). A CSF calibrator was purchased from Tocris Bioscience (UK). All solvents were gradient grade for LC or hypergrade for LC–MS (Merck Millipore, Germany). Argon was obtained from Linde Industrial Gases (Czech Republic). All other used chemicals were purchased from Lach-ner (Czech Republic).

5.2 Study participants

The study was approved by the ethics committee of the Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc. Ethics approval for this study was granted according to University Hospital Olomouc standard SM-L031, and ethics committee reference numbers: 139/10 and 76/15. All participants were informed of the study's purpose and design, and signed informed consent forms. Blood serum and CSF samples were collected, pre-treated, transported, and stored under standardized conditions. Patient

recruitment, sample collection, and laboratory analyses were performed between 2016 and 2022. The study was not pre-registered.

The study included a total of 100 age-matched male and female participants that were divided into HC and six groups representing the following core clinical entities, each with a different clinical diagnosis and presumed type of neurodegenerative proteinopathy: PD, DLB, PSP, CBS, MSA, and AD. All clinical diagnoses were based on established clinical diagnostic criteria [60-67]. The patients underwent thorough neurological examination at the tertiary movement disorders center to establish clinical diagnosis; the other (than neurodegenerative) causes of symptoms were carefully excluded. The 1.5T or 3.0T magnetic resonance imaging (MRI) of the brain and the dopamine transporter DaTScan (^{123}I-ioflupane) imaging were done in all participants, in indicated cases was the positron emission tomography (PET; ^{18}F-flutemetamol) brain imaging done as well. All patients were followed up in the tertiary movement disorders center; the final clinical diagnosis was confirmed at the same time when the blood serum and CSF examinations were done.

In all patients, the vascular origin of neurological symptoms including cognitive deterioration was excluded using imaging studies: T2-weighted, fluid-attenuated inversion recovery (FLAIR) and diffusion-weighted MRI (DWI-MRI), ultrasonography (USG) and transcranial Doppler (TCD) examinations, and using the calculation of Hachinski Ischemic Score (HIS); its value in all patients was less than 3.

The PD and DLB clinical units were combined into a single LBD group because of the high similarity of their basic morphological changes in the histopathological findings defined in the pathological diagnostic criteria [68, 69]. For the same reason, the PSP and CBS clinical units were combined into the 4R-Tau group. None of the patients suffering from corticobasal degeneration had a previous diagnosis of frontotemporal dementia. Patients with other serious comorbidities (e.g., hematological disease, cancer, depression, psychosis, chronic kidney disease, or metabolic derangements) were excluded from the study. Behavioral variant frontotemporal dementia (*bv*FTD) is pathologically extremely heterogeneous entity. Current neuropathological classification of degenerative proteinopathies is based on the presence of predominant pathology. Thus, most cases of FTD are accordingly classified within one of three broad molecular subgroups: frontotemporal lobar degeneration with tau, TDP-43, or FET protein accumulation. Based on the clinical presentation, the relevant pathology cannot be presumed in

most cases of *bv*FTD [70]. This was the principal reason why this disease has been excluded from the cohort.

None of the patients nor controls have been treated with corticosteroids; in the HC group, corticosteroid treatment has been one of the exclusion criteria. The HC group consisted of participants examined for benign conditions (e.g., back pain, carpal tunnel syndrome, or tension headaches) with no evidence of any neurodegenerative disease. The demographic characteristics of each participant group are shown in the supplementary material in Table S1. Twenty-four patients with LBD, three patients with 4R-Tau and nine patients with MSA were treated with antiparkinsonian drugs (levodopa known as L-DOPA, peripheral decarboxylase inhibitors). Members of the AD and HC groups were not medicated. The study was not blinded.

5.3 Sample preparation

Blood and CSF collection was performed at 10:00 am with a prior 18-hour fasting period. Approximately 10 mL of peripheral blood and CSF were collected by venipuncture or lumbal puncture into sterile tubes (no anticoagulant) under standardized conditions [71, 72]. All samples were processed within 10 min of collection. Blood and CSF were centrifuged at 1100 ×g for 10 min at 4 °C. The serum was transferred into dark amber glass vials, heated in a water bath (30 °C for 5 min), sonicated (5 min), and bubbled with a stream of argon (2 min). CSF and serum samples were then immediately stored in the dark at -80 °C until preparation for analysis (long-term stability of the analytes was tested in reference [2]). There was only one freeze-thaw cycle before the analysis.

Levels of neuroactive compounds were determined using a previously published, fully validated, highly sensitive and efficient method [2]. Briefly, cooled CSF or serum samples (100 µL) were spiked with a predefined quantity of stable isotopically labelled internal standards. Samples were placed in a CoolBox™ (Biocision) during all pipetting steps and protected from light during processing. Complete precipitation of proteins was induced by incubating (60 min, -20 °C) the samples on a rotator with ice-cold methanol (-20 °C). The samples were then centrifuged at 6.500 rpm for 7 min at 4 °C. Before further centrifugation (8.000 rpm for 5 min at 4 °C), the supernatant was transferred to a micro-spin centrifuge filter tube with a nylon membrane (pore size: 0.20 µm). The resulting filtrate was evaporated under a stream of nitrogen to dryness. Before analysis, the sample was dissolved in 30 µL of 2% methanol, mixed (30 s), sonicated (5 min), and transferred into a vial insert. Target analytes

were quantitated using matrix-matched calibration curves prepared using artificial serum (4% bovine serum albumin in 10 mM phosphate-buffered saline, pH 7.4) or a CSF calibrator.

5.4 LC–MS/MS conditions

The prepared samples were analysed by UHPLC–MS/MS using an Acquity® UPLC® (Waters) system connected to a triple quadrupole mass spectrometer Xevo® TQ (Waters) with positive electrospray ionization. Samples were stored in an autosampler maintained at 8 °C during analysis and were injected (10 µL) into a reversed-phase chromatography column (Acquity UPLC HSS T3 Column, 100 Å, 1.8 µm, 2.1 × 100 mm; Waters) equipped with the appropriate pre-column (VanGuard™ HSS 1.8 µm; Waters). Mobile phase A was 0.1% formic acid in water, while mobile phase B was methanol. The column was maintained at 30 °C and samples were eluted at a flow rate of 0.3 mL/min using the following gradient: 0–2 min—98:2 (A:B; isocratic elution), 2–10 min—40:60 (A:B; gradient elution). A wash step and equilibration were performed at the end of the gradient. The total analytical run time was 14 min. The mass spectrometer was operated in multi-reaction monitoring mode using the previously reported parameters [2]. Quantitative analysis was performed using the MassLynx™ 4.2 (Waters) and Microsoft Office (Microsoft) software packages.

5.5 Data treatment and statistical analysis

Statistical analyses were performed in GraphPad (version 9.5, San Diego, California, USA), R (version 4.2.0), and TIBCO Statistica® (version 14.0.0, Palo Alto, California, USA). Zero imputation was done using the k-nearest neighbours algorithm as implemented in the R impute package (Hastie T, Tibshirani R, Narasimhan B, Chu G (2023). impute: Imputation for microarray data. R package version 1.72.3) with k (the number of neighbours used for imputation) being set to 5. The raw data were log-transformed to obtain a normal distribution and the Shapiro-Wilk test was used to assess normality. Subsequent analyses were based on box-plots, ANOVA with post hoc testing (Holm-Shidak multiple comparisons test), Pearson correlations, and the Mann Whitney U test (for sample groups where normality was not achieved). The p-value threshold for significance was < 0.05. The study design was planned for a minimum of 10 samples per experimental group to ensure sufficient statistical significance. The power of the study was evaluated and an effect size > 0.89 (Cohen's D) for comparisons between studied groups (n = 10–31) was found to be statistically significant for a two-tailed t-test based on type I error (Alpha = 0.05) and the required Power (1 - beta = 0.8). Statistically

significant correlations in the range of 0.35–0.58 were computed for all studied groups (n = 10–31).

Author Information

***Michal Kaleta**- orcid.org/0000-0003-0875-2912; E-mail: michal.kaleta@upol.cz*

***Eva Hényková**- orcid.org/0000-0003-4033-7786; E-mail: henykova.eva@centrum.cz*

***Kateřina Menšíková**- orcid.org/0000-0003-4834-3160; E-mail: katerina.mensikova@fnol.cz*

***David Friedecký**- orcid.org/0000-0002-3448-9073; E-mail: david.friedecky@upol.cz*

***Aleš Kvasnička**- orcid.org/0000-0001-5973-316X; E-mail: ales.kvasnicka@upol.cz*

***Kateřina Klíčová**- orcid.org/0000-0002-7528-4146; E-mail: katerina.klicova@fnol.cz*

***Dorota Koníčková** - orcid.org/0000-0003-4446-0060; Email: dorota.konickova@upol.cz*

***Miroslav Strnad**- orcid.org/0000-0002-2806-794X; E-mail: miroslav.strnad@upol.cz*

***Petr Kaňovský**- orcid.org/0000-0002-9009-3241; E-mail: petr.kanovsky@fnol.cz*

***Ondřej Novák**- orcid.org/0000-0003-3452-0154; E-mail: novako@ueb.cas.cz*

Author Contributions

M.K. performed the measurements, processed the experimental data, and wrote the manuscript with input from all authors. E.H., K.M., D.F., A.K., K.K., D.K., M.S., P.K., and O.N. reviewed and edited the structure of the article. D.F. and A.K. performed the statistical analyses. All authors have read and agreed to the published version of the manuscript.

Abbreviations

3-OH-AA, 3-hydroxy-anthranilic acid; 3-OH-KYN, 3-hydroxy-L-kynurenine; 4R-Tau, four-repeat tauopathy; 5-MeO-TA, 5-methoxytryptamine; 5-OH-IAA, 5-hydroxyindole-3-acetic acid; 5-OH-TRP, 5-hydroxy-L-tryptophan; 6-OH-M, 6-hydroxymelatonin; AADC, aromatic amino acid decarboxylase; AA, Anthranilic acid; AD, Alzheimer's disease; AFMK, N^1-acetyl-N^2-formyl-5-methoxykynuramine; *bv*FTD, behavioral variant frontotemporal dementia; CBS, Corticobasal syndrome; CSF, Cerebrospinal fluid; DLB, Dementia with Lewy bodies; DWI-MRI, diffusion-weighted MRI; FLAIR, Fluid-Attenuated Inversion Recovery; HC, Healthy control; HIS, Hachinski Ischemic Score; IAA, Indole-3-acetic acid; L-DOPA, Levodopa; KA, Kynurenic acid; KYN, L-kynurenine; LBD, Lewy body disease; M, Melatonin; MRI, magnetic resonance imaging; MSA, Multiple system atrophy; *N*-Ac-S, *N*-acetylserotonin; *N*-Me-S, *N*-

methylserotonin; *N*-Me-TA, *N*-methyltryptamine; PD, Parkinson's disease; PET, positron emission tomography; PSP, Progressive supranuclear palsy; S, Serotonin; TA, Tryptamine; TCD, transcranial Doppler; TRP, L-tryptophan; UHPLC–MS/MS, Ultra-high performance liquid chromatography–tandem mass spectrometry; USG, ultrasonography.

Supporting Information

Table S1. Characteristics of the study participants (PDF)

Acknowledgements

The authors would like to thank Hana Svobodová for her help in preparing the samples for analysis. The graphical abstract was created using the AI system DALL·E 2.

Funding information

This work was financially supported by the European Regional Development Fund – Project ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868), and by the student project IGA_PrF_2023_031 of the Palacky University Olomouc.

Conflicts of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ACS Chemical Neuroscience

Supporting Information

Patients with Neurodegenerative Proteinopathies Exhibit Altered Tryptophan Metabolism in the Serum and Cerebrospinal Fluid

Michal Kaleta[a,b,c*], Eva Hényková[a,b,c], Kateřina Menšíková[b,c], David Friedecký[d], Aleš Kvasnička[d], Kateřina Klíčová[b,c], Dorota Koníčková[b,c], Miroslav Strnad[a,b,c], Petr Kaňovský[b,c], Ondřej Novák[a*]

[a]Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences & Palacky University, Šlechtitelů 27, 783 71, Olomouc, Czech Republic

[b]Department of Neurology, University Hospital Olomouc, 779 00 Olomouc, Czech Republic

[c]Department of Neurology, Faculty of Medicine and Dentistry, Palacky University, 779 00 Olomouc, Czech Republic

[d]Laboratory for Inherited Metabolic Disorders, Department of Clinical Biochemistry, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Zdravotníků 248/7, 779 00 Olomouc, Czech Republic

Correspondence*

Michal Kaleta
E-mail: michal.kaleta@upol.cz

Ondřej Novák
E-mail: novako@ueb.cas.cz

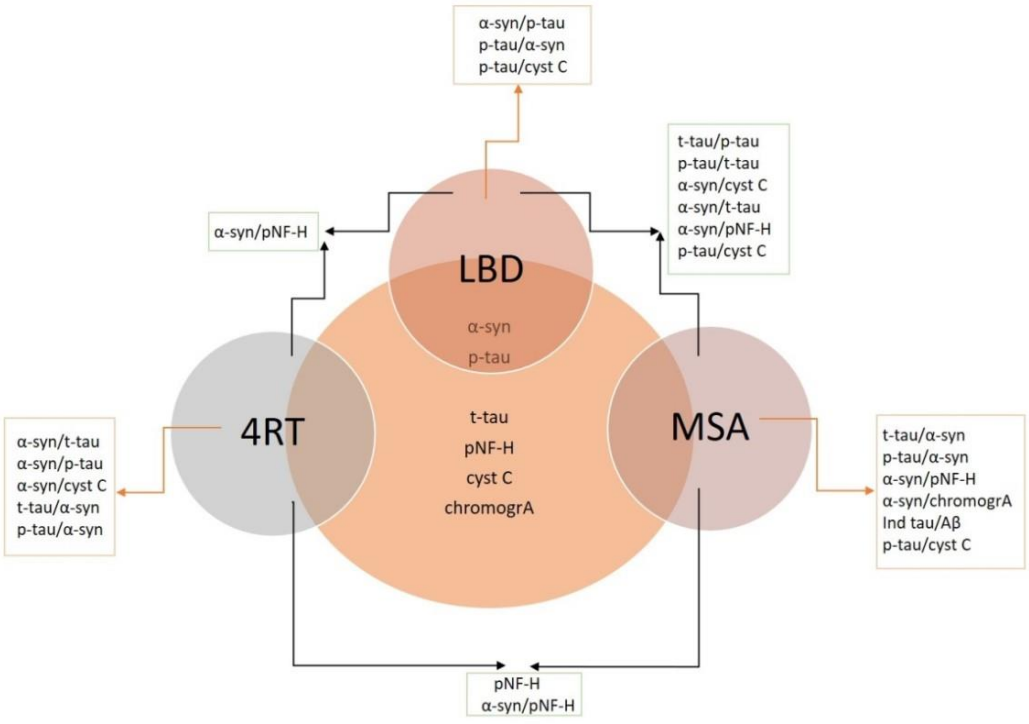
Table S1. Characteristics of the study participants in each group (*n* = 100).

