THE BIOCHEMISTRY OF BILE ACIDS AND THEIR HPLC-MS ANALYSIS

BACHELOR THESIS

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Annotation:

The aim of this thesis was to summarize the biological importance and the physico-chemical properties of bile acids, to investigate the current methods for detection and quantification and to develop a quick and reliable HPLC-MS method to detect bile acids in biological systems, such as urine and plasma.

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Abbreviations

APCI	atmospheric pressure chemical ionization
BA	Bile Acids
BS	Fetal Bovine Serum (stripped with charcoal)
CA	cholic acid
CDCA	chenodeoxycholic acid
DCA	deoxycholic acid
EHC	enterohepatic cycle
ESI	electro-spray ionisation
HPLC	high pressure/performance liquid chromatography
HRMS	high resolution mass spectroscopy
LCA	lithocholic acid
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometer
QqQ	triple quadrupole
RP	reversed phase
TEAS	triethyl amine sulfate
UDCA	Ursodeoxycholic acid
UHPLC	ultra high performance chromatography

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1. Introduction and Aim of the thesis

The Laboratory of Analytical Biochemistry & Metabolomics, Biology Centre CAS has done extensive work in the field of steroid analytics and the qualification and quantification of similar compound in biological matrices. So they gave me the opportunity to research on the steroid homologues group of bile acids. The aim of this work was to gain a basic understanding of the bile acids and their biological and physiological importance and to develop a HPLC-MS method for the qualification and quantification of the bile acids.

2. Theoretical Background

2.1. Bile Acids

2.1.1. Chemical structure and properties

The bile acids (BA) are in principle steroid acids, occuring in bile of various vertebrates. Their structure is formed by a cyclopentanoperhydrophenanthrene sceleton and an C17 alkyl side chain possessing a carboxylic group. These 4 rings, consisting of 3 six-membered rings and one five-membered ring, and the side chain can be seen in Fig. 1.

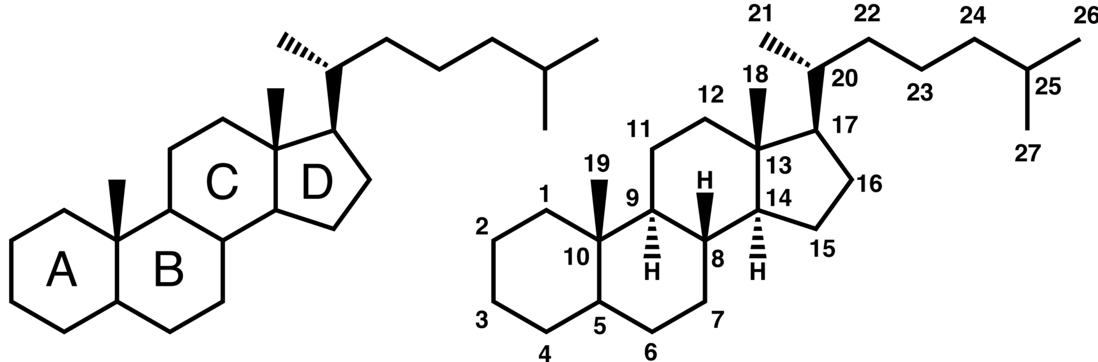
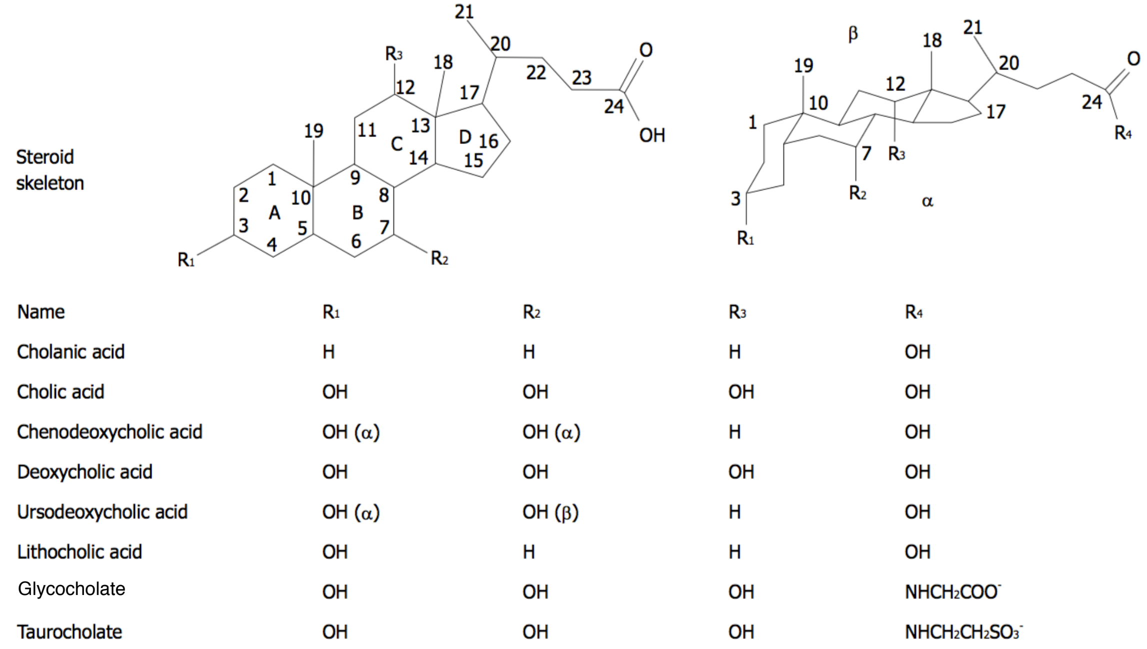


Fig. 1 Standard IUPAC ring and atom numbering of the steroid skeleton (<https://commons.wikimedia.org/w/index.php?curid=5613812>, 13 February 2017)

The various bile acids differ mainly from the side chain, the number and position of the hydroxyl groups and the configuration at the A/B ring junction. In the mammalian bile acids, the configuration at C5 (connection between the rings A and B) is almost always β or cis configured. The types and variations

of bile acids are the results of the evolution from C27 5α-alcohol sulphates to the C24 5β-acids (Fig. 2). So the human and higher mammalian bile acids consist of 24 carbon atoms in contrast to much earlier species (e.g. sharks, amphibians and reptiles) which metabolize BA with 25 to 27 carbons.

In the human bodies, the BA are often conjugated with taurine or glycine at the carboxylic acid site. [1]



Name	R_1	R_2	R_3	R_4
Cholanic acid	H	H	H	OH
Cholic acid	OH	OH	OH	OH
Chenodeoxycholic acid	OH (α)	OH (α)	H	OH
Deoxycholic acid	OH	OH	OH	OH
Ursodeoxycholic acid	OH (α)	OH (β)	H	OH
Lithocholic acid	OH	H	H	OH
Glycocholate	OH	OH	OH	$NHCH_2COO^-$
Taurocholate	OH	OH	OH	$NHCH_2CH_2SO_3^-$

Fig. 2 Structures of the most abundant bile acids in humans, and their glycine and taurine conjugates from Monte MJ [1]

2.1.2. Conjugation of bile acids

In humans most bile acids are conjugated over an amide bond on the carboxylic acid group. Taurine and glycine are the most abundant conjugated species and in the human body the ratio between them is around 3 to 1 (glycine to taurine). This ratio can be different in other mammals such as mice, where 95% of the conjugated BA are taurine conjugates. The conjugation is a useful option increasing the BA polarity and thus solubility in aqueous biological matrices. It also prevents the precipitation of Ca^{2+} salts and hinders passive absorption. The BA conjugates are also much less prone to metabolization by pancreatic carboxypeptidases. [2]

2.1.3. Physiological properties

BA exhibit detergent properties having a hydrophobic and a hydrophilic part. While the basic steroid skeleton with the alkyl side chain is largely hydrophobic, the carboxyl group, the hydroxyl groups and the conjugated moieties are polar. BA thus have capability of forming small micelles consisting of typically 10 BA molecules. This amphipathic nature is then used for the transport and the absorption of fats and lipids in the intestinal system. According to the increased hydrophobicity, BA can be sorted: UDCA > cholic acid (CA) > chenodeoxycholic acid (CDCA) > deoxycholic acid (DCA) > lithocholic acid (LCA) [1]. In general, taurine conjugates are more polar than the glycine analogues [3].