Participants group	Description	Number of participants	Sex ratio (male/female)	Age Median (range)	Mean age at disease onset (years)	Mean duration of disease (years)	L-DOPA therapy (Nr.)	Mean daily L-DOPA dose (mg)
LBD	Parkinson's disease, Dementia with Lewy bodies	31	9/22	69 (38–82)	63.1	4.16	24	577.08
4R-Tau	Progressive supranuclear palsy, Corticobasal syndrome	10	2/8	66 (51–83)	62.6	4.10	3	766.66
MSA	Multiple system atrophy	13	2/11	65 (52–80)	62.2	4.15	9	622.22
AD	Alzheimer's disease	25	3/22	75 (51–90)	70.6	2.64	0	0
HC	Healthy control	21	11/10	57 (37–75)	N/A	N/A	0	0

LBD, Lewy body disease; 4R-Tau, Four-repeat tauopathy; MSA, Multiple system atrophy; AD, Alzheimer's disease; HC, Healthy control; L-DOPA, levodopa (L-3,4-dihydroxyphenylalanine)

Supplement V

Cerebrospinal Fluid and Blood Serum Biomarkers in Neurodegenerative Proteinopathies: A Prospective, Open, Cross-Correlation Study



Received: 14 April 2023 | Revised: 27 June 2023 | Accepted: 2 August 2023

DOI: 10.1111/jnc.15944

Journal of Neurochemistry JNC The Official Journal of the International Society for Neurochemistry WILEY

ORIGINAL ARTICLE

Cerebrospinal fluid and blood serum biomarkers in neurodegenerative proteinopathies: A prospective, open, cross-correlation study

Dorota Koníčková[1,2] | Kateřina Menšíková[1,2] | Kateřina Klíčová[1,2] | Monika Chudáčková[1,2] | Michaela Kaiserová[1,2] | Hana Přikrylová[1,3] | Pavel Otruba[1,2] | Martin Nevrlý[1,2] | Petr Hluštík[1,2] | Eva Hényková[1,2,4] | Michal Kaleta[1,2,4] | David Friedecký[5] | Radoslav Matěj[6] | Miroslav Strnad[1,2] | Ondřej Novák[4] | Lucie Plíhalová[7] | Raymond Rosales[8] | Carlo Colosimo[9] | Petr Kaňovský[1,2]

[1]Department of Neurology, University Hospital Olomouc, Olomouc, Czech Republic

[2]Department of Neurology, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

[3]Neurology Outpatient Clinic "St. Moritz", Olomouc, Czech Republic

[4]Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences, Palacky University, Olomouc, Czech Republic

[5]Laboratory of Inherited Metabolic Disorders, Faculty of Medicine and Dentistry, Palacky University, University Hospital Olomouc, Olomouc, Czech Republic

[6]Department of Pathology and Molecular Medicine, Third Faculty of Medicine, Charles University, Thomayer University Hospital, Prague, Czech Republic

[7]Department of Chemical Biology, Faculty of Science, Palacky University, Olomouc, Czech Republic

[8]Department of Neurology and Psychiatry, Neuroscience Institute, University of Santo Tomas Hospital, Manila, Philippines

[9]Department of Neurology, Santa Maria University Hospital, Terni, Italy

Correspondence
Dorota Koníčková, Department of Neurology, University Hospital Olomouc and Palacky University, Faculty of Medicine and Dentistry Olomouc, 77900 Olomouc, Czech Republic.
Email: dorota.konickova@upol.cz

Funding information
European Regional Development Fund - Project ENOCH, Grant/Award Number: CZ.02.1.01/0.0/0.0/16_019/0000868 ; IGA_PrF_2023_031; IGA-LF-2022-014; NV 19-04-00090

Abstract

Neurodegenerative diseases are a broad heterogeneous group affecting the nervous system. They are characterized, from a pathophysiological perspective, by the selective involvement of a subpopulation of nerve cells with a consequent clinical picture of a disease. Clinical diagnoses of neurodegenerative diseases are quite challenging and often not completely accurate because of their marked heterogeneity and frequently overlapping clinical pictures. Efforts are being made to define sufficiently specific and sensitive markers for individual neurodegenerative diseases or groups of diseases in order to increase the accuracy and speed of clinical diagnosis. Thus said, this present research aimed to identify biomarkers in the cerebrospinal fluid (CSF) and serum (α-synuclein [α-syn], tau protein [t-tau], phosphorylated tau protein [p-tau], β-amyloid

Abbreviations: 4RT, four-repeat tauopathies; Aβ, amyloid beta; BS, blood serum; CBS, corticobasal syndrome; chromogrA, chromogranin A; CNS, central nervous system; CSF, cerebrospinal fluid; cyst C, cystatin C; DaTScan, dopamine transporter scan; DLB, dementia with Lewy bodies; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; Ind tau/Aβ, ratio of tau protein/amyloid beta; IPD, idiopathic Parkinson's disease; IQR, interquartile range; LBD, Lewy body diseases; MRI, magnetic resonance imaging; MSA, multiple system atrophy; MSA-P, multiple system atrophy-parkinsonism; NFH, neurofilament heavy chains; PD, Parkinson's disease; pNF-H, phosphorylated form of neurofilament heavy chains; PSP, progressive supranuclear palsy; p-tau, phosphorylated tau protein; REF, routine neuropsychological battery; t-tau, tau protein; α-syn, α-synuclein.

[Aβ], clusterin, chromogranin A [chromogrA], cystatin C [cyst C], neurofilament heavy chains [NFH], phosphorylated form of neurofilament heavy chains [pNF-H], and ratio of tau protein/amyloid beta [Ind tau/Aβ]) that could help in the differential diagnosis and differentiation of the defined groups of α-synucleinopathies and four-repeat (4R-) tauopathies characterized by tau protein isoforms with four microtubule-binding domains. In this study, we analyzed a cohort of 229 patients divided into four groups: (1) Parkinson's disease (PD) + dementia with Lewy bodies (DLB) ($n = 82$), (2) multiple system atrophy (MSA) ($n = 25$), (3) progressive supranuclear palsy (PSP) + corticobasal syndrome (CBS) ($n = 30$), and (4) healthy controls (HC) ($n = 92$). We also focused on analyzing the biomarkers in relation to each other with the intention of determining whether they are useful in distinguishing among individual proteinopathies. Our results indicate that the proposed set of biomarkers, when evaluated in CSF, is likely to be useful for the differential diagnosis of MSA versus 4RT. However, these biomarkers do not seem to provide any useful diagnostic information when assessed in blood serum.

KEYWORDS
biomarkers, blood serum, cerebrospinal fluid, neurodegenerative diseases, tauopathies, α-synucleinopathies

1 | INTRODUCTION

Neurodegenerative proteinopathies include diseases in which the pathological hallmark is a clearly defined pathological protein in the brain and (or) spinal cord. This pathological protein might be present in the form of an oligomeric chain, a polymeric chain, or fibrillary aggregates either intracellularly or extracellularly; intracellular aggregates frequently form structures called "bodies" like Lewy bodies and Pick bodies (Emamzadeh & Surguchov, 2018). Currently, the pathological taxonomy distinguishes different disease groups named following pathologically changed crucial protein, for example, α-synucleinopathies, tauopathies, TDP-43-proteinopathies, ubiquitinopathies, and FUS-proteinopathies (Koníčková et al., 2022; Kovacs, 2016, 2019; Kovacs et al., 2022; Tarutani et al., 2022). A complete updated pathological classification of neurodegenerative proteinopathies is shown in Table 1.

Neurodegenerative proteinopathies might clinically manifest in various phenotypes; nonetheless, the most frequent is parkinsonian syndrome (Batla et al., 2013; Farníková et al., 2010; Menšíková et al., 2019). In such a situation, the phenotypes are similar and mistakable and the diagnostic process in the initial phase of the disease can be extremely difficult; any tool that might improve the diagnostic accuracy should be used. Such tools have to be validated with regard to their sensitivity and specificity; the first step in this validation process is a prospective, open, cross-correlation study.

One tool is the assessment of blood serum-based (BS) and cerebrospinal fluid-based (CSF) biomarkers. There are two types of body fluid biomarkers: pathological proteins and metabolites of neurotransmitters. Previous studies have provided evidence that determining the presence, concentrations, and mutual ratios of certain pathological proteins in CSF might be highly beneficial for distinguishing particular phenotypes of neurodegenerative proteinopathies (Jabbari et al., 2019; Magdalinou et al., 2015, 2017; Marques et al., 2021; Přikrylová Vranová et al., 2010; Xiang et al., 2022). Based on these studies, biomarkers, that play an important role in the development of individual neurodegenerative proteinopathies have been selected. The presented study was designed to assess whether the key biomarkers in CSF and in serum significantly differ in various types of parkinsonian syndromes and whether they can be examined further with regard to their routine clinical use.

2 | PATIENTS AND METHODS

The study protocol was approved by the IEC of the Faculty of Medicine and Dentistry, Palacky University in Olomouc and University Hospital Olomouc. All patients were informed about the purpose and the design of the study and they all signed informed consent forms. Patient enrollment, data collection, sampling, and laboratory examinations took place between 2016 and 2022. The study was not preregistered. No blinding was performed. Ethics approval for this study was granted according to University hospital Olomouc standard SM-L031, and ethics committee reference numbers: 139/10 and 76/15.

2.1 | Patients

The patients were consecutively recruited from 2016 to 2021 from the tertiary movement disorders outpatient clinic where they had a scheduled consultation. Patients with the following diagnoses

TABLE 1 Pathological classification of neurodegenerative proteinopathies.

Disease group	Protein		Disease type	Form	Genes	Phenotype
AD	Tau, Aβ		AD	SP/GEN	*APP*, *PSEN1*, *PSEN2*, *APOE*, *ABCA7*, *TREM2*	Amnestic late onset AD Behavioral dysexecutive AD lvPPA Posterior cortical atrophy
Tauopathy (FTLD-Tau)	Tau	3R + 4R	FTDP-17T	GEN	*MAPT*	FTD/parkinsonism
			PART (NFT-D)	SP		Cognitive deficit
		3R	PiD	SP		FTD
		4R	PSP	SP		Atypical parkinsonism/FTD
			CBD	SP		Atypical parkinsonism/FTD
			AGD	SP		Cognitive deficit, personality changes
			GGT	SP		FTD, corticospinal tract involvement
α-Synucleinopathy	α-synuclein		PD	SP/GEN	*SNCA*, *PRKN*, *UCHL1*, *PINK1*, *DJ-1*, *LRRK2*, *ATP13A2*, *GIGYF2*, *HTRA2*, *PLA2G6*, *FBXO7*, *VPS35*, *EIF4G1*, *DNAJC6*, *SYNJ1*, *TMEM230*, *CHCHD2*, *VPS13C*, *PSPA*, *GBA*	Typical PD manifesting with two or more of cardinal signs (bradykinesia, rigidity, tremor, postural instability)
			DLB	SP/GEN	*SNCA*, *SCARB2*, *APOE*	Dementia, parkinsonism
			MSA	SP		Autonomic dysfunction, atypical parkinsonism, cerebellar symptoms, pyramidal signs
TDP-43 proteinopathy	TDP-43		FTLD-TDP (type A-D)	SP/GEN	*C9ORF72*, *GRN*, *TARDBP*, *DCTN1*, *VCP*, *TBK1*	bvFTD nfvPPA svPPA IBMPFD UMN and LMN involvement
			MND-TDP	SP/GEN		UMN and LMN involvement
			FTLD-MND-TDP	SP/GEN		bvFTD + UMN and LMN involvement
FUS proteinopathy	FUS		aFTLD-U	SP	*FUS*	Aggressive early onset bvFTD
			BIBD	SP		Pure ALS ALS + dementia Pure FTD ALS + FTD + parkinsonism
			NIFID	SP		Heterogeneous phenotype, including atypical dementia, pyramidal and extrapyramidal signs presenting at a young age
			MND-FUS	GEN		ALS

TABLE 1 (Continued)

Disease group	Protein	Disease type	Form	Genes	Phenotype
Prion disease	PrP	sCJD, VPSPr, sFI iCJD vCJD Kuru gCJD, GSS, FFI, PrP-CAA	SP ACQ ACQ ACQ GEN GEN	*PRNP*	Various combinations of rapidly progressing dementia, visual, cerebellar extrapyramidal pyramidal dysfunction, myoclonus and sleep disorder in FFI

Note: The table summarizes pathological classification of neurodegenerative proteinopathies.

Abbreviations: 3R, three repeats isoform of tau protein; 4R, four repeats isoform of tau protein; ABCA7, ATP-binding cassette subfamily A member 7 gene; ACQ, acquired; AD, Alzheimer disease; aFTLD-U, atypical FTLD with ubiquitinated inclusions; AGD, Argyrophilic grain disease; ALS, amyotrophic lateral sclerosis; APOE, apolipoprotein E; APP, amyloid precursor protein; ATP13A2, ATPase 13A2; Aβ, amyloid beta; BIBD, Basophilic inclusion body disease; bvFTD, behavioral variant of frontotemporal dementia; CAA, sporadic cerebral amyloid angiopathy; CBD, Corticobasal degeneration; CHCHD2, coiled-coil-helix-coiled-coil-helix domain containing 2gene; CJD, Creutzfeldt-Jakob disease (i: iatrogenic, s: sporadic, v: variant, g: genetic); DCTN1, Dynactin subunit 1; DLB, dementia with Lewy bodies; DNAJC6, DnaJ heat shock protein family (Hsp40); EIF4G1, eukaryotic translation initiation factor; FBX07, F-box protein 7; FFI, fatal familial insomnia; FTD, frontotemporal dementia; FTDP-17T, frontotemporal dementia and parkinsonism linked to chromosome 17 caused by mutations in the MAPT (tau) gene; FTLD, frontotemporal lobar degeneration; FTLD-TDP, FTLD with ubiquitin and TDP-43 positive, tau negative and FUS negative inclusions; FUS, Fused in sarcoma protein; GBA, glucosylceramidase beta; GEN, genetic; GGT, globular glial tauopathy; GIGYF2, GRB10 interacting GYF protein 2; GRN, gene encoding progranulin; GSS, Gerstmann-Sträussler-Scheinker disease; HTRA2, Htr A serine peptidase 2; IBMPFD, inclusion body myopathy with early-onset Paget disease and frontotemporal dementia; LMN, lower motoneuron; LRRK2, Leucin rich repeat kinase2 gene; lvPPA, logopedic variant of primary progressive aphasia; MAPT, Microtubule-associated protein tau; MND, motor neuron disease; MND-FUS, motor neuron disease with FUS protein inclusions; MND-TDP, motor neuron disease with TDP-43 positive inclusions; MSA, multiple system atrophy; NFT-D, neurofibrillary tangle–predominant dementia; nfvPPA, nonfluent variant of primary progressive aphasia; NIFID, Neurofilament intermediate filament inclusion disease; PART, Primary age-related tauopathy; PD, Parkinson's disease; PiD, Pick disease; PINK1, PTEN-induced putative kinase 1; PLA2G6, phospholipase A2 group VI gene; PRNP, prion protein gene; PrP, prion protein; PSAP, prosaposin; PSEN1, Presenilin-1; PSEN2, Presenilin-2; PSP, Progressive supranuclear palsy; SCARB2, Scavenger receptor class B member 2; sFI, sporadic fatal insomnia; SNCA, Synuclein alpha; SP, sporadic; svPPA, semantic variant of primary progressive aphasia; SYNJ1, synaptojanin 1; TARDBP, TAR DNA-binding protein; TBK1, TANK-binding kinase 1; TDP-43, Transactive response (TAR) DNA-binding protein 43; TMEM230, transmembrane protein 230; TREM2, Triggering receptor expressed on myeloid cells 2 gene; UCHL1, Ubiquitin carboxyl-terminal hydrolase isozyme L1; UMN, upper motoneuron; VCP, Valosin-containing protein; VPS13C, vacuolar protein sorting 13 homolog C; VPS35, VPS35 retromer complex component; VPSPr, variably protease-sensitive prionopathy.

were included in the study: diagnosis of idiopathic Parkinson's disease (IPD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), progressive supranuclear palsy (PSP), or corticobasal syndrome (CBS) made on the basis of the current form of validated clinical diagnostic criteria (Alexander et al., 2014; Armstrong et al., 2013; Bhidayasiri et al., 2019; Boxer et al., 2017; Gilman et al., 2008; Höglinger et al., 2017; McKeith et al., 2020; Postuma et al., 2015), ability to undergo examinations in the study protocol, adherence to follow-up, and absence of any other serious illness for which the treatment would interfere with the planned examination (i.e. cancer, hematological disease, depression, psychosis). The healthy control group consisted of individuals who had undergone a routine examination for benign conditions, that is, low back pain, carpal tunnel, and tension headache. The mean age of the patients was 64 years.

All patients with any form of the above-mentioned neurodegenerative disorders were admitted to the ward and underwent complete clinical neurological examination. Brain magnetic resonance imaging (MRI) at 1.5T was performed in all the patients and the findings were interpreted by an experienced neuroradiologist. A dopamine transporter scan (DaTScan) was also done in all the patients, as well as a neurophysiological examination that involved scalp electroencephalography and recorded multimodal evoked potentials including transcranial magnetic stimulation with motor evoked potentials; electromyography to confirm or rule out the presence of polyneuropathy was done as well. The patients were examined by an ophthalmologist, and the presence of orthostatic hypotension was tested in a dedicated laboratory. The patients were assessed using a routine neuropsychological battery (REF) to confirm the presence or absence of cognitive deterioration. A logopedic assessment was done to confirm the presence or absence of any disorder of higher nervous functions. In case of any reported urological problems, the patients were fully examined by urologist.

The diagnoses were initially formulated based on the above-mentioned diagnostic criteria; in the last year of the study, the diagnoses were verified according to the current (and mostly updated) clinical diagnostic criteria.

The patients diagnosed with proteinopathy were divided into groups based on their clinical diagnoses and clinical-pathological taxonomy. There were 74 patients with PD, 8 patients with DLB, 25 patients with multiple system atrophy-parkinsonism (MSA-P), 23 patients with PSP, and 7 patients with CBS; in addition to this, there were 92 subjects in a control group. Based on a very close clinical picture, we divided the subjects into 4 groups: (1) patients with Lewy body diseases (LBD) (PD and DLB), (2) patients with MSA, and (3) patients with 4R tauopathies (PSP and CBS) and (4) the control group. These groups were not subject to neuropathological verification. The detailed patient demographics are shown in Table 2.

2.2 | Biochemical testing

Samples were obtained from lumbar puncture CSF and from venipuncture BS, performed under standard sterile conditions. About 10 mL of CSF and approximately 10 mL of peripheral blood were collected in sterile tubes; both CSF and serum were morphologically assessed and then centrifuged at 1100×*g* for 10 min at 4°C. Each sample was frozen at −80°C. The biochemical analysis was done at the laboratory of the accredited Department of Clinical Biochemistry of the University Hospital Olomouc (CSN ISO 15189:2013; subject No. 8254; certificate No. 220/2021 valid until 9 April 2026).

The samples were diluted according to the manufacturer's instructions (α-synuclein – 4×; clusterin – 100×; cystatin C (CSF) – 1600×; cystatin C (BS) – 4×; phosphorylated form of neurofilament heavy chains in CSF – 2×; other measured biomarkers without dilution). The biomarker concentrations were measured by analytical methods used for quantitative determination- by sandwich ELISA (enzyme-linked immunosorbent assay) (CE-IVD) and Atellica® CH analyzer (Siemens, USA; SEKK certified). Both are based on a highly specific interaction between antigen and antibody (heterogeneous immunoassay), with an enzyme covalently bound to one of these partners. The assay is performed spectrophotometrically or fluorescently, with the product concentration being proportional to the concentration of antigen or antibody in the sample.

A sandwich ELISA was evaluated for quantitative determination of biomarkers: clusterin (BioVendor); tau protein (Euroimmun); phosphorylated tau protein (Euroimmun); beta-amyloid$_{1-42}$ (Euroimmun); α-synuclein (IBL); phosphorylated form of neurofilament heavy chains (BioVendor); chromogranin A (EPITOPE Diagnostic) and cystatin C (BioVendor).

Serum cystatin C levels were analyzed by Atellica® CH analyzer (Atellica CH Cystatin C_2 [CYSC_2], Siemens). The results were assessed by reference ranges: clusterin (0.05–16 mg/L), tau protein (0–614 ng/L), phosphorylated tau protein (0–61 ng/L), beta-amyloid 1–42 (>550 ng/L), α-synuclein (0.03–10 μL/L), phosphorylated form of neurofilament heavy chains (62.5–4000 pg/mL), chromogranin

TABLE 2 Demographic data about patients and healthy controls.

	Healthy controls		MSA		PD + DLB		PSP + CBS		Test's statistic	*p*-value
Gender	Nr.	Proportion	Nr.	Proportion	Nr.	Proportion	Nr.	Proportion		
Female	36	39.1%	20	80%	46	56.1%	17	56.7%	14.964	**0.002**
Male	56	60.9%	5	20%	36	43.9%	13	43.3%		

Note: The table summarizes demographic data about patient groups and a healthy control group regarding the number and proportion of patients. The last column shows the significant value of Fisher's exact test. A significance value of $p = 0.002$ is less than the 0.05 level meaning that the compared groups differ statistically significantly in gender; $df = 3$.

A (2–850 μg/L), cystatin C (0.25–10000 μg/L) and serum cystatin C (0.63–1.03 mg/L).

2.3 | Statistical analysis

The levels of examined candidate biomarkers (defined protein and protein groups) were mutually compared and statistically significant differences were sought. IBM SPSS Statistics version 23 statistical software (IBM Corp.) was used for the data analysis. The non-parametric Kruskal–Wallis test was used to compare the groups concerning their quantitative parameters, and the Mann–Whitney *U* test with Bonferroni significance correction for multiple comparisons was used as a post hoc test. The qualitative parameters of the groups were compared using Fisher's exact test with the Bonferroni significance correction for significant determination between the groups with individual correction of multiple comparison. The normality of the data was assessed using the Shapiro–Wilk test. The median and interquartile range (IQR) were used to describe the baseline characteristics of the defined indices in the comparison groups. All tests were performed at a 0.05 level of significance. Because of the non-normal distribution of the measured parameters, the median and percentile range were used. No sample size calculation was performed. The number of subjects used for the study was determined based on our patients´ database, generated during the 5 years of project duration.

3 | RESULTS

3.1 | Examined proteins

In this study phase, we focused on assessing the usefulness of defining a set of blood-based and cerebrospinal fluid-based biomarkers in a differential diagnosis of neurodegenerative proteinopathies. The analyzed groups were defined based on the pathological taxonomy of the compared proteinopathies. Thus, the statistical evaluation of the results included a comparison of the above-mentioned parameters, examined in the BS and CSF of 137 patients with parkinsonian syndromes. There were 82 patients with LBD, 30 patients with 4R-tauopathy (4RT), and 92 gender- and age-matched healthy controls.

In these groups, the levels of α-synuclein (α-syn), tau protein (tau), phosphorylated tau protein (p-tau), β-amyloid (Aβ), clusterin, chromogranin A (chromogrA), cystatin C (cyst C), neurofilament heavy chains (NFH), phosphorylated form of neurofilament heavy chains (pNF-H), and ratio of tau protein/amyloid beta (Ind tau/Aβ) were examined. The mean levels of biomarkers in the BS and CSF were mutually compared and statistically significant differences were calculated (Table 3).

If the *p*-value was below the 0.05 level, it was necessary to compare the groups further pair by pair and identify between which groups there was a significant difference. The Mann–Whitney *U* test with the Bonferroni correction for multiple comparisons was used as a post hoc test to compare the groups pairwise. The resulting *p*-values of the Mann–Whitney post hoc test with the Bonferroni correction for multiple comparisons are shown in the following table (Table 4).

This comparison (Table 4) shows significantly higher values of CSF pNF-H in the LBD than in the control group. In the MSA patients, the values of the pNF-H CSF parameter were significantly higher than in the control group; there were significantly higher values of pNF-H CSF parameter than in the 4RT; significantly higher values of Ind tau/Aβ than in the control group; significantly lower CSF α-syn values than in the control group; and significantly lower BS-pNF values than in the control group. The differences in BS biomarker levels were significant when pNF-H values were compared between MSA and the controls. The differences in CSF biomarker levels were significant when the pNF-H values were compared between MSA and the controls, DLB and the controls, and MSA and 4RT. They were also significant when the α-syn values were compared between MSA and the controls and DLB and the controls, and when the Ind tau/Aβ was compared between MSA and the controls. In other mutual comparisons, the statistical differences were not significant.

3.2 | Examined protein ratios

Statistical analysis was performed to follow the defined ratios to detect their significant levels. The CSF levels of α-syn, t-tau, and p-tau were benchmarked against the other biomarkers described above.

3.2.1 | Levels of α-synuclein

The levels of α-syn (Table 5) were consequently compared with other biomarkers such as tau, p-tau, pNF-H, clusterin, chromogrA, and cyst C. The basic characteristics of the defined ratios were described, and the groups were compared pairwise to find any significant differences (Table 6). In this multiple comparison (Mann–Whitney post hoc test with Bonferroni correction), significantly lower values for all ratios were found in the MSA compared to the control group. Significantly lower values of α-syn/t-tau, α-syn/pNF-H, α-syn/chromogrA, and α-syn/cyst C ratios were found in the MSA compared to the LBD. Significantly lower values of α-syn/t-tau, α-syn/p-tau, α-syn/pNF-H, and α-syn/cyst C ratios were found in the 4RT compared with the control group. In the 4RT, α-syn/cyst C ratio was lower compared with the control group, and significantly lower values of the α-syn/pNF-H ratio were found in the 4RT compared with the LBD. Significantly lower α-syn/p-tau ratio values were found in the LBD compared with the control group.

3.2.2 | Levels of tau protein

Table 7 shows a comparison of tau protein levels with related biomarkers. The Mann–Whitney post hoc test with Bonferroni

TABLE 3 Statistical analysis of examined biomarker.

	Healthy controls	MSA	PD + DLB	PSP + CBS	Test's statistic	Kruskal–Wallis test *p*-value
clusterin (mg/L)						
Median	3.45	3.11	4.29	3.20	3.212	0.360
Percentile 25	2.30	2.05	2.28	2.05		
Percentile 95	16.0	10.3	1144	11.3		
chromogrA (μg/L)						
Median	62.2	59.5	61.0	56.7	0.442	0.932
Percentile 25	43.1	41.6	50.9	42.1		
Percentile 95	113.5	185.1	136.8	146.8		
pNF-H (ng/L)						
Median	134	329	218	307	17.213	**0.001**
Percentile 25	71	247	75	164		
Percentile 95	2261	586	933	2236		
p-tau (ng/L)						
Median	34.6	28.0	36.3	30.8	4.449	0.217
Percentile 25	24.5	21.9	28.5	27.7		
Percentile 95	72.3	74.4	72.9	73.8		
tau (ng/L)						
Median	227	292	247	275	6.308	0.098
Percentile 25	178	223	190	217		
Percentile 95	555	694	566	564		
Ind tau/Beta (ng/L)						
Median	0.217	0.291	0.250	0.268	12.075	**0.007**
Percentile 25	0.165	0.240	0.170	0.220		
Percentile 95	0.520	1.390	1.150	0.900		
α-syn (μg/L)						
Median	1.180	0.520	0.940	0.880	11.557	**0.009**
Percentile 25	0.720	0.280	0.560	0.560		
Percentile 95	3.200	2.400	3.160	2.840		
cyst C (mg/L)						
Median	3.55	3.63	3.15	4.00	7.683	0.053
Percentile 25	2.63	2.89	2.54	3.32		
Percentile 95	9.99	6.56	6.14	6.80		
Aβ (ng/L)						
Median	1038	869	975	851	7.124	0.068
Percentile 25	798	561	660	680		
Percentile 95	1576	1464	1563	1342		
BS_α-syn (μg/L)						
Median	6.64	4.05	6.53	4.86	5.930	0.115
Percentile 25	3.37	3.28	4.05	3.39		
Percentile 95	35.28	10.00	35.80	10.00		
BS_cystC (mg/L)						
Median		1.04	1.00	0.95	1.396	0.497
Percentile 25		0.82	0.87	0.88		
Percentile 95		1.55	1.83	1.29		

TABLE 3 (Continued)

	Healthy controls	MSA	PD + DLB	PSP + CBS	Test's statistic	Kruskal–Wallis test *p*-value
BS_pNF (ng/L)						
Median	23.5	23.5	23.5	23.5	12.236	**0.007**
Percentile 25	23.5	23.5	23.5	23.5		
Percentile 95	368.8	908.3	185.0	484.3		
BS_chromograA (μg/L)						
Median	10.6	2.2	4.2	4.7	4.659	0.199
Percentile 25	2.00	2.00	2.00	2.00		
Percentile 95	89.4	85.5	152.6	231.6		

Note: The table summarizes elementary statistical comparison in the defined proteinopathy groups of patients. We monitored levels of biomarkers—clusterin, chromogranin A (chromograA), phosphorylated form of neurofilament heavy chains (pNF-H), phosphorylated tau protein (p-tau), tau protein (tau), ratio of tau protein/beta-amyloid (Ind tau/Aβ), α-synuclein (α-syn), cystatin C (cyst C), β-amyloid (Aβ), and phosphorylated neurofilaments (pNF) in blood serum (BS) and cerebrospinal fluid (CSF). Because of the non-normal distribution of values of the measured parameters, the median and the so-called range, that is, minimum–maximum, were suitable for description. In the last column, the significance values of the Kruskal–Wallis test were given. Serum biomarkers are distinguished by mark "BS." The tests were performed at the significance level of 0.05; $df = 3$.

TABLE 4 Resulting *p*-values of Mann–Whitney Post hoc test with Bonferroni correction for multiple comparison.

	CSF pNF-H (ng/L)	CSF Ind tau/Beta (ng/L)	CSF α-syn (μg/L)	BS pNF (ng/L)
Control versus MSA	**0.007**	**0.007**	**0.021**	**0.042**
Control versus PD + DLB	**0.040**	0.073	0.161	0.068
Control versus PSP + CBS	0.991	1.000	0.959	1.000
MSA versus PD + DLB	1.000	1.000	1.000	1.000
MSA versus PSP + CBS	**0.014**	0.413	0.163	0.368
PD + DLB versus PSP + CBS	0.236	1.000	1.000	0.392

Note: The table summarizes the comparison of statistically significant values generated by comparing the *p*-values from the previous table. Serum biomarkers are distinguished by mark "BS." The tests were performed at the significance level of 0.05.

Abbreviations: Ind tau/Aβ, ratio tau protein/beta-amyloid; pNF, phosphorylated neurofilaments; pNF-H, phosphorylated form of neurofilament heavy chains; α-syn, α-synuclein.

correction was used to demonstrate significant differences between the groups (Table 8). Table 8 shows significantly higher values of t-tau/α-syn ratio and t-tau/p-tau ratio than in the control group. Significantly higher values of t-tau/α-syn ratio and t-tau/p-tau ratio were found in the MSA than in the LBD and significantly higher t-tau/α-syn ratio values were found in the 4RT than in the control group.