This scheme also approaches to the Ca^{2+} absorption capability of BAs. Although the use of hydrophilicity/hydrophobicity is helpful it is by far not the only answer to the different properties of the different BAs. [1]

2.2.Bile Acid Metabolism

2.2.1. Bile Acid biosynthesis in the Human Body

The bile acids represent the final product of the cholesterol metabolism. The structural similarity can be seen in the comparison of of cholesterols and BAs is shown in Fig. 3.

Fig. 3 Structure of cholesterol and cholic acid (https://commons.wikimedia.org/w/index.php?curid=645994,15 February 2017 ;https://commons.wikimedia.org/wiki/File:Cholsäure.svg,11 May 2018)

The BA biosynthesis proceeds in the liver from cholesterol. It involves complicated pathways which include several enzymatic steps. The double bond on the B - ring is saturated, at the C7 a hydroxyl group is added, the 3-hydroxyl group is epimerized and 3 carbons at the side chain are oxidatively cleaved off. Afterwards the BA molecule structure is modified by conjugation of various species, mainly Glycine or Taurine. The biosynthetic patways for BAs are shown in Fig.4. The classic pathways shown in Fig. 5 takes place in the liver tissue. CHIANG states: “Conversion of cholesterol to bile acids involves 17

distinct enzymes located in the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes [2]“.

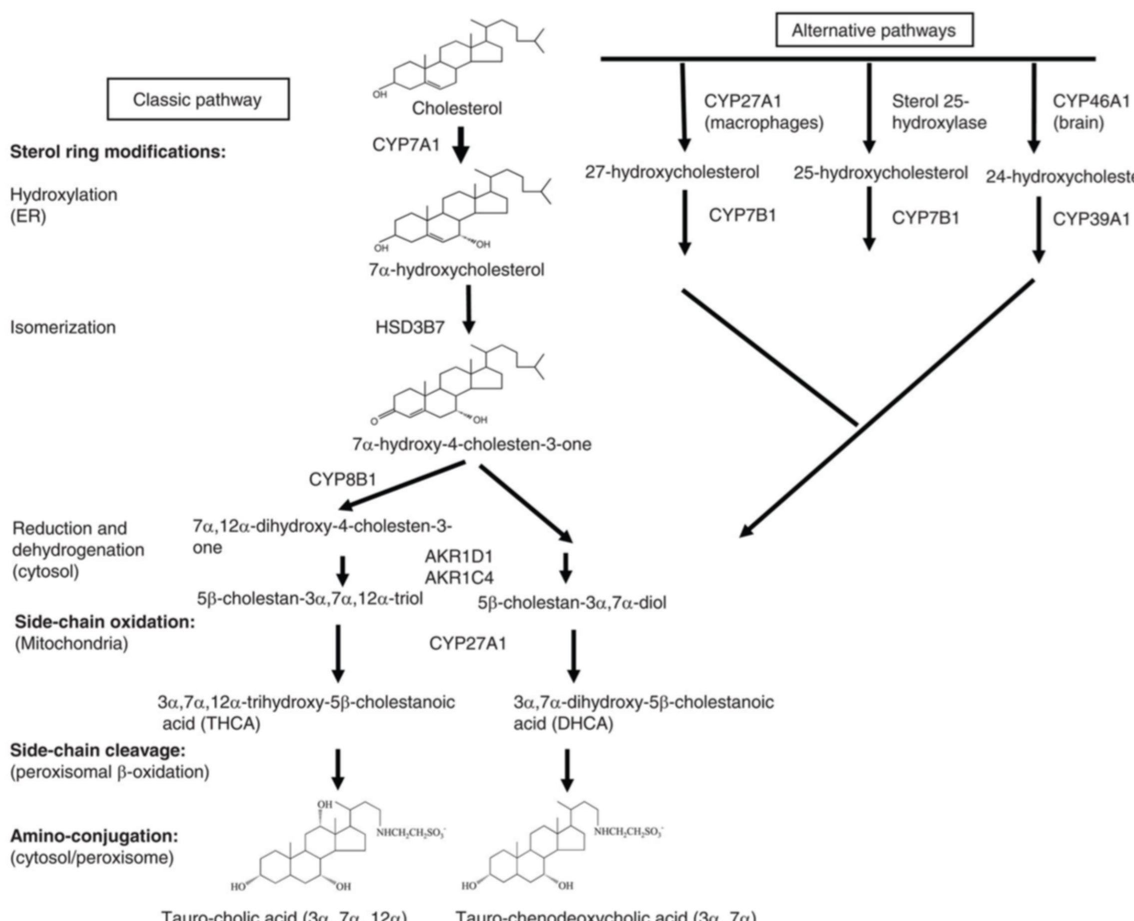


Fig. 4 The biosynthetic pathways of bile acids in the human body, From Chiang JYL [2]

The rate limiting and also the initiating enzyme in the “classic“ pathway is the cholesterol 7α-hydroxylase or also known as cytochrome P450 7A1 (CYP7A1). The alternative pathways are initiated by a different enzyme from the mitochondria called sterol 27-hydroxylase (CYP27A1). These alternative pathways are taking place in most tissues of the body and DUANE [4] pointed out that this pathway contributed to around 9% (range 3% to 18%) of the bile acid production in the human body.[4]

The liver of a human adult produces around 200-600 mg of bile acids which is around 5% of the total amount of bile acids in the digestive system.

2.2.2. Production of secondary BA out of primary BA

Cholic acid (CA) and Chenodesoxycholic acid(CDCA) are produced in the liver, then conjugated with either taurine or glycine and stored in the gallbladder. These are the so called primary BA. These conjugated primary BA are excreted into the intestine where they can get deconjugated and modified by bacteria. For example, with the help of bacterial 7α-dehydroxylase the CA and CDCA can be converted to the two secondary bile acids called deoxycholic acid (DCA) or lithocholic acid (LCA). The 7-hydroxyl group of CDCA can also be epimerized from α to β to form ursedeoxycholic acid (UDCA).

The LCA is mostly excreted with the feces but a small amount is transported back to the liver where it gets quickly sulfated and excreted into the bile again. The addition of this sulfate conjugation at the carboxylic group is a mechanism to detoxify the hydrophobic LCA. The metabolic pathways of the formation of primary and secondary BA are depicted in Fig. 5. [2]

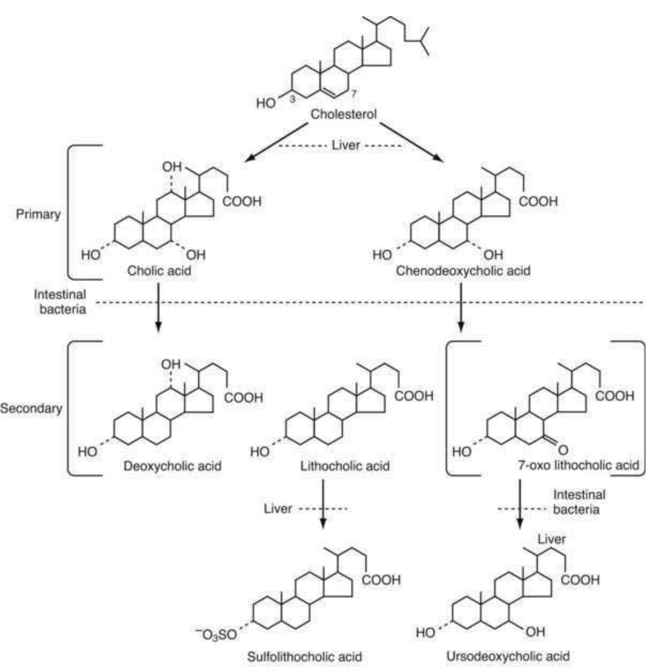


Fig. 5 major primary and secondary bile acids and their sites of synthesis and metabolism, from Carey MC [3]