3.2.3 | Levels of phosphorylated tau protein

The results of phosphorylated tau protein comparison with other biomarkers are in Table 9. The basic characteristics of defined *p*-value indexes lower than 0.05 were compared pairwise (Table 10). Significantly higher values of p-tau/α-syn ratio, lower values of p-tau/t-tau ratio, and p-tau/pNF-H ratio were found in the MSA compared with the control group. In the MSA, we also found significantly lower p-tau/t-tau, p-tau/pNF-H, and p-tau/cyst C ratio values compared with the LBD. Significantly higher values of p-tau/α-syn ratio and p-tau/cyst C ratio were found in the PD than in the control group. Finally, significantly higher values of p-tau/α-syn ratio were found in the 4RT than in the control group.

4 | DISCUSSION

The diagnostic process in the initial phase of parkinsonian syndromes might be extremely difficult; the phenotypes are often largely similar and mistakable. Therefore, the assessment of blood-based and cerebrospinal fluid-based biomarkers could be highly useful in differentiating among particular phenotypes of neurodegenerative proteinopathies. The degenerative proteinopathies form a heterogenous group of disorders and their overlaps are common rather than rare (Kovacs, 2019); therefore, our analyses tried to combine different biomarker relationships.

CSF is a biological fluid that directly reflects both physiological and pathological processes in the central nervous system (CNS). It represents an ideal matrix that provides a biological fingerprint and allows early (hopefully also preclinical) diagnoses of disease. Nevertheless, a more accessible option, such as blood plasma or serum,

TABLE 5 Statistical analysis of proteinopathy groups in the ratios of α-synuclein parameters.

	Healthy controls	MSA	PD + DLB	PSP + CBS	Test's statistic	Kruskal–Wallis test *p*-value
α-syn/t-tau						
Median	0.0048	0.0018	0.0033	0.0027	33.249	**<0.0001**
Percentile 25	0.0029	0.0012	0.0024	0.0018		
Percentile 95	0.0139	0.0048	0.0096	0.0125		
α-syn/p-tau						
Median	0.0355	0.0160	0.0248	0.0234	21.993	**0.0001**
Percentile 25	0.0230	0.0129	0.0147	0.0152		
Percentile 95	0.0954	0.0413	0.0692	0.0721		
α-syn/pNF-H						
Median	0.0062	0.0017	0.0051	0.0026	29.775	**<0.0001**
Percentile 25	0.0029	0.0006	0.0026	0.0014		
Percentile 95	0.0391	0.0097	0.0176	0.0149		
α-syn/clusterin						
Median	0.3037	0.1418	0.2455	0.2242	10.330	**0.016**
Percentile 25	0.1818	0.0745	0.1208	0.1559		
Percentile 95	1.2116	1.0000	0.8681	0.5286		
α-syn/chromograA						
Median	0.0175	0.0078	0.0142	0.0124	25.154	**<0.0001**
Percentile 25	0.0124	0.0056	0.0081	0.0089		
Percentile 95	0.0713	0.0977	0.0555	0.0600		
α-syn/cyst C						
Median	0.2948	0.1313	0.3039	0.1957	24.044	**<0.0001**
Percentile 25	0.1990	0.1004	0.1681	0.1488		
Percentile 95	0.9205	0.4040	0.9559	0.5194		

Note: The table shows comparisons of alpha synuclein (α-syn) levels with other biomarkers important for univariate identification. The last column of the table shows the significance value of the Kruskal–Wallis test. The tests were performed at the significance level of 0.05; $df = 3$.

Abbreviations: chromograA, chromogranin A; cyst C, cystatin C; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, total tau protein.

TABLE 6 Resulting *p*-values of Mann–Whitney Post hoc test with Bonferroni correction for multiple comparison.

	α-Syn/t-tau	α-Syn/p-tau	α-Syn/pNF-H	α-Syn/clusterin	α-Syn/chromograA	α-Syn/cyst C
Control versus MSA	**<0.0001**	**0.001**	**0.0001**	**0.037**	**<0.0001**	**0.0003**
Control versus PSP + CBS	**0.002**	**0.025**	**0.004**	0.294	0.159	**0.016**
Control versus PD + DLB	0.128	**0.035**	1.000	0.375	0.081	1.000
MSA versus PSP + CBS	0.208	0.672	1.000	1.000	0.057	0.456
MSA versus PD + DLB	**0.0002**	0.086	**0.0003**	0.630	**0.006**	**0.001**
PD + DLB versus PSP + CBS	0.251	1.000	**0.022**	1.000	1.000	0.052

Note: The table summarizes the results of pairwise comparison between the groups of statistically significant values generated by comparing the *p*-values from the previous table. The tests were performed at the significance level of 0.05.

Abbreviations: chromograA, chromogranin A; cyst C, cystatin C; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, total tau protein; α-syn, α-synuclein.

is needed; both are less invasive and more affordable alternatives (Gaetani et al., 2020; Koníčková et al., 2022; Swift et al., 2021). The clinical utility of serum biomarkers definitely requires extensive further research (Li et al., 2022). In terms of CSF examination, attention was paid to "classical" biomarkers: α-synuclein, clusterin, chromogranin A, tau protein and its hyperphosphorylated form, beta-amyloid, and cystatin C. Since neurofilament light chains have been described as a suitable biomarker in many cases, our focus was additionally on investigating neurofilament heavy chains (and their phosphorylated form). These biomarkers were examined also

TABLE 7 Statistical analysis of proteinopathy groups in the ratios of total tau protein parameters.

	Healthy controls	MSA	PD + DLB	PSP + CBS	Test's statistic	Kruskal–Wallis test *p*-value
t-tau/α-syn						
Median	209.5	561.1	298.8	365.4	33.249	**<0.0001**
Percentile 25	15.6	397.0	175.9	289.0		
Percentile 95	1417	1613	784	1200		
t-tau/p-tau						
Median	6.36	9.38	7.08	8.09	20.171	**0.0002**
Percentile 25	5.49	8.62	5.40	5.97		
Percentile 95	13.9	16.3	11.9	18.1		
t-tau/pNF-H						
Median	1.78	1.00	1.28	1.10	8.561	**0.036**
Percentile 25	0.71	0.59	0.71	0.48		
Percentile 95	6.56	2.25	5.59	3.41		
t-tau/clusterin						
Median	63.6	94.9	57.3	75.1	8.512	**0.037**
Percentile 25	42.8	67.3	42.8	44.1		
Percentile 95	251	243	144	343		
t-tau/chromogrA						
Median	3.65	4.79	3.68	4.47	7.292	0.063
Percentile 25	2.75	3.90	2.81	3.18		
Percentile 95	35.9	8.0	10.5	25.3		
t-tau/cyst C						
Median	67.3	81.7	78.0	72.7	8.710	**0.033**
Percentile 25	47.1	67.4	53.4	42.6		
Percentile 95	131	153	157	158		

Note: The table shows comparisons of total tau protein (t-tau) levels with other biomarkers important for univariate identification. The last column of the table shows the significance value of the Kruskal–Wallis test. The tests were performed at the significance level of 0.05; $df = 3$.

Abbreviations: chromogrA, chromogranin A; cyst C, cystatin C; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, total tau protein; α-syn, α-synuclein.

TABLE 8 Resulting *p*-values of Mann–Whitney Post hoc test with Bonferroni correction for multiple comparison.

	t-tau/α-syn	t-tau/p-tau	t-tau/pNF-H	t-tau/clusterin	t-tau/cyst C
Control versus MSA	**<0.0001**	**0.0001**	0.124	0.122	0.080
Control versus PSP + CBS	**0.002**	0.759	0.252	1.000	1.000
Control versus PD + DLB	0.128	1.000	1.000	1.000	0.091
MSA versus PSP + CBS	0.208	0.118	1.000	1.000	1.000
MSA versus PD + DLB	**0.0002**	**0.0003**	0.315	0.060	1.000
PD versus PSP + CBS	0.251	1.000	0.712	0.589	1.000

Note: The table summarizes the results of pairwise comparison between the groups of statistically significant values generated by comparing the *p*-values from the previous table. The tests were performed at the significance level of 0.05.

Abbreviations: cyst C, cystatin C; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, total tau protein; α-syn, α-synuclein.

in serum with the intention to obtain the same results with less invasive sampling (Aguirre et al., 2018; Andersen et al., 2017; Angelopoulou et al., 2020; Atik et al., 2016; Mavroudis et al., 2019).

As shown in Figure 1, LBD is characterized by a specific "subset" of CSF biomarkers (α-syn, p-tau, pNF-H, cyst C, and their ratios), which is different than in MSA or 4RT. The dedicated set of marker ratios (α-syn/p-tau, p-tau/α-syn, p-tau/cyst C) is significantly different in LBD as compared to controls. CSF α-syn level ratios with many other biomarkers levels are different in individual proteinopathies, but particularly in LBD and MSA. The α-syn CSF level ratio with NFH

TABLE 9 Statistical analysis of proteinopathy groups in the ratios of phosphorylated tau protein parameters.

	Healthy controls	MSA	PD + DLB	PSP + CBS	Test's statistic	Kruskal–Wallis test *p*-value
p-tau/α-syn						
Median	28.13	62.50	40.30	42.76	21.993	**0.0001**
Percentile 25	21.40	43.10	27.30	33.80		
Percentile 95	203	137	133	211		
p-tau/Aβ42						
Median	0.0354	0.0375	0.0378	0.0402	4.309	0.230
Percentile 25	0.0266	0.0243	0.0282	0.0289		
Percentile 95	0.0625	0.1153	0.1487	0.1277		
p-tau/t-tau						
Median	0.157	0.107	0.141	0.125	20.171	**0.0002**
Percentile 25	0.107	0.081	0.102	0.105		
Percentile 95	0.236	0.152	0.309	0.481		
p-tau/pNF-H						
Median	0.253	0.088	0.193	0.131	20.136	**0.0002**
Percentile 25	0.101	0.060	0.106	0.064		
Percentile 95	0.821	0.213	0.563	0.498		
p-tau/clusterin						
Median	9.18	9.14	8.35	12.03	0.532	0.912
Percentile 25	5.15	5.96	5.34	5.13		
Percentile 95	26	35	28	58		
p-tau/chromograA						
Median	0.50	0.44	0.55	0.61	3.318	0.345
Percentile 25	0.43	0.40	0.39	0.40		
Percentile 95	2.4	0.83	1.22	3.7		
p-tau/cyst C						
Median	9.22	8.09	11.33	8.82	15.662	**0.001**
Percentile 25	7.49	6.53	8.22	6.45		
Percentile 95	16.6	17.4	25.4	22.2		

Note: The table shows comparisons of phosphorylated tau protein (p-tau) levels with other biomarkers important for univariate identification. The last column of the table shows the significance value of the Kruskal–Wallis test. The tests were performed at the significance level of 0.05; $df = 3$.

Abbreviations: Aβ42, amyloid beta, hyperphosphorylated isoform 42; chromograA, chromogranin A; cyst C, cystatin C; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, total tau protein; α-syn, α-synuclein.

TABLE 10 Resulting *p*-values of Mann–Whitney Post hoc test with Bonferroni correction for multiple comparison.

	p-tau/α-syn	p-tau/t-tau	p-tau/p-NF-H	p-tau/cyst C
Control versus MSA	**0.001**	**0.0001**	**0.002**	0.906
Control versus PSP + CBS	**0.025**	0.759	0.060	1.000
Control versus PD + DLB	**0.035**	1.000	1.000	**0.010**
MSA versus PSP + CBS	0.672	0.118	0.934	1.000
MSA versus PD+ DLB	0.086	**0.0003**	**0.001**	**0.015**
PD versus PSP + CBS	1.000	1.000	0.171	0.127

Note: The table summarizes the results of pairwise comparison between the groups of statistically significant values generated by comparing the *p*-values from the Table 8. The tests were performed at the significance level of 0.05.

Abbreviations: cyst C, cystatin C; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, total tau protein; α-syn, α-synuclein.

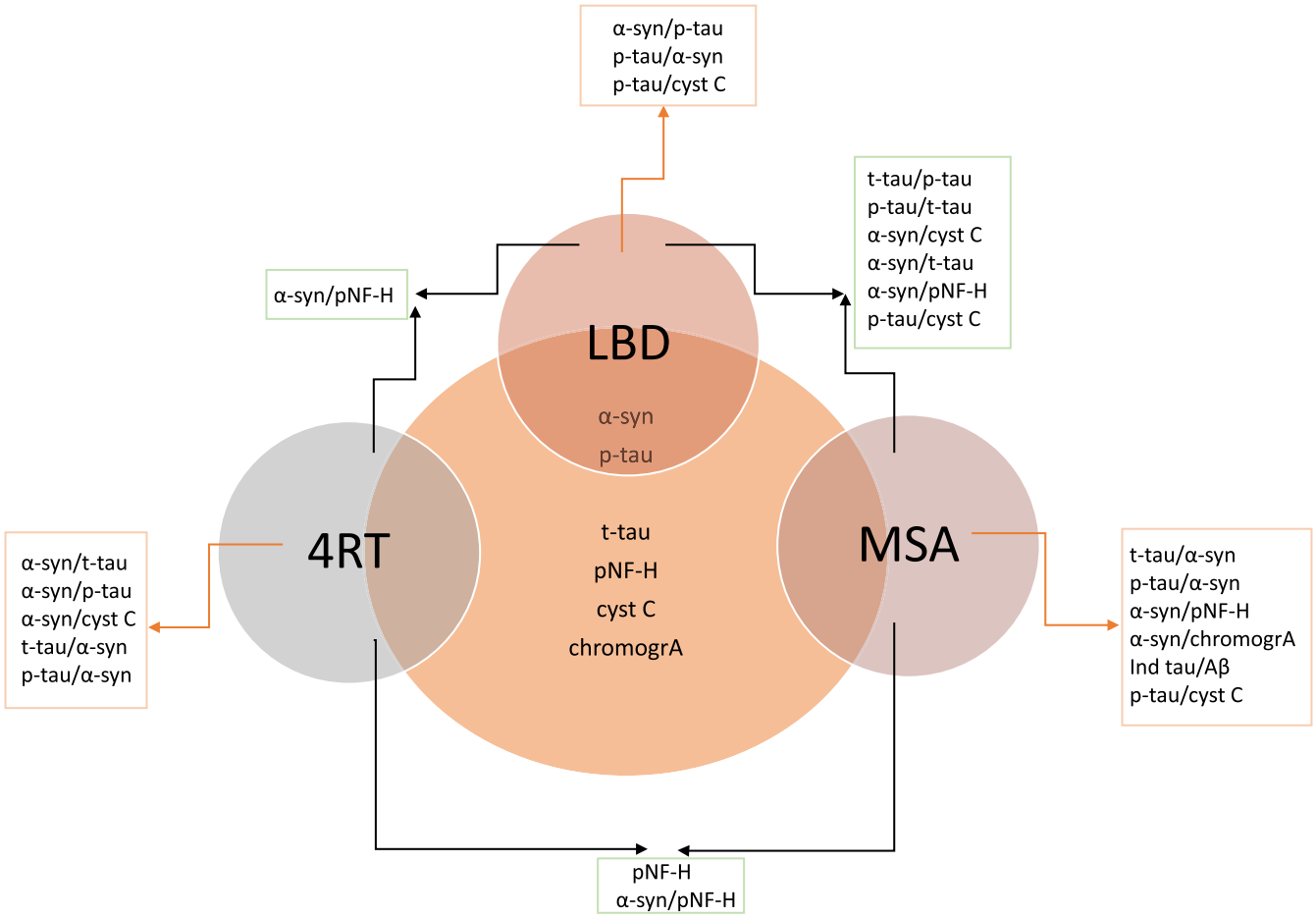


FIGURE 1 Mutual relationships of CSF biomarkers. The figure shows a mutual relationship of examined CSF biomarkers. The three small lateral circles represent the different groups of proteinopathies: the LBD, the 4RT, and the MSA. The central circle represents the biomarkers that are common to proteinopathies and occur in certain proportions in all disease groups. Biomarkers, that overreach the lateral LBD circle are suggested as "discriminating biomarkers" for LBD, but they are irreplaceable in other groups as well. Black arrows illustrate the co-occurrence of biomarkers from each disease. The output of the black arrows into the green boxes shows the individual biomarker ratios that are different in proteinopathies groups. An orange arrow emerges from each lateral circle, defining the boxes in which are noted the biomarkers and their relative ratios characteristics for specific group of disease (when compared to control group). 4RT, 4R tauopathies; chromogrA, chromogranin A; cyst C, cystatin C; Ind tau/Aβ, index of tau protein/beta amyloid; LBD, Lewy body diseases; MSA, multiple system atrophy; pNF-H, phosphorylated form of neurofilament heavy chains; p-tau, phosphorylated tau protein; t-tau, tau protein; α-syn, α-synuclein.

is different in LBD and 4RT. These biomarkers were described by Schulz et al. (2021); however, they reported significantly increased NFH levels in all groups (except DLB). Andersen et al. (2017) postulated the ability of NFH to distinguish PD from 4RT with high sensitivity and specificity and suggested its ratio with cyst C to support the diagnosis of LBD. Our mutual comparison of examined CSF biomarkers demonstrated p-tau might be a discriminative biomarker of PD, and, in combination with other biomarkers, its level might certainly distinguish between LBD and MSA, but not between LBD and 4RT. The results in the examined cohort confirmed our previous finding that chromogrA levels are not significantly different in PD and MSA (Kaiserova et al., 2021).

MSA is characterized by a specific "subset" of CSF biomarkers (α-syn, t-tau, p-tau, pNF-H, cyst C, Ind tau/Aβ, and their ratios) as proteinopathy, and it can be also differentiated from LBD or 4RT. Consistent with previous reports, we found an increase in the t-tau/p-tau ratio and a decrease in the p-tau/t-tau ratio in the MSA as compared to LBD (Barba et al., 2022; Cong et al., 2021; Schulz et al., 2021; Xie et al., 2021). Also, as reported by Parnetti et al. (2019), we observed a t-tau/p-tau ratio that was different in MSA and LBD. Moreover, our statistical analyses showed that ratios of α-syn with all other examined biomarkers were significantly different in LBD, MSA, and 4RT. Significant differences were also observed for the p-tau/cyst C ratio; their values were significantly different when MSA was compared to LBD (and also to the PD phenotype of LBD). Other combinations of biomarkers (i.e., lower values of α-syn ratios with t-tau, pNF-H, cyst C, chromogrA; higher t-tau/α-syn ratio or higher p-tau/α-syn) could also specifically characterize MSA.

4RT is also characterized by a specific "subset" of CSF biomarkers (α-syn, t-tau, p-tau, pNF-H, cyst C, and their combined ratios),

which is different when compared to LBD and MSA. A special α-syn/pNF-H ratio is significantly different in 4RT and LBD; also, other combinations of biomarker levels (decreased values of α-syn/t-tau, α-syn/p-tau, and α-syn/cyst C and increased values of t-tau/α-syn and p-tau/α-syn) could uniquely characterize 4RT.

5 | CONCLUSIONS

Our effort to examine BS and CSF biomarkers has shown that the proposed "set" of biomarkers, when assessed in CSF, may be potentially useful for differential diagnosis of MSA versus 4RT and of LBD versus MSA but not between LBD and 4RT. It seems that these biomarkers do not possess a diagnostic value when they are assessed in BS. By contrast, α-syn in combination with NFH might be able to distinguish α-synucleinopathies from each other, and specifically between LBD and 4RT.

There is still a need for closer examination that would enable the diagnosis of diseases with higher accuracy than is currently possible when the diagnosis is based on the established clinical diagnostic criteria. Our results clearly indicate that we are not yet able to meaningfully use the studied BS biomarkers to differentiate neurodegenerative proteinopathies. Finding BS biomarkers that can predict the manifestation of proteinopathy itself or that will help to more clearly differentiate neurodegenerative diseases in their initial stages will be an important future research step.

AUTHOR CONTRIBUTIONS

Dorota Koníčková and Petr Kaňovský involved in conceptualization; Dorota Koníčková wrote the original draft preparation; Kateřina Menšíková, Petr Kaňovský, Radoslav Matěj, David Friedecký, Petr Hluštík, Raymond Rosales, Carlo Colosimo, Kateřina Klíčová, Monika Chudáčková, Michaela Kaiserová, Hana Přikrylová, Pavel Otruba, Martin Nevrlý, Michal Kaleta, Miroslav Strnad, Ondřej Novák, and Lucie Plíhalová involved in review and editing; Kateřina Klíčová provided the resources; Petr Kaňovský and Kateřina Menšíková involved in supervision; Eva Hényková administered the project. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

Authors thank for the language and format editing to Veronika Glogarová, MSc, and Anne Johnson, MSc.

FUNDING INFORMATION

This work was supported by the European Regional Development Fund – Project ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868), by the grant IGA-LF-2022-014, IGA_PrF_2023_031 and grant NV 19-04-00090.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jnc.15944.

DATA AVAILABILITY STATEMENT

Detailed information is provided in tables (Tables 1–10) and Figure 1 used in this study. All tables and figures are enclosed.

ORCID

Dorota Koníčková https://orcid.org/0000-0003-4446-0060
Kateřina Menšíková https://orcid.org/0000-0003-4834-3160
Kateřina Klíčová https://orcid.org/0000-0002-7528-4146
Monika Chudáčková https://orcid.org/0000-0001-6873-0059
Michaela Kaiserová https://orcid.org/0000-0003-4776-9055
Hana Přikrylová https://orcid.org/0000-0001-9949-4036
Pavel Otruba https://orcid.org/0000-0002-6994-5112
Martin Nevrlý https://orcid.org/0000-0001-7160-5283
Petr Hluštík https://orcid.org/0000-0002-1951-0671
Eva Hényková https://orcid.org/0000-0003-4033-7786
Michal Kaleta https://orcid.org/0000-0003-0875-2912
David Friedecký https://orcid.org/0000-0002-3448-9073
Radoslav Matěj https://orcid.org/0000-0002-6152-6343
Miroslav Strnad https://orcid.org/0000-0002-2806-794X
Ondřej Novák https://orcid.org/0000-0003-3452-0154
Lucie Plíhalová https://orcid.org/0000-0001-8177-6844
Raymond Rosales https://orcid.org/0000-0001-6387-1389
Carlo Colosimo https://orcid.org/0000-0002-2216-3973
Petr Kaňovský https://orcid.org/0000-0002-9009-3241

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SUPPORTING INFORMATION

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How to cite this article: Koníčková, D., Menšíková, K., Klíčová, K., Chudáčková, M., Kaiserová, M., Přikrylová, H., Otruba, P., Nevrlý, M., Hluštík, P., Hényková, E., Kaleta, M., Friedecký, D., Matěj, R., Strnad, M., Novák, O., Plíhalová, L., Rosales, R., Colosimo, C., & Kaňovský, P. (2023). Cerebrospinal fluid and blood serum biomarkers in neurodegenerative proteinopathies: A prospective, open, cross-correlation study. *Journal of Neurochemistry*, *167*, 168–182. https://doi.org/10.1111/jnc.15944

Comparison of groups in gender

The table shows the number and percentage of females (F) and males (M) in the comparison groups. The last column shows the significance value of Fisher's exact test. A significance value of p = 0.002 is less than the 0.05 level meaning that the compared groups differ statistically significantly in gender.

Table 2. *Demographic Data about Patients and Healthy Controls*

Gender	**Healthy controls**		**MSA**		**PD+DLB**		**PSP+CBS**		**Test´s statistic**	**p-value**
	Nr.	Proportion	Nr.	Proportion	Nr.	Proportion	Nr.	Proportion		
Female	36	39.1%	20	80%	46	56.1%	17	56.7%	14,964	**0,002**
Male	56	60.9%	5	20%	36	43.9%	13	43.3%		

Table 2 summarizes demographic data about patient groups and a healthy control group regarding the number and proportion of patients. The last column shows the significant value of Fisher's exact test. A significance value of p = 0.002 is less than the 0.05 level meaning that the compared groups differ statistically significantly in gender; df = 3.

To determine between which groups there is a significant difference, it is necessary to perform a pairwise comparison of the groups. For this comparison, Fisher's exact test is suitable (as a post-hoc test). The resulting p-values for individual comparisons must be corrected for multiple comparisons. The following table shows the results of pairwise comparisons of the gender groups. The p-values were corrected using the Bonferroni correction.

Table 2.1. *Post-hoc tests - pairwise group comparisons*

Gender parameters	Corrected p-value	Uncorrected p-value
HC vs. MSA	**0,002**	0,0003
HC vs. PD	0,151	0,0252
HC vs. PSP+CBS	0,555	0,0924
MSA vs. PD	0,188	0,0314
MSA vs. PSP+CBS	0,398	0,0663
PD vs. PSP+CBS	1,000	0,9571

Table 2.1. shows the results of the pairwise comparison of the gender groups. The p-values were corrected using the Bonferroni correction. A significant difference in gender was found only between the MSA and the control groups. (There were significantly more females in the MSA group compared to the CONTROL group (p = 0.002)).

3.1. Examined proteins

Because of the non-normal distribution of values of measured parameters, the median and the percentile range was used. In the last column the significance values of the Kruskal-Wallis test are given. In case the p-value came out less than the 0.05 level, it is necessary to further compare the groups pair by pair and find out between which groups there is a significant difference.

Table 3. *Statistical Analysis of Examined Biomarker*

		Healthy controls	MSA	PD+DLB	PSP+CBS	Test's statistic	Kruskal-Wallis test p-value
clusterin [mg/l]	Median	3.45	3.11	4.29	3.20		
	Percentile 25	2.30	2.05	2.28	2.05	3.212	0.360
	Percentile 95	16.0	10.3	1144	11.3		
chromogrA [µg/l]	Median	62.2	59.5	61.0	56.7		
	Percentile 25	43.1	41.6	50.9	42.1	0.442	0.932
	Percentile 95	113.5	185.1	136.8	146.8		
pNF-H [ng/l]	Median	134	329	218	307		
	Percentile 25	71	247	75	164	17.213	**0.001**
	Percentile 95	2261	586	933	2236		
p-tau [ng/l]	Median	34.6	28.0	36.3	30.8		
	Percentile 25	24.5	21.9	28.5	27.7	4.449	0.217
	Percentile 95	72.3	74.4	72.9	73.8		
tau [ng/l]	Median	227	292	247	275		
	Percentile 25	178	223	190	217	6.308	0.098
	Percentile 95	555	694	566	564		
Ind tau/Beta [ng/l]	Median	0.217	0.291	0.250	0.268		
	Percentile 25	0.165	0.240	0.170	0.220	12.075	**0.007**
	Percentile 95	0.520	1.390	1.150	0.900		
α-syn [µg/l]	Median	1.180	0.520	0.940	0.880		
	Percentile 25	0.720	0.280	0.560	0.560	11.557	**0.009**
	Percentile 95	3.200	2.400	3.160	2.840		
cyst C [mg/l]	Median	3.55	3.63	3.15	4.00		
	Percentile 25	2.63	2.89	2.54	3.32	7.683	0.053
	Percentile 95	9.99	6.56	6.14	6.80		
Aβ [ng/l]	Median	1038	869	975	851		
	Percentile 25	798	561	660	680	7.124	0.068
	Percentile 95	1576	1464	1563	1342		
BS_α-syn [µg/l]	Median	6.64	4.05	6.53	4.86		
	Percentile 25	3.37	3.28	4.05	3.39	5.930	0.115
	Percentile 95	35.28	10.00	35.80	10.00		
BS_cystC [mg/l]	Median		1.04	1.00	0.95		
	Percentile 25		0.82	0.87	0.88	1.396	0.497
	Percentile 95		1.55	1.83	1.29		
BS_pNF [ng/l]	Median	23.5	23.5	23.5	23.5		
	Percentile 25	23.5	23.5	23.5	23.5	12.236	**0.007**
	Percentile 95	368.8	908.3	185.0	484.3		
BS_chromogrA [µg/l]	Median	10.6	2.2	4.2	4.7		
	Percentile 25	2.00	2.00	2.00	2.00	4.659	0.199
	Percentile 95	89.4	85.5	152.6	231.6		

Table 3 summarizes elementary statistical comparison in the defined proteinopathy groups of patients. We monitored levels of biomarkers - clusterin, chromogranin A (chromogrA), phosphorylated form of neurofilament heavy chains (pNF-H), phosphorylated tau protein (p-tau), tau protein (tau), ratio of tau protein/beta amyloid (Ind tau/Aβ), α-synuclein (α-syn), cystatin C (cyst C), β-amyloid (Aβ) and phosphorylated neurofilaments (pNF) in blood serum (BS) and cerebrospinal fluid (CSF). Due to the non-normal distribution of values of the measured parameters, the median and the so-called range, i.e. minimum - maximum, were suitable for description. In the last column, the significance values of the Kruskal-Wallis test were given. Serum biomarkers are distinguished by mark "BS". The tests were performed at the significance level of 0.05; df = 3.

The Mann-Whitney U test with Bonferroni's significance correction for multiple comparisons was used as a post-hoc test to compare groups pairwise.

The final p-values of the Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons are shown in the following table.

***Table 4**. Resulting p-Values of Mann-Whitney Post-Hoc Test with Bonferroni Correction for Multiple Comparison*

	CSF pNF-H [ng/l]	CSF Ind tau/Beta [ng/l]	CSF α-syn [µg/l]	BS pNF [ng/l]
Control vs. MSA	**0.007**	**0.007**	**0.021**	**0.042**
Control vs. PD+DLB	**0.040**	0.073	0.161	0.068
Control vs. PSP+CBS	0.991	1.000	0.959	1.000
MSA vs. PD+DLB	1.000	1.000	1.000	1.000
MSA vs. PSP+CBS	**0.014**	0.413	0.163	0.368
PD+DLB vs. PSP+CBS	0.236	1.000	1.000	0.392

Table 4 summarizes the comparison of statistically significant values generated by comparing the p-values from the previous table. Phosphorylated form of neurofilament heavy chains (pNF-H); ratio tau protein/beta amyloid (Ind tau/Aβ); α-synuclein (α-syn); phosphorylated neurofilaments (pNF). Serum biomarkers are distinguished by mark "BS". The tests were performed at the significance level of 0.05.

Corrected p-values in the table less than the 0.05 level indicate a significant difference between the compared groups.