2.2.3. The enterohepatic cycle

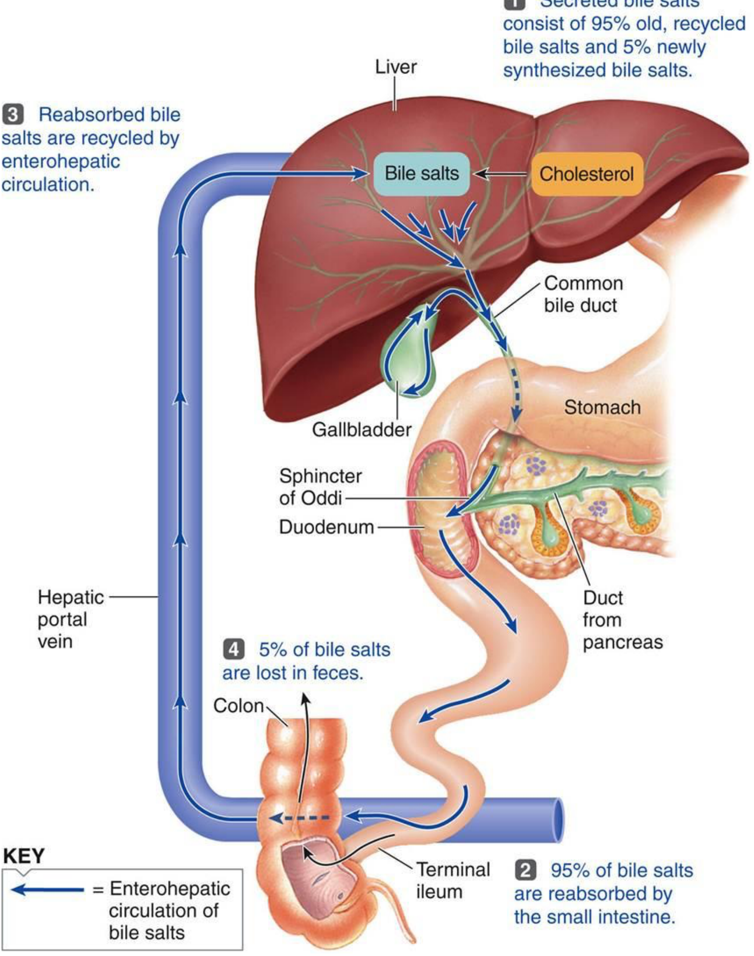


Fig. 6 Schematic of the enterohepatic cycle (blue arrows) (http://codigovisual.com.ar/wp-content/enterohepaticcirculation-of-bile-salts-501.jpg, 15 February 2017)

The enterohepatic cycle (EHC) is a very important and very efficient mechanism by the human body to recycle the bile acids produced in the liver. The EHC is important for the homeostasis of the digestive system. The whole enterohepatic cycle is schematically depicted in Fig. 6.

The BA are always stored in the gallbladder. When the intestine signals incoming food, with the hormone cholecystokinin, the gallbladder is activated and releases the bile with the stored BA into the small intestine. There the BA do their actual work in emulsifying the fats. The upper small intestine can reabsorb the bile acids passively only to a very small extent, due to the formation of the BA conjugates.

On the other hand, 95 % of primary BAs are reabsorbed in the terminal Ileum (last part of the small intestine) and transported back to the liver via the hepatic portal vein. The DCA is reabsorbed in the colon and also recycled to the liver. As also mentioned, the LCA is mainly excreted by the feces. Only traces get back to the liver. The loss of BAs through the feces is compensated with the new produced primary BAs in the liver. The whole amount of 3g of BA consists of ~40% CA, 40% CDCA, 20% DCA, and trace amount of LCA [2]. It is also important to mention that very small BA amounts (0.5 mg/d) are excreted in urine even though the kidney itself also partially recycles BAs. [2]

2.2.4. Regulation of the bile acid synthesis

The rate limiting CYP7A1 is the key enzyme in the BA regulation in the body. The production of this enzyme is influenced by different factors such as the BA concentrations themselves, insulin, cholesterol, thyroid hormones and glucocorticoids. The circadian rhythms also play a role in the CYP7A1 activity. Through experiments with rats, a negative feedback mechanism have been confirmed. The rats that failed to recycle the BA properly, showed elevated CYP7A1 activity while lower CYP7A1 activity was observed after the feeding the rats with BAs. The BA regulation mechanism is supposed to work on a transcriptomic level. The true mechanism has yet to be found but CHIANG ET AL[2] claim that there could be a connection between the mRNA half-life and the BA through certain elements in the 3'-untranslated regions. The post-translational mechanisms have barely been investigated and more research is needed to make valid statements. [2]

A main regulatory effect on the BA synthesis renders the food intake. After every nutrient intake the postprandial liver metabolism is triggered and the enzyme activity gets elevated. The CPY7A1 enzyme enzyme is a very specific hydroxylase which accepts only cholesterol and adds a hydroxyl group to the 7α-position. CYP7A1 was mostly found in the endoplasmic reticulum, where the cholesterol

concentration is rather low. Therefore the concentration of cholesterol in the ER directly regulates the activity of the hydroxylase. CHIANG suggests that there is evidence that the CYP7A1 prefers newly synthesized cholesterol which would directly indicate a link between *de novo* synthesis of Cholesterol and BA. Increased BA synthesis would decrease the ER-concentraion of cholesterol which in turn would trigger more *de novo* cholesterol synthesis via a specific pathway. [2]

2.2.5. Bile acid contents in the healthy human body

The typical levels of bile acids in human serum and urine described in literature can be seen in Table 1.

Table 1: Bile acid levels in body fluids of healthy humans [5, 6]

Compound	Full Name	Concentration in Urine (mean ± sem) [nM]	Concentration in Serum (mean ± sem) [nM]
CA	Cholic acid	194.2 ± 30.2	181.56 ± 83.1
CDCA	Chenodeoxycholic acid	3.52 ± 0.25	256.8 ± 56.3
DCA	Deoxycholic acid	20.1 ± 1.2	386.7 ± 66.0
GCA	Glycocholic acid	69.8 ± 5.5	233.0 ± 56.0
GCDCA	Sodium Glycochenodeoxycholate	7.73 ± 0.54	771.5 ± 111.9
GDCA	Sodium Glycodeoxycholate	10.7 ± 0.6	246.2 ± 42.5
GLCA	Glycolithocholic acid	0.36 ± 0.03	16.3 ± 4.1
GUDCA	Glycoursodeoxycholic acid	35.4 ± 3.8	-

Compound	Full Name	Concentration in Urine (mean ± sem) [nM]	Concentration in Serum (mean ± sem) [nM]
LCA	Lithocholic acid	0.15 ± 0.02	12.8 ± 1.8
TCA	Sodium Taurocholic acid	9.78 ± 0.8	179.7 ± 47.0
TDCA	Sodium Taurodeoxycholic acid	1.88 ± 0.1	44.9 ± 11.8
TLCA	Sodium Taurolithocholic acid	0.32 ± 0.03	23.4 ± 3.6
TUDCA	Tauroursodeoxycholic acid	2.15 ± 0.13	5.0 ± 1.1
UDCA	Ursodeoxycholic acid	3.46 ± 0.25	137.6 ± 25.1

2.3. Current Methods for Sample Preparation in BA analysis by HPLC-MS

HPLC/MS is an efficient tool for the detailed, comprehensive BA analysis. If the BA content is high as in the bile, then a simple sample dilution and or a simple clean-up is sufficient.

When the BA concentration is high, as it is in bile, there is not really a need for sample preparation. Most of the time a dilution and clean up may be sufficient.

However, the BA analysis in complex biological materials requires a carefully evaluated sample preparation method. The major aim of this step is to get rid of the salts, proteins and lipids in the sample matrix. The BAs may also be bound to proteins and lipids and must be released prior the HPLC/MS analysis. Griffiths and Sjovall [7] remarked that the solvent extraction may be inferior to the octadecylsilane substituted (ODS) silica column based sorbent extraction.[7].

Eva Wahlèn et al.(1994) experimented with different preparation and extraction methods for human urine. The best results in terms of recovery and efficiency were shown when diluting 10 ml sample with 10 ml of 0,5M triethyl

amine sulfate(TEAS) (pH = 7) before passing it through the C18 - column. The TEAS medium is efficient for the BA extraction minimizing loss of some BAs. [8]

Urine and plasma are most commonly used and relatively easily manageable for BA analysis. If feces or tissue are to be analyzed, the extraction step is more complicated, the method validation difficult and more labor intensive.

2.4. Current HPLC-MS Methods in Context of BA Analysis

The HPLC-MS is currently a preferred method for the BA determination and that it has been utilized for: “human and rodent urine, plasma/serum, bile, cecal content, liver, feces, and more surprisingly brain.“ [7]

Using the HPLC-MS technique, multiple BA can be analyzed in one continuous process and the sensitivity is fairly high. If set up correctly the structure of the BA can also be obtained and determined. A big advantage of LC-MS over GC-MS is that no lengthy and work intensive derivatization is necessary.

2.4.1. HPLC

RP-HPLC is most widely used method for BA separation. The columns used have been usually standard C18 and the column diameter and the flow-rates can vary from 1 ml/min (d = 4.6 mm) to 1 μl/min (d = 180μm). In the BA analysis, aqueous methanol or acetonitrile solutions are commonly used as the mobile phase. Griffiths (2010) analyzes the current mobile phases in use and concludes that some methods use a neutral pH mobile phase so that the BA are present in their deprotonated form and other methods use an acidic pH (~ 4) to provide the BA (except taurine conjugates) in the protonated form. The pH is buffered e.g. with ammonium acetate and formic or acetic acid. For the commonly used electrospray ionization technique (ESI) it is very important to avoid non-volatile compounds in the mobile phase. [7]

2.4.2. Ionisation Techniques

The most common HPLC-MS systems rely on atmospheric ionization techniques. The most widely used methods are electro-spray ionization (ESI, Fig. 7) and atmospheric pressure chemical ionization (APCI). For LC-MS systems, there are a lot of different ionization techniques available but only ESI and APCI are relevant for the BA determination. These two techniques are both working with similar principles and are both very good applicable for the BA ionization and detection. Both can be run in positive or negative ion detection mode. Lee, Kochhar & Shim [9] were comparing the two techniques for cholesterol esters. They concluded that the ESI is ionizing the cholesterol esters far more effective than the APCI system. Wegner et al [10] used an ESI in positive and negative ion mode for BA analysis and got satisfactory results. In the experiments with the BA of mice Alnouti et al. [11] used the ESI in positive Ion mode for glycine conjugates and in negative for taurine conjugates and unconjugated BA. [7, 9-11]

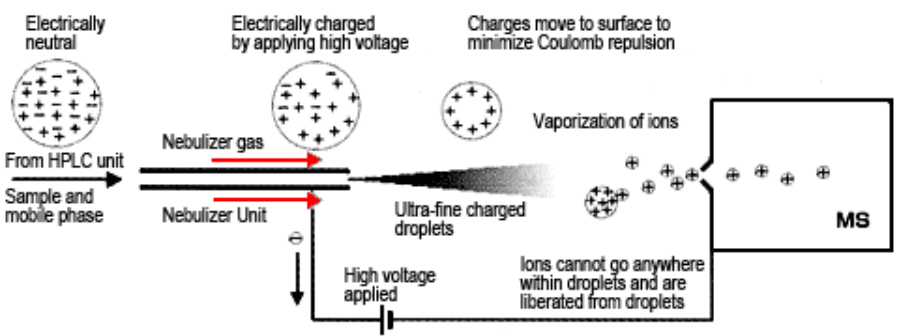


Fig. 7: Schematic Illustration of the ESI (https://www.shimadzu.com/an/hplc/support/lib/lctalk/47/47intro.html, 28 March 2018)

2.4.3. Mass Spectrometry in the bile acid analysis

The type of mass spectrometer varies between the different methods described in literature. The criteria for the application involve the required sensitivity and range, the need of structural clarification, the budget and last but not least the availability in the laboratory. The research group Wegner et al. [10] for example are using a hybrid triple quadrupole/linear ion trap mass spectrometer. With modern on-column

tandem mass spectrometers (MS/MS) like this the detection limits can be in the order of 20–50 pg[10]. Alnouti et al. [11] determined 18 BA in a 20min run with an UHPLC-ESI-QqQ-MS system. Griffith & Stovall [7] suggest to run the QqQ-MS in a multi reaction monitoring (MRM) scheme to optimize the sensitivity. Which means that the MS1 and/or the MS3 are set to select specific m/z ratios.
Another current MS method is the high-resolution MS (HRMS). The current most common HRMS systems represent an orbital ion trap (Orbitrap) and time-of flight analyser (TOF). Both systems are capable to acquire HRMS spectra with a measured mass accuracy below 1 ppm and thus estimate elemental compostion of ions with sufficient confidance. The availability of HRMS and tandem MS/MS options is very useful for the estimation of a variety of structurally diverse BAs. These problems have been considerably improved by the manufacturers over the last years. The author also points out that especially in BA analysis the additional information provided by the full scan of the HRMS is very important and desirable. This is due to the many different configurations and huge variety of the BA.
[7, 10-12]

3. Materials and Methods

3.1. Laboratory Equipment

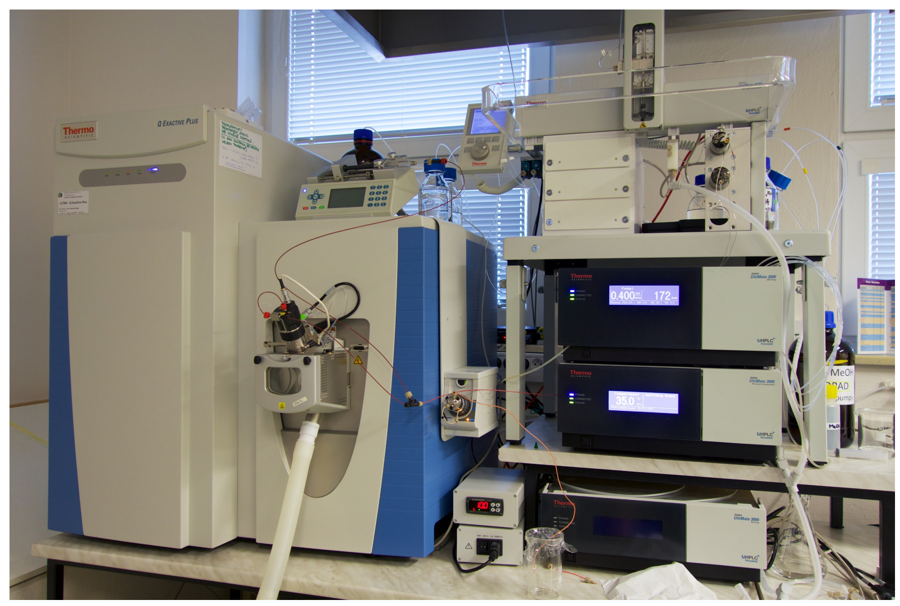


Fig. 8: UHPLC-Orbitrap System used in this study (laboratory BC-CAS)

Fig 9: UHPLC-Triple Quadrupole System used in this study (laboratory BC-CAS)

Their were two HPLC - MS systems in use in this thesis. The first system used was a Thermo Fischer TSQ Quantum Ultra (UHPLC-Triple Quadrupole). The UHPLC - System used in front of the triple quadrupole was a CTC Analytics HTS Pal autosampler, a Flux Instruments Rheos Allegro UHPLC pump, and a LC-MS Packing Thermos TM column thermostat.
The second system was a Thermo Fischer Q-Exactive Plus (UHPLC - Orbitrap). The Orbitrap UHPLC system was a Thermo Scientific Dionex Ultimate 3000 including the corresponding autosampler system.
Both mass spectrometers used electro spray as the ionization technique and were operated in positive and negative ion mode.

3.2. Chemicals

All the standards used were purchased from various suppliers and were delivered in solid form and with a purity stated in Table 1. In addition to the 14 different bile acids in the table four deuterated BAs were used as internal standards. The standards were obtained from Cayman Chemical (DCA, GCA, GLCA, CDCA, LCA, TDCA, TCA, TUDCA), Sigma Aldrich (CA, GDCA, GCDCA, TLCA, UDCA), CIL - Cambridge Isotope Laboratories (GUDCA, GUDCA d4, TCA d4), CDN Isotopes (DCA d4) and TRC - Toronto Research Chemicals (UDCA d5).

Table 1: List of Standards

Compound	Full Name	Structure	Molecular mass [g/mol]	Purity
CA	Cholic acid		408.57	98 %
CDCA	Chenodeoxycholic acid		392.60	95 %
DCA	Deoxycholic acid		392.60	95 %
GCA	Glycocholic acid		465.60	95 %
GCDCA	Sodium Glycochenodeoxycholate		471.61	97 %

Compound	Full Name	Structure	Molecular mass [g/mol]	Purity
GDCA	Sodium Glycodeoxycholate		471.61	97 %
GLCA	Glycolithocholic acid		433.60	98 %
GUDCA	Glycoursodeoxycholic acid		449.62	98 %
LCA	Lithocholic acid		376.60	95 %
TCA	Sodium Taurocholic acid		537.70	95 %
TDCA	Sodium Taurodeoxycholic acid		521.70	95 %
TLCA	Sodium Taurolithocholic acid		505.69	100 %
TUDCA	Tauroursodeoxycholic acid		499.70	95 %
UDCA	Ursodeoxycholic acid		392.57	99 %

The given standards were all weighed in and diluted in 5 ml methanol measured with volumetric flasks to obtain approximately 1 μmol*ml^{-1} stock solutions. To homogenize and ensure that everything is dissolved the solutions were put into an ultrasonic bath for 5min. The exact weights and concentrations used within the study are summarized in Table 2.