***Table 4.1**. Uncorrected p-values*

	CSF pNF-H [ng/l]	CSF Ind tau/Beta [ng/l]	CSF α-syn [µg/l]	BS pNF [ng/l]
Control vs. MSA	0,0012	0,0011	0,0035	0,0069
Control vs. PD+DLB	0,0066	0,0121	0,0269	0,0113
Control vs. PSP+CBS	0,1652	0,2283	0,1598	0,2436
MSA vs. PD+DLB	0,3933	0,4883	0,2364	0,6983
MSA vs. PSP+CBS	0,0024	0,0688	0,0272	0,0613
PD vs. PSP+CBS	0,0393	0,2850	0,3861	0,0654

Table 4.1 summarized the uncorrected p-values. The final p-values of the Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons are shown in the Table 4. The Mann-Whitney U test with Bonferroni's significance correction for multiple comparisons was used as a post-hoc test to compare groups pairwise.

3.2. Examined protein ratios

The median and IQR were used to describe the baseline characteristics of the defined indices in the comparison groups.

The last column of the table shows the significance value of the Kruskal-Wallis test. In the case where the p-value is less than the 0.05 level, it is necessary to compare the groups pairwise to determine between which groups there is a significant difference.

***Table 5**. Statistical Analysis of Proteinopathy Groups in the Ratios of α-synuclein Parameters*

		Healthy controls	MSA	PD+DLB	PSP+CBS	Test's statistic	Kruskal-Wallis test p-value
α-syn/ t-tau	Median	0.0048	0.0018	0.0033	0.0027	33.249	< **0.0001**
	Percentile 25	0.0029	0.0012	0.0024	0.0018		
	Percentile 95	0.0139	0.0048	0.0096	0.0125		
α-syn/ p-tau	Median	0.0355	0.0160	0.0248	0.0234	21.993	**0.0001**
	Percentile 25	0.0230	0.0129	0.0147	0.0152		
	Percentile 95	0.0954	0.0413	0.0692	0.0721		
α-syn/ pNF-H	Median	0.0062	0.0017	0.0051	0.0026	29.775	< **0.0001**
	Percentile 25	0.0029	0.0006	0.0026	0.0014		
	Percentile 95	0.0391	0.0097	0.0176	0.0149		
α-syn/ clusterin	Median	0.3037	0.1418	0.2455	0.2242	10.330	**0.016**
	Percentile 25	0.1818	0.0745	0.1208	0.1559		
	Percentile 95	1.2116	1.0000	0.8681	0.5286		
α-syn/ chromogrA	Median	0.0175	0.0078	0.0142	0.0124	25.154	< **0.0001**
	Percentile 25	0.0124	0.0056	0.0081	0.0089		
	Percentile 95	0.0713	0.0977	0.0555	0.0600		
α-syn/ cyst C	Median	0.2948	0.1313	0.3039	0.1957	24.044	< **0.0001**
	Percentile 25	0.1990	0.1004	0.1681	0.1488		
	Percentile 95	0.9205	0.4040	0.9559	0.5194		

Table 5 shows comparisons of alpha synuclein (α-syn) levels with other biomarkers important for univariate identification. The last column of the table shows the significance value of the Kruskal-Wallis test. Total tau protein (t-tau); phosphorylated tau protein (p-tau); phosphorylated form of neurofilament heavy chains (pNF-H); chromogranin A (chromograA); cystatin C (cyst C). The tests were performed at the significance level of 0.05; df = 3.

Results of pairwise group comparison

Resulting p-values of Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons.

***Table 6**. Resulting p-Values of Mann-Whitney Post-Hoc Test with Bonferroni Correction for Multiple Comparison*

	α-syn/t-tau	α-syn/p-tau	α-syn/pNF-H	α-syn/ clusterin	α-syn/chromogrA	α-syn/cyst C
Control vs. MSA	< **0.0001**	**0.001**	**0.0001**	**0.037**	< **0.0001**	**0.0003**
Control vs. PSP+CBS	**0.002**	**0.025**	**0.004**	0.294	0.159	**0.016**
Control vs. PD+DLB	0.128	**0.035**	1.000	0.375	0.081	1.000
MSA vs. PSP+CBS	0.208	0.672	1.000	1.000	0.057	0.456
MSA vs. PD+DLB	**0.0002**	0.086	**0.0003**	0.630	**0.006**	**0.001**
PD+DLB vs. PSP+CBS	0.251	1.000	**0.022**	1.000	1.000	0.052

Table 6 summarizes the results of pairwise comparison between the groups of statistically significant values generated by comparing the p-values from the previous table. α-synuclein (α-syn); total tau protein (t-tau); phosphorylated tau protein (p-tau); phosphorylated form of neurofilament heavy chains (pNF-H); chromogranin A (chromograA); cystatin C (cyst C). The tests were performed at the significance level of 0.05. ***(Corrected p-values are shown in the table, p less than 0.05 indicates a significant difference between the compared groups).***

***Table 6.1**. Uncorrected p-values*

	α-syn/t-tau	α-syn/p-tau	α-syn/ p-NF-H	α-syn/ clusterin	α-syn/ chromogr A	α-syn/ cystC
Control vs. MSA	0,0000022	0,0001	0,000011	0,0062	0,0000037	0,000052
Control vs. PSP+CBS	0,0004	0,0042	0,00060	0,0491	0,0266	0,0027
Control vs. PD+DLB	0,0213	0,0059	0,1666	0,0625	0,0135	0,7791
MSA vs. PSP+CBS	0,0346	0,1121	0,1989	0,1874	0,0095	0,0759
MSA vs. PD+DLB	0,000037	0,0144	0,000054	0,1050	0,0010	0,00017
PD vs. PSP+CBS	0,0418	0,4066	0,0036	0,7311	0,7206	0,0086

Table 6.1. summarized *the uncorrected p-values. The final p-values of the Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons are shown in the Table 6. The Mann-Whitney U test with Bonferroni's significance correction for multiple comparisons was used as a post-hoc test to compare groups pairwise.*

The last column of the table shows the significance value of the Kruskal-Wallis test. In the case the p-value is less than the 0.05 level, a pairwise comparison of the groups is necessary to determine between which groups there is a significant difference.

Table 7. *Statistical Analysis of Proteinopathy Groups in the Ratios of Total Tau Protein Parameters*

		Healthy controls	MSA	PD+DLB	PSP+CBS	Test's statistic	Kruskal-Wallis test p-value
t-tau/ α-syn	Median	209.5	561.1	298.8	365.4		
	Percentile 25	15.6	397.0	175.9	289.0	33.249	< **0.0001**
	Percentile 95	1417	1613	784	1200		
t-tau/ p-tau	Median	6.36	9.38	7.08	8.09		
	Percentile 25	5.49	8.62	5.40	5.97	20.171	**0.0002**
	Percentile 95	13.9	16.3	11.9	18.1		
t-tau/ pNF-H	Median	1.78	1.00	1.28	1.10		
	Percentile 25	0.71	0.59	0.71	0.48	8.561	**0.036**
	Percentile 95	6.56	2.25	5.59	3.41		
t-tau/ clusterin	Median	63.6	94.9	57.3	75.1		
	Percentile 25	42.8	67.3	42.8	44.1	8.512	**0.037**
	Percentile 95	251	243	144	343		
t-tau/ chromogrA	Median	3.65	4.79	3.68	4.47		
	Percentile 25	2.75	3.90	2.81	3.18	7.292	0.063
	Percentile 95	35.9	8.0	10.5	25.3		
t-tau/ cyst C	Median	67.3	81.7	78.0	72.7		
	Percentile 25	47.1	67.4	53.4	42.6	8.710	**0.033**
	Percentile 95	131	153	157	158		

Table 7 shows comparisons of total tau protein (t-tau) levels with other biomarkers important for univariate identification. The last column of the table shows the significance value of the Kruskal-Wallis test. α-synuclein (α-syn); phosphorylated tau protein (p-tau); phosphorylated form of neurofilament heavy chains (pNF-H); chromogranin A (chromograA); cystatin C (cyst C). The tests were performed at the significance level of 0.05; df = 3.

Table 8. *Resulting p-Values of Mann-Whitney Post-Hoc Test with Bonferroni Correction for Multiple Comparison*

	t-tau/ α-syn	t-tau/ p-tau	t-tau/ pNF-H	t-tau/ clusterin	t-tau/ cyst C
Control vs. MSA	< **0.0001**	**0.0001**	0.124	0.122	0.080
Control vs. PSP+CBS	**0.002**	0.759	0.252	1.000	1.000
Control vs. PD+DLB	0.128	1.000	1.000	1.000	0.091
MSA vs. PSP+CBS	0.208	0.118	1.000	1.000	1.000
MSA vs. PD+DLB	**0.0002**	**0.0003**	0.315	0.060	1.000
PD vs. PSP+CBS	0.251	1.000	0.712	0.589	1.000

Table 8 summarizes the results of pairwise comparison between the groups of statistically significant values generated by comparing the p-values from the previous table. α-synuclein (α-syn); total tau protein (t-tau); phosphorylated tau protein (p-tau); phosphorylated form of neurofilament heavy chains (pNF-H); cystatin C (cyst C). The tests were performed at the significance level of 0.05. ***(Corrected p-values are shown in the table, p less than 0.05 indicates a significant difference between the compared groups).***

Table 8.1. *Uncorrected p-values*

	t-tau/ α-syn	t-tau/ p-tau	t-tau/ p-NF-H	t-tau/ clusterin	t-tau/ cystC
Control vs. MSA	0,00000	0,00002	0,02067	0,02031	0,01338
Control vs. PSP+CBS	0,00040	0,12652	0,04202	0,19700	0,49415
Control vs. PD+DLB	0,02133	0,54290	0,35348	0,54492	0,01513
MSA vs. PSP+CBS	0,03461	0,01967	0,83926	0,46733	0,19307
MSA vs. PD+DLB	0,00004	0,00006	0,05255	0,00992	0,58439
PD vs. PSP+CBS	0,04177	0,37716	0,11866	0,09825	0,42942

Table 8.1. summarized *the uncorrected p-values. The final p-values of the Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons are shown in the Table 8. The Mann-Whitney U test with Bonferroni's significance correction for multiple comparisons was used as a post-hoc test to compare groups pairwise.*

Table 9*. Statistical Analysis of Proteinopathy Groups in the Ratios of Phosphorylated Tau Protein Parameters*

		Healthy controls	**MSA**	**PD+DLB**	**PSP+CBS**	**Test's statistic**	**Kruskal-Wallis test p-value**
p-tau/ α-syn	Median	28.13	62.50	40.30	42.76		
	Percentile 25	21.40	43.10	27.30	33.80	21.993	**0.0001**
	Percentile 95	203	137	133	211		
p-tau/ Aβ42	Median	0.0354	0.0375	0.0378	0.0402		
	Percentile 25	0.0266	0.0243	0.0282	0.0289	4.309	0.230
	Percentile 95	0.0625	0.1153	0.1487	0.1277		
p-tau/ t-tau	Median	0.157	0.107	0.141	0.125		
	Percentile 25	0.107	0.081	0.102	0.105	20.171	**0.0002**
	Percentile 95	0.236	0.152	0.309	0.481		
p-tau/ pNF-H	Median	0.253	0.088	0.193	0.131		
	Percentile 25	0.101	0.060	0.106	0.064	20.136	**0.0002**
	Percentile 95	0.821	0.213	0.563	0.498		
p-tau/ clusterin	Median	9.18	9.14	8.35	12.03		
	Percentile 25	5.15	5.96	5.34	5.13	0.532	0.912
	Percentile 95	26	35	28	58		
p-tau/ chromogrA	Median	0.50	0.44	0.55	0.61		
	Percentile 25	0.43	0.40	0.39	0.40	3.318	0.345
	Percentile 95	2.4	0.83	1.22	3.7		
p-tau/ cyst C	Median	9.22	8.09	11.33	8.82		
	Percentile 25	7.49	6.53	8.22	6.45	15.662	**0.001**
	Percentile 95	16.6	17.4	25.4	22.2		

Table 9 shows comparisons of phosphorylated tau protein (p-tau) levels with other biomarkers important for univariate identification. The last column of the table shows the significance value of the Kruskal-Wallis test. α-synuclein (α-syn); amyloid beta, hyperphosphorylated isoform 42 (A642); total tau protein (t-tau); phosphorylated form of neurofilament heavy chains (pNF-H); chromogranin A (chromogrA); cystatin C (cyst C). The tests were performed at the significance level of 0.05; df =3.

Table 10. *Resulting p-Values of Mann-Whitney Post-Hoc Test with Bonferroni Correction for Multiple Comparison*

	p-tau/ α-syn	p-tau/ t-tau	p-tau/ p-NF-H	p-tau/ cyst C
Control vs. MSA	**0.001**	**0.0001**	**0.002**	0.906
Control vs. PSP+CBS	**0.025**	0.759	0.060	1.000
Control vs. PD+DLB	**0.035**	1.000	1.000	**0.010**
MSA vs. PSP+CBS	0.672	0.118	0.934	1.000
MSA vs. PD+ DLB	0.086	**0.0003**	**0.001**	**0.015**
PD vs. PSP+CBS	1.000	1.000	0.171	0.127

Table 10 summarizes the results of pairwise comparison between the groups of statistically significant values generated by comparing the p-values from the Table 8. α-synuclein (α-syn); total tau protein (t-tau); phosphorylated tau protein (p-tau); phosphorylated form of neurofilament heavy chains (pNF-H); cystatin C (cyst C). The tests were performed at the significance level of 0.05. **(Corrected p-values are shown in the table, p less than 0.05 indicates a significant difference between the compared groups).**

Table 10.1. *Uncorrected p-values*

	p-tau/ α-syn	p-tau/ t-tau	p-tau/ p-NF-H	p-tau/ cystC
Control vs. MSA	0,00008	0,00002	0,00039	0,15092
Control vs. PSP+CBS	0,00424	0,12652	0,01004	0,52080
Control vs. PD+DLB	0,00591	0,54290	0,25522	0,00162
MSA vs. PSP+CBS	0,11207	0,01967	0,15565	0,54285
MSA vs. PD+DLB	0,01437	0,00006	0,00018	0,00255
PD vs. PSP+CBS	0,40659	0,37716	0,02843	0,02121

Table 10.1. summarized the uncorrected p-values. The final p-values of the Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons are shown in the Table 10. The Mann-Whitney U test with Bonferroni's significance correction for multiple comparisons was used as a post-hoc test to compare groups pairwise.

PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Laboratory of Growth Regulators

Mgr. Michal Kaleta

Summary of the Doctoral Thesis

STUDY OF ENDOGENOUS NEUROACTIVE SUBSTANCES IN BIOLOGICAL MATERIAL

P0511D030004 – Experimental Biology

Supervisor

Prof. Mgr. Ondřej Novák, Ph.D.

OLOMOUC
2023

This Ph.D. thesis was realized in the Laboratory of Growth Regulators within the framework of internal Ph.D. Study of Experimental Biology, guaranteed by the Laboratory of Growth Regulators, Faculty of Science, Palacký University in Olomouc, between the years 2019-2023.

Ph.D. candidate: **Mgr. Michal Kaleta**

Supervisor: **Prof. Mgr. Ondřej Novák, Ph.D.**
Laboratory of Growth, Faculty of Science of Palacký University & Institute of Experimental Botany of the Czech Academy of Sciences, Olomouc, Czech Republic

Consultant: **Mgr. Jana Oklešťková, Ph.D.**
Laboratory of Growth, Faculty of Science of Palacký University & Institute of Experimental Botany of the Czech Academy of Sciences, Olomouc, Czech Republic

Oponents: **Ing. Martin Hill, Ph.D., DrSc.**
Institute of Endocrinology, Department of Steroids and Proteofactors, Prague, Czech Republic

Mgr. Radana Brumarová, Ph.D.
Laboratory for Inherited Metabolic Disorders, Department of Clinical Biochemistry, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czech Republic

The evaluation of this Ph.D. thesis was written by **Prof. Ing. Miroslav Strnad, CSc. DSc.**, Laboratory of Growth Regulators, Faculty of Science, Palacký University in Olomouc.