Table 2: Standard Stock Solutions

Compound	Purity	Molar mass / g/mol	Theoretical weight / mg	Theoretical conc. / µmol/ml	V / ml	Real weight / mg	Real concentration / µmol/ml
DCA d4	100 %	396.60	1.983	1	5	2.00	1.009
GUDCA d4	97 %	453.65	2.338	1	5	2.25	0.962
TCA d4 (Na)	98 %	541.71	2.764	1	5	2.54	0.919
UDCA d5	100 %	397.60	1.988	1	5	1.83	0.921
CA 98%	98 %	408.57	2.085	1	5	2.06	0.988
CDCA	95 %	392.60	2.066	1	5	1.99	0.963
DCA	95 %	392.60	2.066	1	5	2.30	1.113
GCA	95 %	465.60	2.451	1	5	2.37	0.967
GCDCA (Na)	97 %	471.61	2.431	1	5	2.49	1.024
GDCA (Na)	97 %	471.61	2.431	1	5	2.50	1.028
GLCA	98 %	433.60	2.212	1	5	2.06	0.931
GUDCA	98 %	449.62	2.294	1	5	2.69	1.173
LCA	95 %	376.60	1.982	1	5	2.07	1.044
TCA (Na)	95 %	537.70	2.830	1	5	2.88	1.018
TDCA (Na)	95 %	521.70	2.746	1	5	2.64	0.961
TLCA (Na)	100 %	505.69	2.528	1	5	2.56	1.012
TUDCA	95 %	499.70	2.630	1	5	2.76	1.049
UDCA	99 %	392.57	1.983	1	5	1.98	0.999

For the initial measurements and HPLC-MS method adjustments, the standards were diluted 1:10. Later out of the stock solutions standard-mixtures were prepared. At first a 200 nmol*ml^{-1} was prepared by transferring 800μl of each standardard stock solutions into a vial and evaporating the solvent completely. Then the BAs were redissolved in 4 ml of methanol. The obtained 200 nmol*ml^{-1} standard was then subsequently diluted to obtain the other standard mixtures shown in Table 3.

Table 3: Standard mixtures

Mixture Concentrations / nmol/ml	amount left / µl	Amount Stock solution added / µl	Stock-Solution used for the dilution
200	3070.7	800	BA Std. 1 µmol/ml
150	500	375	200 nmol/ml mixture
100	500	250	200 nmol/ml mixture
50	500	125	200 nmol/ml mixture
25	383.2	62.5	200 nmol/ml mixture
5	400	80	25 nmol/ml mixture
1	400	16	25 nmol/ml mixture
0.8	400	12.8	25 nmol/ml mixture
0.5	400	8	25 nmol/ml mixture

3.3. Biological Material

Serum and Urine were used for the testing of the developed HPLC/MS method. Human serum was a pooled anonymous serum of healthy patients (n=10) was obtained from hospital Ceske Budejovice (a courtesy of Miroslav Verner, MD). A fresh urine sample was collected in the morning before the experiment from myself.

3.4.Preparation of real samples (urine and serum)

The BA in the real samples were extracted via liquid-liquid extraction. The manual was obtained from my colleague Dr. Petra Berkovà who used this technique on other steroid samples.

First 100 µl of sample were diluted in 400 µl of Methanol inside a Eppendorf vial and blended with a Vortex and homogenized in an ultrasonic bath for 5 minutes. This precipitated the proteins in the sample. The vial was then centrifuged (10,000 RPM for 10 min at 5°C) and the supernatant was transferred into a new vial. Then 200 µl of methanol were added to the precipitate, vortexed, homogenized and centrifuged again. The new supernatant was added to the previously obtained one and 200 µl of methanol were again added to the precipitate. Then the extraction was conducted one more time. The combined supernatants were than fully evaporated and the

obtained extracted sample was redissolved in 100 μl MeOH and centrifuged again. The supernatant was transferred into an HPLC vial.

3.5.HPLC Analysis

In both systems a guard column was used to protect the valuable analytical column. The analytical column used was a Phenomenex Kinetex® C18 100 Å (150 mm x 3 mm x 2.6 μm). The same Kinetex® column was used on both LC-MS systems.

The mobile phase consisted of Water(A) and 3 parts of acetonitrile with 1 part of methanol (B). Both components of the mobile phase were modified with 0.1% formic acid and 20mM ammonium acetate. The used solvents were all HPLC grade. The mobile phase gradient was determined experimentally and will be mentioned and described in a later chapter. The methanol (LC-MS Grade) and the acetonitrile (LC-MS Grade) were obtained from Merck and the water was purified in the lab with a Thermo Scientific™Barnstead™ Nanopure™System.

3.6. Optimization of the electrospray ionization conditions

After a good separation was achieved in the HPLC, the ESI conditions had to be set to the right temperature to ensure good ionization and optimal sensitivity. The temperatures were changed in the range of 200 to 300°C for the vaporizer temperature and 200 to 350°C for the capillary temperature temperature. To determine the right ESI - temperatures the 20 nmol*ml^{-1} mixture used in the HPLC - Separation experiments was analyzed using different temperatures. To find out which are the optimal parameters the intensity of the molecular peaks were compared. All the other parameter were kept the same and are stated in the results section.

3.7. Optimization of the SRM conditions for the QqQ-MS/MS System

For the efficient HPLC-MS analysis, the SRM tandem mass spectrometric conditions must be determined for each of the 14 BA analytes.

First it was recognized that the negative ion mode detection is the better choice for the studied BAs. The tested BAs produced distinct, stable [M-H]- ions.
After that each precursor ion was decomposed in a collision cell and the conditions optimized for the most intensive product ion fragments. This was done by injecting the pure standard solutions directly into the mass spectrometer.

3.8. Determination of HPLC conditions

In the beginning a good separation had to be ensured. All the separation experiments were conducted with a mixture of all bile acid standards (approx. 20 nmol/L for each BA). In these experiments the flow rate, the gradient program and the used column were determined.

3.9. Measurement of the Standard Mixtures and Data Processing

The sample mixtures were prepared in different concentrations as described above in the Chemicals section. These dilution series were used to determine the linearity of the signal response. For these experiments just the unlabeled standards were investigated. They were measured the predetermined and optimized HPLC-MS program and the samples were injected with the help of the autosampler system. This experiment was conducted with the triple quadrupole MS as well as with the orbitrap instrument.
The matrix has a large influence on the measured peak intensity and therefore this influence must be investigated separately. To do so a second standard calibration series was done using charcoal stripped fetal bovine serum (Sigma Aldrich) instead of the methanol. The standards were added into the serum and then processed with the above described sample preparation. This bovine serum was used to emulate a typical biological sample matrix. The obtained calibration curves were then compared to the ones obtained earlier using clean standards dissolved in methanol.
The data processing throughout the thesis was done with the Thermo XCalibur software (Version 5.1).

4. Results and Discussion

4.1. Fragmentation Patterns of the BAs and the optimized SRM Conditions

The obtained results of the SRM - optimization experiments are summarized in the Table 4. Some of the BAs are very stable and therefore hard to fragment, at least in negative ion mode. On the other hand, [M+H]+ ions were easier decomposed providing therefore providing more structural information. However, the negative ion mode provided much better sensitivity and thus detection limits.

The unconjugated bile acids were the hardest to detect in negative ion mode. They fragmented somewhere along the sidechain. For the conjugated BA a sufficient fragmentation could be achieved because the conjugates (Glycine, Taurine and Sulphate) where easily cleaved of and the formed Ions appears very stable.

Problems were observed with UCDA and LCA, because no fragmentation pattern could be obtained, in accordance with the findings of of WEGNER et. al. [10]. A SIM method was therefore implemented, which means the CID was disabled and the parent Ions were measured directly.

Table 4: The experimentally found SRM - Settings and Fragmentation Patterns on a QqQ TSQ Ultra Thermo mass spectrometer.

Bile Acid	Base peak / m/z	Product ions / m/z	Tube Lens / V	Collision energy / V
LCA	375.5	375.5	140	0
DCA	391.3	345.3	158	34
		347.3	158	30
CDCA	391.31	373.7	163	34
UDCA	391.32	391.3	150	0
DCA-D4 IS	395.3	349.6	140	35
UDCA-D5 IS	396.1	396.1	140	0
CA	407.3	251.5	158	36
		289.5	158	39
GLC	432.3	74.5	141	37

Bile Acid	Base peak / m/z	Product ions / m/z	Tube Lens / V	Collision energy / V
GDCA, GCDCA, GUDCA	448.3	74.4	140	37
GUDCA-D4 IS	452.4	74.4	124	39
GCA	464.3	74.5	146	40
TLCA	482.3	80.5	135	63
		124.3	135	52
TDCA	498.3	107.3	153	54
		124.4	153	52
TUDCA	498.31	80.4	166	65
		124.3	166	52
TCA	514.3	80.3	155	68
		107.3	155	56
TCA-D4 IS	518.3	80.4	130	70
		107.3	130	57

4.2. Optimization of ESI temperature for BAs

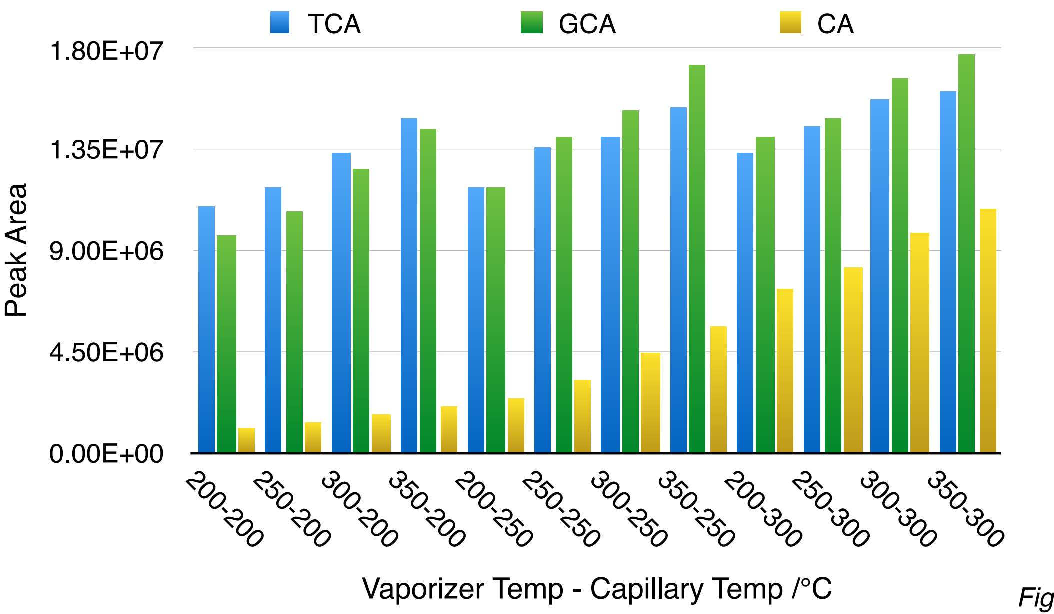


Fig.

Fig. 10: TCA/GCA/CA at different ESI temperatures

The signal intensity largely depends on the temperature on the ESI heated capillary.This parameter was therefore optimized for each examined BA analyze. The results obtained for CA, TCA, GCA are shown in Fig. 10. The sensitivity can be improved by increasing the temperature of the vaporizer

and the capillary temperature to 350 and 300°C respectively. A similar trend as in Fig. 10 was observed for all other BAs examined in this study.

4.3.HPLC - Gradient and Flow conditions

At the start, experimental conditions reported by the paper by WEGNER ET. AL. [10] were examined.

In addition to the above mentioned Kintex® C18 column, a Sherzo SM - C18 column was tested in preliminary experiments and showed bad peak shape and worse separation and resolution in comparison to the Kinetex® column. The flow rate was set to 0.2 ml*min^{-1} The HPLC program started with a mobile phase mixture of 70% A (aqueous) and had a total run time of 52 min. The mobile phase gradient versus time is shown in Fig. 12.

An adequate separation of all examined BAs and their well shaped peaks were obtained, so the Kinetex C18 Column was used in further experiments. The mobile phase composition was further adapted to 50 % of A and the isocratic phase at the beginning was shortened to 1 minute instead of 5 minutes. This was done due to the fact that no bile acids eluted earlier than 10 min into the original program. The separation of all analytes was satisfactory and importantly the analysis time shortened by 12 minutes. A higher proportion of B at the start was ruled out because it worsened the peak shape considerably.

Next the second isocratic step at 100% B was shortened and the gradient was prolonged and steepened at the end. This optimized separation and shortened the analysis time further. Finally the flow rate was increased to 0.4 ml*min^{-1} to further shorten the overall analysis time to 26 minutes. The final HPLC program is shown in Fig. 12 and Table 5.

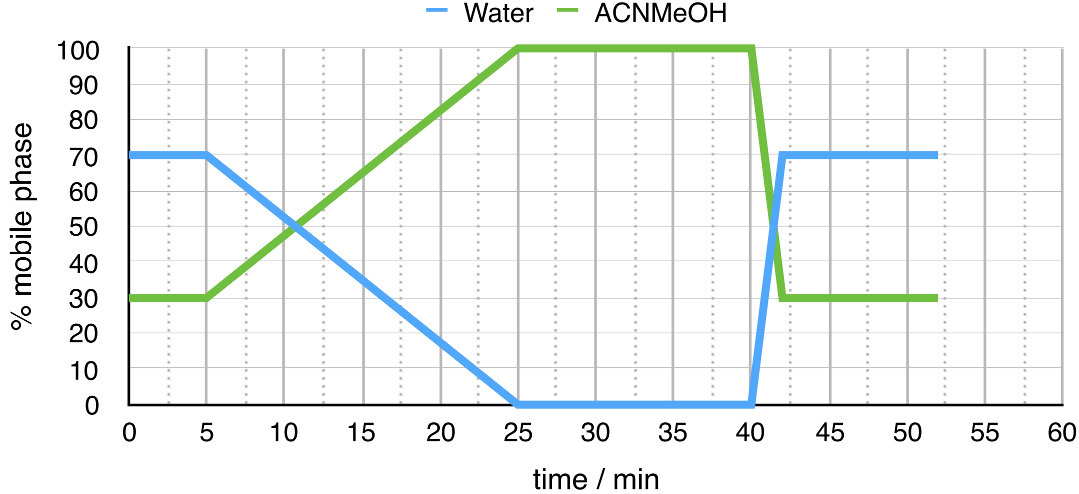


Fig. 11: First attempt for a mobile phase HPLC-program

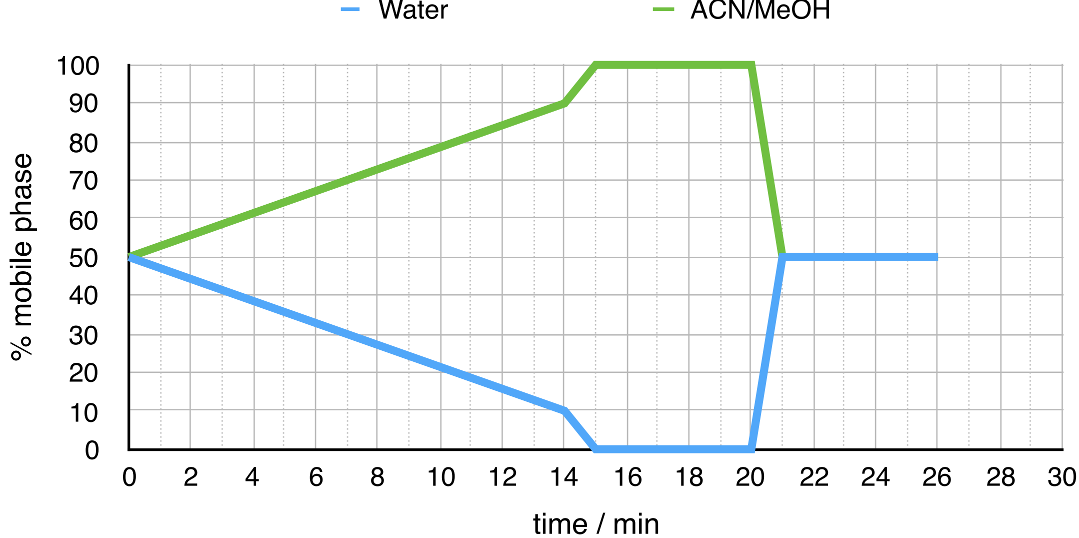


Fig. 12: Final optimized HPLC mobile phase gradient program

Table 5: Final optimized HPLC mobile phase gradient program

Time / min	Water %	ACN/MeOH %
0	50	50
14	10	90
15	0	100
20	0	100
21	50	50
26	50	50
Flow rate = 0.4 ml/min		

The final result of the experiments with the gradients and flow rates can be seen in Fig. 13 and 14.