The oral defence will take place on 29.01.2024 before the Commission for the Ph.D. thesis of the Study Program Experimental Biology, room ……., Šlechtitelů 27, Olomouc – Holice.

The Ph.D. thesis and expert reviews will be available 14 days before the defence in the Study Department of Faculty of Science (Mgr. M. Karásková), Palacký University, 17. listopadu 12, Olomouc.

After the defense, the Ph.D. thesis will be stored in the Library of the Biological Departments of Faculty of Science, Palacký University, Šlechtitelů 27, Olomouc – Holice.

Prof. Ing. Miroslav Strnad, CSc. DSc.
Chairman of the Commision for the Ph.D. thesis,
Study Program Experimental Biology,
Faculty of Science, Palacký University in Olomouc

CONTENT

1. INTRODUCTION

The increasing human lifespan is, unfortunately, associated with a growing proportion of age-related neurodegenerative diseases (among others) (Sorgdrager *et al.* 2019). Perhaps unsurprisingly, the most common neurodegenerations are Parkinson's (PD) and Alzheimer's diseases (AD) (Klatt *et al.* 2021). Worldwide, several million people suffer from these diseases. It should not be forgotten that this growing medical and socio-economic problem of an aging population is also a major burden on the healthcare systems (Ayeni *et al.* 2022; Van Schependom & D'Haeseleer 2023). An early detection of these conditions is very important, preferably before the full onset of the disease (Shusharina *et al.* 2023).

The nervous system is the target but also the source of a wide variety of neuroactive substances of different chemical natures. The vast majority of available predictive, prognostic, or diagnostic biomarkers of neurodegenerative diseases are proteins, i.e. high molecular weight markers (alpha-synuclein, tau protein, etc.) (Koníčková *et al.* 2022; Koníčková *et al.* 2023). However, low molecular weight substances are also an interesting, albeit insufficiently explored area (Hényková *et al.* 2022; Kaleta *et al.* 2021; Kaleta *et al.* unpublished A and B). One of the groups of these substances that have attracted interest in recent years are certainly the neuroactive steroids (NASs). These substances, capable of modulating the function and development of the nervous system, are being intensively studied, including their use as potential drugs for various diseases of the nervous system (Blanco *et al.* 2018; Reddy & Estes 2016; Melcangi *et al.* 2016). Several synthetic analogues have undergone clinical trials. Some compounds may also be directly involved in the pathogenetic processes of several neuropathological entities or may be potentially useful in their pharmacotherapy. In this respect, tryptophan (TRP) metabolites are a very interesting and not so thoroughly studied group (Poeggeler *et al.* 2022; Hényková *et al.* 2016; Kaleta *et al.* unpublished B). They include neuroprotective and neurotoxic substances, which makes them particularly interesting in the context of the neurodegenerative process.

This Ph.D. thesis describes the development and validation of an analytical method based on ultra-high performance liquid chromatography combined with tandem mass spectrometry (UHPLC–MS/MS) for the determination of selected NASs in human serum. In addition, metabolic profiling of TRP metabolites and analysis of candidate protein biomarkers in several neurodegenerative pathologies are also presented.

2. AIMS AND SCOPE

The presented Ph.D. thesis focuses mainly on the study of two groups of neuroactive substances, namely NASs and TRP-related substances, in human body fluids. Sensitive methods based on UHPLC–MS/MS were used to study these analytes. The last part of this thesis is devoted to selected candidate protein biomarkers of neurodegeneration.

The main aims of the work described in this thesis were as follows:

- preparation of an overview of studied neurodegenerative diseases, neuroactive substances, and classical and modern methods of their analysis,
- development and validation of a purification protocol and a detection UHPLC–MS/MS method for profiling a selected group of steroid analytes with neuroactive properties in human blood serum,
- metabolic profiling of TRP and TRP-related analytes by UHPLC–MS/MS and analysis of protein candidate biomarkers in human cerebrospinal fluid (CSF) and blood serum samples from a healthy control group and several neuropathological cohorts; mapping changes in TRP metabolism and interpretation of results.

3. MATERIAL AND METHODS

3.1 Chemicals

- Unlabeled standards and stable isotopically labeled internal standards (mostly deuterated) were obtained from Sigma–Aldrich (Germany), Fluka (Netherlands), National Measurement Institute (Australia), Cambridge Isotope Laboratories, Inc. (USA), C/D/N Isotopes (Canada), Olchemim Ltd. (Czech Republic), and Toronto Research Chemicals (Canada). Some of them were synthesized in the Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany ASCR, Olomouc.
- All chemicals and solvents for sample preparation and analysis were purchased from Merck Millipore (Germany), Fluka (USA), Sigma-Aldrich (USA), Lach-ner (Czech Republic), Linde Industrial Gases (Czech Republic), and Tocris Bioscience (UK). Ultrapure water was produced using the Direct-Q® 3 UV Water Purification System (Merck Millipore, Germany).

3.2 Biological material

Human serum and CSF samples were provided by the Department of Neurology, University Hospital Olomouc, Czech Republic. Peripheral blood and CSF were collected, processed, transported, and stored according to the standardised protocol of the Department of Neurology. All samples (i.e. CSF and serum obtained) were stored in the dark at -80 °C until analysis.

This biological material was used following an approval of the ethics committee of the Faculty of Medicine and Dentistry, Palacký University Olomouc and University Hospital Olomouc. Ethical approval was granted according to the standard of the University Hospital Olomouc SM-L031 and the reference numbers of the ethics committee: 139/10 and 76/15. All volunteers were informed about the purpose of the study and signed an informed consent.

3.3 Instrumentation

- ACQUITY™ UPLC™ H-Class PLUS System (Waters, USA) connected to a triple quadrupole mass spectrometer (MS) Xevo® TQ-S micro (Waters, UK)
- ACQUITY® UPLC® (Waters, USA) system connected to a triple MS Xevo® TQ (Waters, UK)
- Kinetex® Biphenyl column (100 × 2.1 mm, 1.7 µm, 100 Å; Phenonemex, USA), ACQUITY Column In-Line Filter kit (Waters, UK)
- ACQUITY UPLC® HSS T3 column (100 × 2.1 mm, 1.8 µm, 100 Å; Waters, UK), ACQUITY UPLC® HSS T3 VanGuard™ pre-column (5 × 2.1 mm, 1.8 µm, 100Å; Waters, UK)
- Atellica® CH analyzer (Siemens, USA; SEKK certified)
- Enzyme-Linked ImmunoSorbent Assays (ELISA) kits (CE-IVD; BioVendor, Euroimmun, EPITOPE Diagnostic)

3.4 Methods

3.4.1 Extraction and purification methods

- Serum samples (150 µL) for steroid analysis were subjected to a protocol involving precipitation of serum proteins and extraction of steroid analytes using ice-cold acetonitrile (ACN; 595 µL, -20 °C) containing 0.05% butylated hydroxytoluene (prevention of oxidation). The protocol included incubation of samples on a rotator (1 h, -20 °C) with the addition of ACN, methanol (MeOH; 45 µL, instead of standard solution in the calibration), and internal standards (5 µL), centrifugation, filtration (centrifuge filter microtubes, nylon, 0.2 µm), and evaporation under a stream of nitrogen. Before analysis, the samples were reconstituted in 100% MeOH (50 µL) and filtered again (centrifuge filter microtubes, nylon, 0.2 µm). The quantification of the analytes was performed using matrix-matched calibration curves prepared from artificial serum: 4% bovine serum albumin (BSA) in 10 mmol/L phosphate-buffered saline (PBS), pH 7.4. The same purification and extraction protocol was used for calibration samples and serum samples.
- TRP-related analytes were analyzed in blood serum and CSF samples (100 µL). The samples were protected from light during processing and placed in a CoolBox™ (Biocision). The corresponding isotopically labeled internal standards were added to

each sample at the beginning of the extraction (listed in Hényková *et al.* 2016). The purification protocol included protein precipitation with 100% MeOH for 1 h at -20 °C, centrifugation, filtration (centrifuge filter tubes, nylon, 0.2 µm), and evaporation (under nitrogen). Samples were reconstituted in 2% aqueous MeOH (30 µL) before LC–MS/MS analysis. The quantification was performed using serum (4% BSA in 10 mmol/L PBS) and CSF (CSF calibrator; Tocris Bioscience, UK) matrix-matched calibration curves.

3.4.2 UHPLC–MS/MS analysis

- Selected steroid analytes were determined by ACQUITY™ UPLC™ H-Class PLUS System (Waters, USA) connected to a triple quadrupole MS Xevo® TQ-S micro (Waters, UK) with electrospray ionisation (ESI). The samples were injected (2 µL) onto a reversed-phase chromatography column (Kinetex® Biphenyl column; 100 × 2.1 mm, 1.7 µm, 100 Å; Phenonemex, USA) maintained at 40 °C. Analytes were eluted with 100% MeOH (A) and 7.5 mmol/L aqueous formic acid (B) as mobile phases at a flow rate of 0.5 mL/min using the following gradient: 0–10 min, 60–75% A; 10–12 min, 75–85% A; 12–12.25 min, 85–99% A; 12.25–12.75 min, 99% A; 12.75–13 min, 99%–60% A; 13–15 min, 60% A. The tandem MS with positive ESI was operated in multiple reaction monitoring (MRM) mode using quantification and confirmation transitions. Based on the expected retention times of the analytes, data acquisition was divided into five separate MRM scan segments. The defined addition of isotopically labeled (deuterated) internal standards enabled the quantification of analytes by the isotopic dilution method.
- UHPLC–MS/MS analysis of targeted TRP and TRP-related analytes was performed by ACQUITY® UPLC® (Waters, USA) system connected to a triple MS Xevo® TQ (Waters, UK). The analytes (sample injection 10 µL) were separated using an ACQUITY UPLC® HSS T3 column (100 × 2.1 mm, 1.8 µm, 100 Å, Waters, UK; temperature 30 °C) equipped with a pre-column ACQUITY UPLC® HSS T3 VanGuard™ (5 × 2.1 mm, 1.8 µm, 100Å; Waters, UK) and were eluted in a gradient (0–2 min, 98% A; 2–10 min 40% A) of aqueous 0.1% formic acid (A) and 100% MeOH (B) at a flow rate of 0.3 mL/min. A wash and equilibration steps were performed at the end of the gradient. The MS instrument with ESI source was operated in MRM mode.

3.4.3 Biochemical determination of protein analytes

- The biochemical analyses were performed in the accredited laboratory of the Department of Clinical Biochemistry, University Hospital Olomouc (CSN ISO 15189:2013; subject No. 8254; certificate No. 220/2021 valid until 9 April 2026).
- The sandwich ELISAs (CE-IVD) were used to determine: clusterin, tau protein, phosphorylated tau protein, beta-amyloid $_{1\text{-}42}$, alpha-synuclein, phosphorylated form of neurofilament heavy chains, chromogranin A, and cystatin C. Serum cystatin C was analyzed using an Atellica® CH analyzer (Siemens, USA). Detection was performed spectrophotometrically or fluorometrically.

4. SURVEY OF RESULTS

Certain pathologies of the nervous system, among others, can affect metabolic pathways, thereby altering the levels of certain metabolites. Knowledge of specific metabolic profiles or patterns is essential for several reasons, for example, it may provide a basis for the discovery of new biomarkers or therapeutic targets. However, highly reliable analytical techniques are needed to monitor and map these changes (reviewed in Kaleta *et al.* 2021, Hényková *et al.* 2022) Therefore, a sensitive method based on UHPLC–MS/MS has been developed that allows the determination of some representatives of NASs in human serum (Kaleta *et al.* unpublished A). Moreover, alterations in TRP metabolism at the level of the kynurenine, methoxyindole, kynuramine, and intestinal bacterial indole pathways have been comprehensively mapped in several degenerative proteinopathies (Kaleta *et al.* unpublished B). Serum and CSF levels of several candidate protein biomarkers were also analyzed in the same neurodegenerative cohorts (Koníčková *et al.* 2023).

4.1 Method development for NAS determination

A complex method allowing simultaneous detection and quantification of nine selected steroids with neuroactive effects in human blood serum has been developed and validated. This method combines a relatively time-effective and simple purification protocol and a sensitive detection method based on UHPLC–MS/MS. The analytes included representatives of progestins (pregnenolone, PREG; allopregnanolone, ALLO; progesterone, PROG; 5α-dihydroprogesterone, DHP) and androgens (testosterone, T; 5α-dihydrotestosterone, DHT; androstenedione, ANDRO; epiandrosterone, EPIA; dehydroepiandrosterone, DHEA).

For more detailed information see:

Kaleta, M., Oklestkova, J., Strnad, M., Novák O. Simultaneous Determination of Selected Steroids with Neuroactive Effects in Human Serum by Ultra-High Performance Liquid Chromatography–Tandem Mass Spectrometry (In preparation, A).

4.1.1 Extraction and purification protocol

- A purification and extraction technique consisting of three main steps, namely serum protein precipitation, filtration, and evaporation, was proposed for the processing of

blood serum samples. This simple arrangement makes the resulting sample processing relatively rapid and simple. The use of ice-cold MeOH and ACN (-20 °C) in combination without and with filtration (micro-spin filter tubes with 0.2 µm porous mebranes) was tested. The best process efficiency values were achieved for ACN in combination with filtration. Filtration optimisation resulted in the selection of filters with a nylon-based membrane (other filters tested were Bio-Inert modified nylon, polytetrafluoroethylene, and polyvinylidene fluoride). Butylated hydroxytoluene (0.05%) was added to the extraction agent to protect the analytes from oxidation. A proportion of MeOH was also added to the serum samples so that their processing corresponds to the preparation of matrix-matched calibration samples (steroid standards prepared in MeOH).

4.1.2 UHPLC–MS/MS method

- The final reversed-phase chromatographic separation (Kinetex® Biphenyl column, 100 × 2.1 mm, 1.7 µm, 100 Å, Phenomenex, USA) of one sample was performed within 15 min with a gradient consisting of MeOH and 7.5 mmol/L aqueous formic acid at a flow rate of 0.5 mL/min. Analytes were eluted from 4.16 min (DHEA) to 10.83 min (DHP). A triple quadrupole MS in positive ESI mode was used to detect the target steroid analytes. The MS instrument operated in MRM mode with protonated $[M+H]^+$ or $[M-H_2O+H]^+$ molecules formed by the loss of water molecules as precursor ions. The values of collision energy and cone voltage were optimised to obtain specific product ions with high abundance and to ensure the highest possible sensitivity. Two MRM mass transitions were selected for each analyte, one was used for quantification and the other for confirmation. The dwell time values (0.050–0.250 ms) were set to achieve at least 15 scan points per chromatographic peak width. The MS parameters (MRM transitions, cone voltages, collision energies, dwell times, etc.) for each analyte and the corresponding internal standards are listed in Kaleta *et al. unpublished A.*

4.1.3 Analytical method validation

- The developed method was validated based on the European Medicines Agency (EMA) guideline (EMA 2011). Validation experiments were performed using four quality control levels: low (LQ; 0.0569 pmol/inj.), medium (MQ; 0.18 pmol/inj.), high (HQ; 1.8 pmol/inj.), and ultra-high (UHQ; 5.7 pmol/inj.).