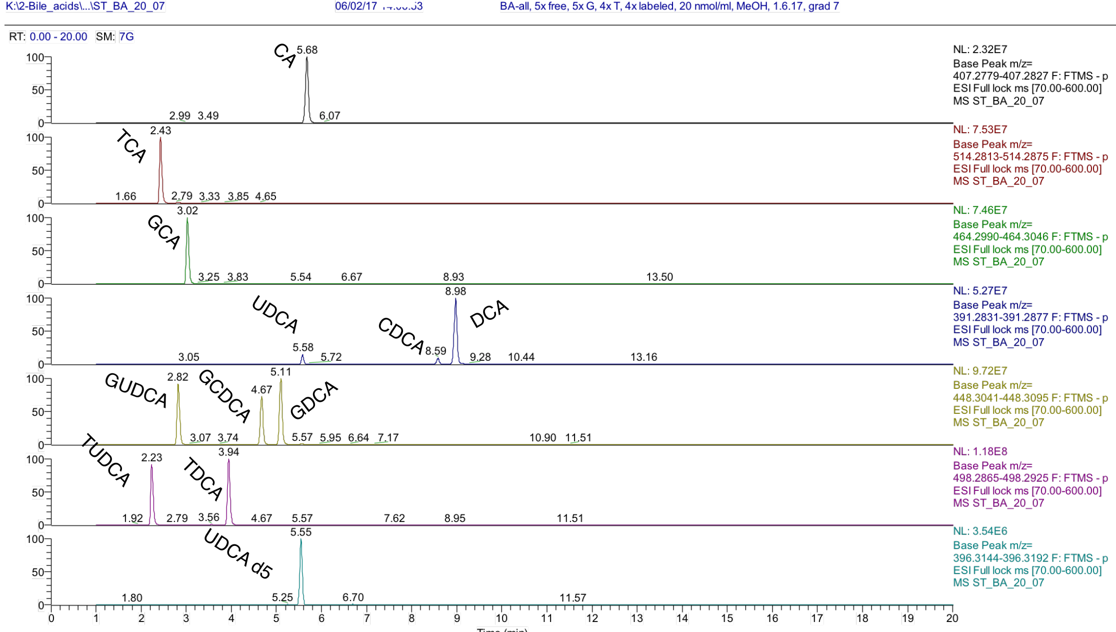


Fig. 13: Chromatogram of the improved separation gradient - Part 1

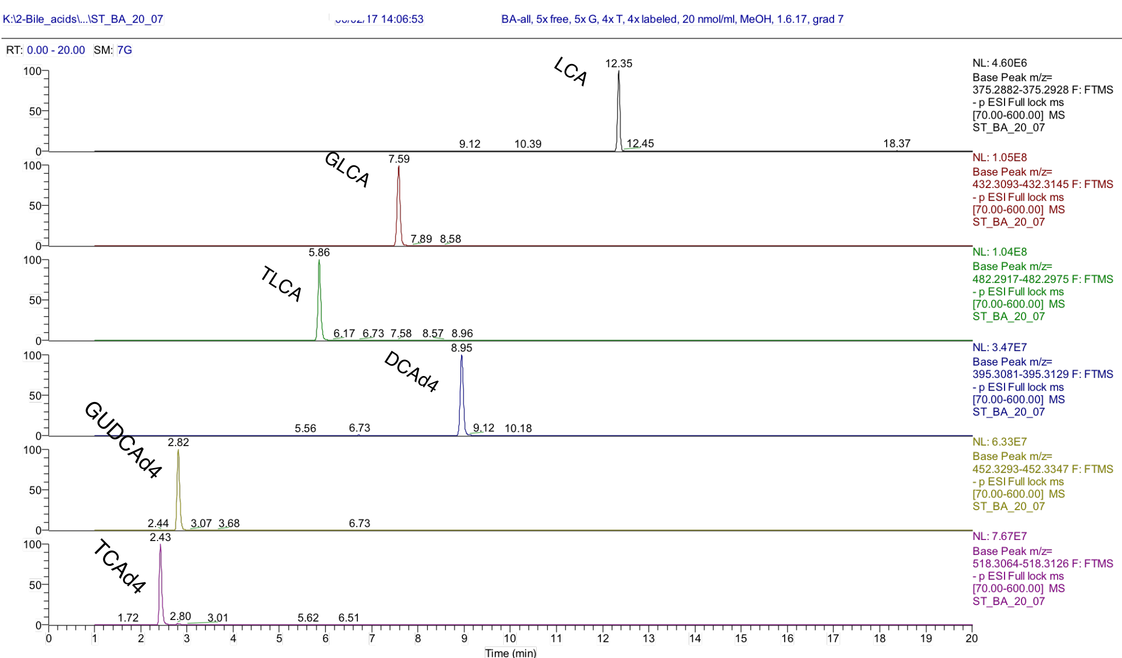


Fig. 14: Chromatogram of the improved separation gradient - Part 2

4.4. External standard calibration and measurement of real samples

The standard mixtures were prepared according to Table 3 (Section Chemicals) in methanol. The standard series was prepared in a concentration range of 0.5 to 100 nmol/ml. The result of this can be seen in the three Diagrams (Fig. 15, 16 & 17). The calibration equations and coefficients of determination are all in a usable range and therefore the calibration can be

used for the real samples. The collected calibration curves equations can be seen in Table 6.

Overall most of the examined BAs showed good linear responses and were also detected easily in the tested concentration range. Although the data shows that the lowest concentration (approx. 0.5 nmol/ml) maybe doesn't respond as linear as desired. To determine the definitive limit of detection further experiments would be necessary. On the other hand the limit of detection for most of the studied BAs is probably below the used concentrations, but for a definitive determination of the detection limit also further experiments must be conducted. It has to be mentioned as well that the LoD and LoQ should be investigated in the matrix as well to obtain reliable results and analytic parameters.

As mentioned the linearity was good for most of the BAs studied. LCA on the other hand showed poor peak intensity and could not even be detected above 1 nmol/ml. The results for show that the method doesn't work as well for LCA as for all the other examined BAs.

These findings also indicate that although the BA are all very similar in structure, the formed ions and fragments are very different and this causes the different sensitivity, as seen in the different slopes of the different bile acids. The obtained results in this experiments are only valid for clean methanol standard solutions, the situation in a real sample matrix could be entirely different. These experiments and their results are described in the next sections.