- The quantification of analytes was performed using matrix-matched calibration curves prepared in an artificial matrix (4% BSA in 10 mmol/L PBS, pH 7.4) spiked with unlabeled steroid standards (from 0.18 fmol/inj. to 57 pmol/inj.). Defined additions of stable isotopically labeled deuterated standards allowed the quantification of analytes by the isotope dilution method. An overview of the individual analytes, the corresponding deuterated internal standards, and their optimised additions are given in Kaleta *et al. unpublished A*. At least seven-point calibration curves with coefficients of determination (r^2) ≥0.9989 were obtained for all analytes. The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined as a signal-to-noise ratio of ≥3 and ≥5, respectively. The lowest detectable levels of some steroids were below 1 fmol/inj., while their LLOQs ranged from 0.0018 to 0.0569 pmol/inj.
- Within- and between-run precision and accuracy were determined using sample sets of neat solutions (100% MeOH) spiked with unlabeled standards at four quality control levels (LQ, MQ, HQ, and UHQ) and a defined addition of internal standards. Within-run precision and accuracy were also determined using the participants' pool serum. Each quality control level was represented by five replicates. The measured mean concentrations for most analytes in neat solution and serum did not differ from the reference values by more than ±15%. The coefficient of variation values ranged from 0.2 to 14.1%. These results are in line with EMA recommendations (EMA 2011).
- Method recovery, matrix effect, and internal standard-normalised matrix effect were determined using blood serum from several individual participants. Two sets of serum samples were prepared: spiked at four quality control levels before and after extraction. Analytical method recoveries of steroids in serum samples were between 66 and 102%. However, as the accuracy and precision determination has shown, the use of internal standards compensates for these process losses. The higher standard deviation values, especially for DHEA, are probably cause by the use of serum from several participants and their individual characteristics. The strongest matrix effect in terms of ion suppression was observed for DHP. Values of the absolute and internal standard-normalised matrix effect ranged from 19 to 24% and from 27 to 33%, respectively. Such a strong matrix effect may have several explanations: the elution of DHP at the end of the gradient together with a high proportion of contaminants interfering with its ionisation efficiency and the under-compensation of matrix effect

(use of an internal standard eluting at a different retention time). However, the quantification of the analytes is accurate and precise, which is ensured using matrix-matched calibration curves.

- Finally, the developed and validated method was successfully applied to the analysis of 16 female and male serum samples. The determined endogenous concentrations of the target analytes corresponded to the expected levels.

4.2 Metabolic profiling of tryptophan-related metabolites

The endogenous levels of 18 TRP-related neuroactive compounds were profiled by a high-throughput and sensitive UHPLC–MS/MS method (Hényková *et al.* 2016) in time-linked serum and CSF of 100 participants. They were divided into five cohorts based on clinical diagnoses: Lewy body disease (LBD), four-repeat tauopathy (4R-Tau), multiple system atrophy (MSA), Alzheimer's disease (AD), and healthy controls (HC). The basic characteristics of the cohorts are shown in *Table 1*.

For more detailed description of experimental design and results see:

Kaleta, M., Hényková, E., Menšíková, K., Friedecký, D., Kvasnička, A., Klíčová, K., Koníčková, D., Strnad, M., Kaňovský, P., Novák O. Patients with Neurodegenerative Proteinopathies Exhibit Altered Tryptophan Metabolism in the Serum and Cerebrospinal Fluid. ACS Chemical Neuroscience (Submitted, B).

Table 1. Characteristics of the study participants (*n* = 100).

Participants group	Description	Number of participants	Sex ratio (M/F)	Age median (range)
LBD	Parkinson's disease, Dementia with Lewy bodies	31	9/22	69 (38–82)
4R-Tau	Progressive supranuclear palsy, Corticobasal syndrome	10	2/8	66 (51–83)
MSA	Multiple system atrophy	13	2/11	65 (52–80)
AD	Alzheimer's disease	25	3/22	75 (51–90)
HC	Healthy control	21	11/10	57 (37–75)

LBD, Lewy body disease; 4R-Tau, Four-repeat tauopathy; MSA, Multiple system atrophy; AD, Alzheimer's disease; HC, Healthy control

4.2.1 Alterations of tryptophan metabolism in selected neurodegenerations

- Serum and CSF levels of eight analytes were below the LOQ or LOD in all or most participants: *N*-methylserotonin, tryptamine, *N*-methyltryptamine, 5-methoxytryptamine, *N*-acetylserotonin, 6-hydroxymelatonin, melatonin, and N_1-acetyl-N_2-formyl-5-methoxykynuramine. For this reason, these analytes were excluded from further statistical evaluations. The remaining ten analytes were successfully quantified, these are TRP, 3-hydroxykynurenine (3-OH-KYN), serotonin, kynurenine (KYN), 5-hydroxy-L-tryptophan (5-OH-TRP), 3-hydroxy-anthranilic acid, 5-hydroxyindole-3-acetic acid, kynurenic acid, anthranilic acid, and indole-3-acetic acid. These analytes were subjected to statistical analyses.
- The most statistically significant differences (p-values of ≤ 0.05 to ≤ 0.0001) between the study cohorts were observed for 3-OH-KYN and 5-OH-TRP in serum, and KYN in CSF. A significant increase in serum 5-OH-TRP was found in the LBD, 4R-Tau, and MSA groups compared to HC and/or AD. The serum 3-OH-KYN levels were significantly different in the LBD and AD groups, as were CSF KYN concentrations in the LBD and HC groups.
- Furthermore, it was suggested that the effect of antiparkinsonian treatment (levodopa and peripheral decarboxylase inhibitors) led to a significant increase in 5-OH-TRP and 3-OH-KYN levels in the treated LBD group. A similar trend, but without statistical significance, was also observed in the case of MSA (limited number of samples). The effect of treatment could not be evaluated in the 4R-Tau group due to the limited number of patients.
- The increase in 5-OH-TRP in the LBD group may be due to substrate competition between levodopa and 5-OH-TRP at the aromatic amino acid decarboxylase (EC 4.1.1.28) (see *Figure 1*). The effect of a peripheral inhibitor may also contribute to the increase. A similar trend was also observed for MSA, but the mechanism of the increase in 5-OH-TRP may be different.
- The effect of antiparkinsonian treatment was also observed in the case of 3-OH-KYN (unknown mechanism). The benefit of complementary treatments targeting the synthesis of this neurotoxic metabolite has been suggested.

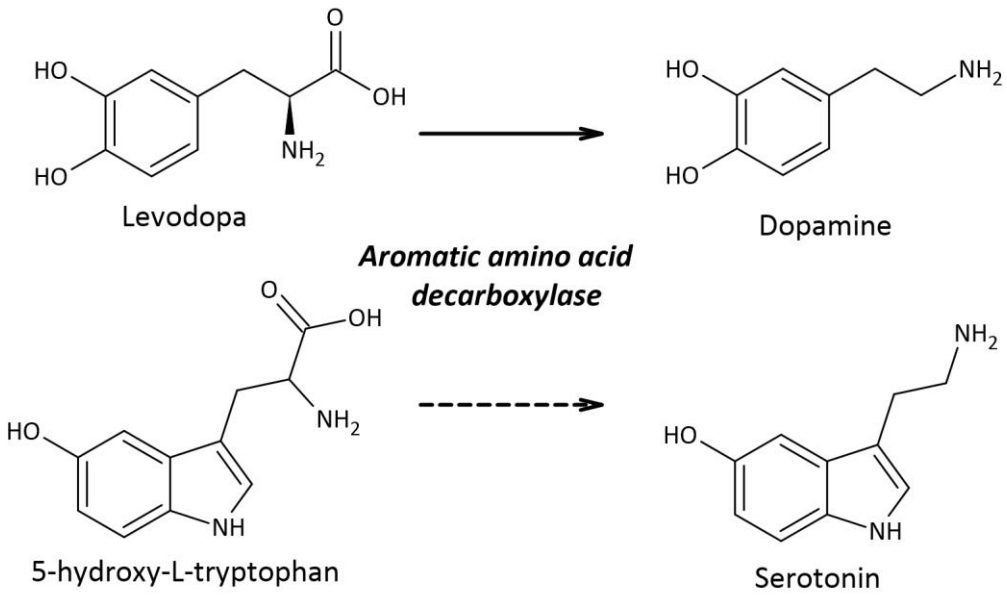

Figure 1. Metabolism of levodopa and 5-hydroxy-L-tryptophan.

- Pearson correlation analysis revealed an increased proportion of negative correlations between serum and CSF analytes in the 4R-Tau compared with the other cohorts. This specific correlation pattern could be a first step in developing a reliable tool to distinguish between tauopathies (4R-Tau) and synucleinopathies (LBD and MSA). Alterations in different phases of TRP metabolism may influence the neurotoxicity of protein aggregates and thus contribute to the development of different types of neurodegenerative proteinopathies.

4.3 Identification of serum and CSF protein biomarkers

Selected candidate protein biomarkers (alpha-synuclein, tau protein, phosphorylated tau protein, beta-amyloid, clusterin, chromogranin A, cystatin C, neurofilament heavy chains, phosphorylated form of neurofilament heavy chains, and ratio of tau protein/beta-amyloid) were determined in serum and CSF of several selected neurodegenerative proteinopathies. The study cohorts were LBD, MSA, 4R-Tau, and HC. Diagnostic criteria, patient recruitment, and more detailed demographic characteristics of participants are provided in (Koníčková *et al.* 2023).

- The results suggest that the determination of these specific biomarkers in blood serum, compared to CSF, does not provide any diagnostic benefit.

- The CSF collection, despite its complications (e.g. the invasiveness of the procedure for the patient), is still an important source of diagnostic information.
- The proposed panel of biomarkers in CSF could potentially be useful for the differentiation of MSA and 4R-Tau, LBD and MSA, but not LBD and 4R-Tau.

For more detailed information see:

Koníčková, D., Menšíková, K., Klíčová, K., Chudáčková, M., Kaiserová, M., Přikrylová, H., Otruba, P., Nevrlý, M., Hluštík, P., Hényková, E., Kaleta, M., Friedecký, D., Matěj, R., Strnad, M., Novák, O., Plíhalová, L., Rosales, R., Colosimo, C., Kaňovský, P. (2023) Cerebrospinal Fluid and Blood Serum Biomarkers in Neurodegenerative Proteinopathies: A Prospective, Open, Cross-Correlation Study. Journal of Neurochemistry 167, 168-182.

5. CONCLUSION AND PERSPECTIVES

Given the ongoing challenges associated with diagnosing neurodegenerative diseases, the pursuit of novel, highly reliable, specific, and sensitive biomarkers enabling their accurate and accelerated diagnosis is currently a significant area of research. In response to this, a comprehensive mapping of changes in TRP metabolism was conducted across several neurodegenerative proteinopathies. Furthermore, protein candidate biomarker analysis was also conducted within the same cohorts. Another highly intriguing group of substances, possessing neuroactive effects, and thus holding potential significance in terms of their involvement in pathogenesis or potential therapeutic applications, are steroid compounds. In this context, a new analytical method has been developed to enable the simultaneous profiling of several of their representatives. The most important outcomes of the work are:

- A purification and UHPLC–MS/MS detection method enabling metabolic profiling of nine selected representatives of progestins and androgens with neuroactive effects in human serum has been developed and validated.
- The determination of 18 TRP-related substances in serum and CSF in four types of neurodegenerations has been performed. Significant differences between groups were determined for serum 5-OH-TRP, 3-OH-KYN, and CSF KYN. It has been suggested that the effect of antiparkinsonian treatment may contribute to changes in their levels. A specific correlation pattern of TRP metabolites was found in the 4R-Tau. This observation may guide the development of tools for the differential diagnosis of tauopathies and synucleinopathies.
- In the same cohorts of patients, the levels of some protein biomarkers were determined. The suggested CSF biomarker platform could prove valuable in distinguishing between MSA and 4R-Tau, as well as between LBD and MSA.

In summary, reliable profiling of candidate molecule levels using sensitive analytical methods can help to better understand metabolic changes under physiological and pathological conditions. The knowledge gained may contribute to the discovery of new biomarkers (predictive, diagnostic, prognostic, etc.). In addition, new therapeutic strategies could be designed. However, finding sensitive and specific serum biomarkers is still a major challenge, if only because of the higher invasiveness of CSF sampling.

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[1] E.H. and M.K. contributed equally to the presented paper.

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8. SOUHRN (SUMMARY, IN CZECH)

Název disertační práce:

Studium endogenních neuroaktivních substancí v biologickém materiálu

Tato dizertační práce se zaměřuje na studium dvou skupin neuroaktivních látek, a to neuroaktivních steroidů a látek souvisejících s tryptofanem, v lidských tělních tekutinách. Ke studiu těchto analytů byly použity citlivé metody založené na ultra-vysokoúčinné kapalinové chromatografii kombinované s tandemovou hmotnostní spektrometrií (UHPLC–MS/MS). Poslední část práce je věnována vybraným kandidátním proteinovým biomarkerům neurodegenerace.

Vzhledem k přetrvávajícím problémům spojeným s diagnostikou neurodegenerativních onemocnění je v současné době významnou oblastí výzkumu hledání nových, vysoce spolehlivých, specifických a citlivých biomarkerů, které by umožnily jejich přesnější a rychlejší diagnostiku. V reakci na to bylo provedeno komplexní mapování změn v metabolismu tryptofanu u několika vybraných neurodegenerativních proteinopatií. Kromě toho byla v rámci stejných kohort provedena také analýza kandidátních proteinových biomarkerů. Další velmi zajímavou skupinou látek, které mají neuroaktivní účinky, a mají tak potenciální význam z hlediska jejich zapojení do patogeneze nebo možného terapeutického využití, jsou steroidní sloučeniny. V této souvislosti byla vyvinuta nová analytická metoda, která umožňuje současné profilování několika jejich zástupců.

Nejdůležitější výsledky práce:

- Byla vyvinuta a validována metoda purifikace a UHPLC–MS/MS detekce umožňující metabolické profilování devíti vybraných zástupců progestinů a androgenů s neuroaktivními účinky v lidském séru.
- Bylo provedeno stanovení 18 metabolitů tryptofanu v séru a mozkomíšním moku u čtyř typů neurodegenerací. Významné rozdíly mezi skupinami byly stanoveny pro 5-hydroxytryptofan a 3-hydroxykynurenin v krevním séru a kynurenin v mozkomíšním moku. Předpokládá se, že ke změnám jejich hladin může přispívat účinek antiparkinsonické léčby. Specifický korelační vzorec metabolitů tryptofanu byl zjištěn u 4R-Tauopatií (4R-Tau). Toto pozorování může být vodítkem pro vývoj nástrojů pro diferenciální diagnostiku tauopatií a synukleinopatií.

- Ve stejných kohortách pacientů byly stanoveny hladiny některých kandidátních proteinových biomarkerů. Využití navrhované platformy likvorových biomarkerů by mohlo být cenné při rozlišování mezi multisystémovou atrofií (MSA) a 4R-Tau, stejně jako mezi nemocí s Lewyho tělísky (LBD) a MSA.

Spolehlivé profilování hladin kandidátních molekul pomocí citlivých analytických metod může pomoci lépe pochopit metabolické změny za fyziologických i patologických stavů. Získané poznatky mohou přispět k objevu nových biomarkerů. Kromě toho by mohly být také navrženy nové terapeutické strategie. Vzhledem k vyšší invazivitě odběrů vzorků mozkomíšního moku je však nalezení citlivých a specifických biomarkerů v krevním séru stále velkou výzvou.