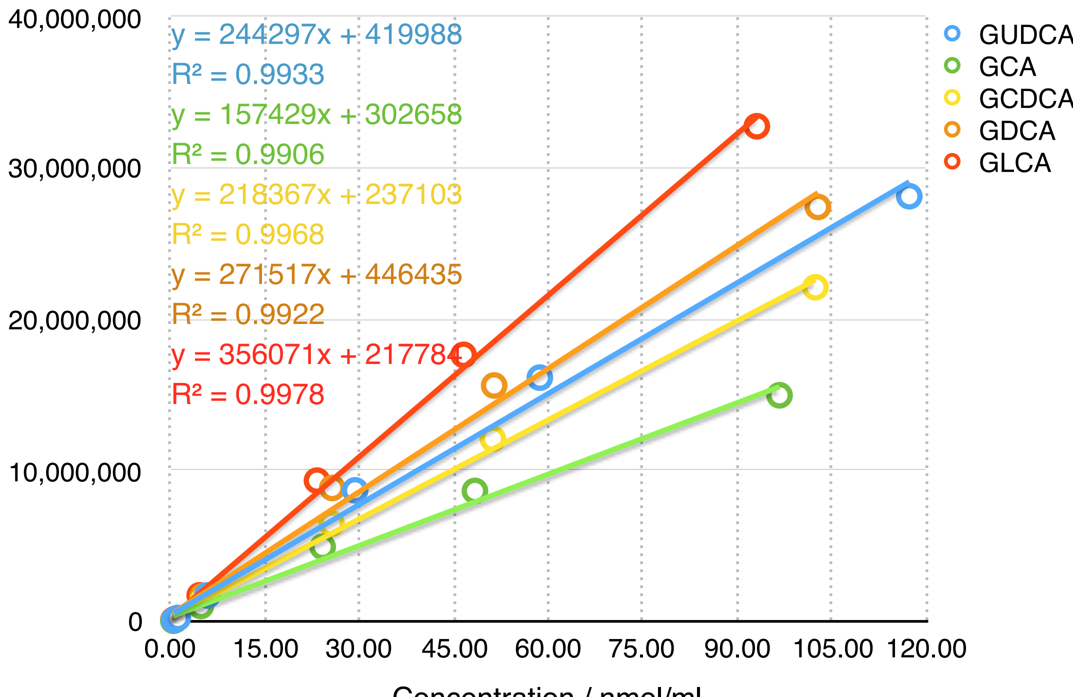


Fig. 15: Calibration curve of the gylcine conjugated bile acids

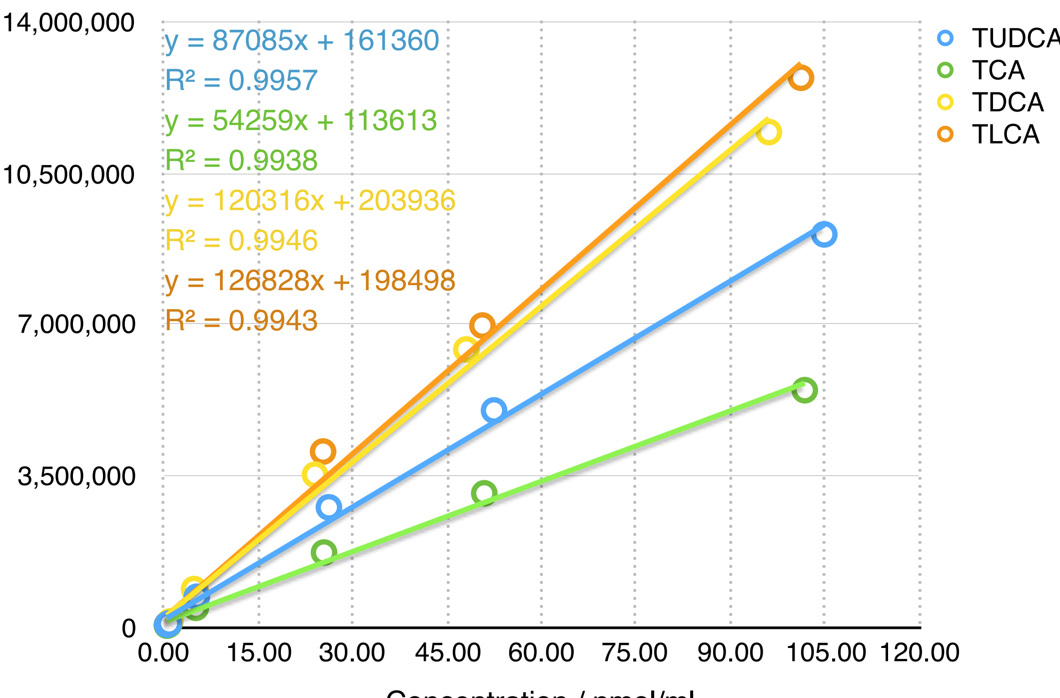


Fig. 16: Calibration curve of the taurine conjugated bile acids

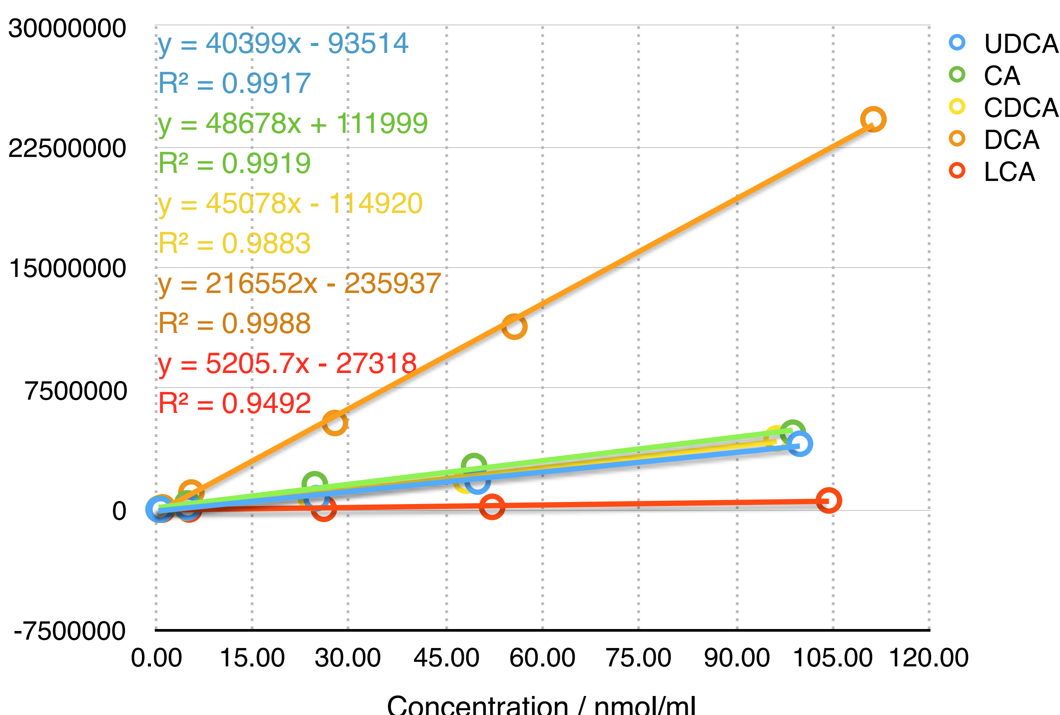


Fig. 17: Calibration curve of the unconjugated bile acids

Table 6: Collected calibration curve equations.

	k	d	R^2
UDCA	40399	-93514	0.9917
CA	48678	111999	0.9919
CDCA	45078	-114920	0.9883
DCA	216552	-235937	0.9988
LCA	5206	-27318	0.9492
GUDCA	244297	419988	0.9933
GCA	157429	302658	0.9906
GCDCA	218367	237103	0.9968
GDCA	271517	446435	0.9922
GLCA	356071	217784	0.9978
TUDCA	87085	161360	0.9957
TCA	54259	113613	0.9938
TDCA	120316	203936	0.9946
TLCA	126828	198498	0.9943

4.5. Measurements of the Biological Samples

The developed HPLC-MS/MS SRM method was applied to a model urine and serum sample. Nevertheless, the real BA levels in the human urine a serum are lower than the calibration range available on the used HPLC/MS

instrument. This responds very well with the literature research, were it was stated that the concentration in the plasma and urine of a healthy human should be extremely low[5, 6]. The chromatograms for the plasma can be seen in Fig. 21-23. They were assigned with the help of the measurements of the stripped bovine serum spiked with the standards. As an example for the free BA the peaks marked as 1 and 2 in Fig. 18 correspond to the bile acids CA and DCA respectively. We can see that there are detectable peaks in the plasma but the intensity of the peaks are way too low in comparison to the clean standards and the noise in the measurement. In fact the peak intensity of the exemplary peaks is around 1000 times weaker than the 100nmol*ml^{-1} standards which means if linearity is assumed that the concentration is in the region of 0.1nmol*ml^{-1}. Thus, to determine these peaks reliably even more diluted standards need to be prepared but under 500 pmol*ml^{-1} it is not sure that the chosen method is still sensitive enough. The same is true for the glycine and taurine conjugated bile acids shown in Fig. 19 and 20. Comparison of the three Figures it can also be seen that the free bile acids are much less efficiently ionized than their conjugated counterparts and thus their determination requires a use of more sensitive instrument.
As stated earlier the absolute LoD and LLoQ needs to be determined in the used sample matrix, however this would exceed the scope of this thesis. The use of the deuterated standards (mentioned in the Chemicals Section) was also omitted because it was not necessary for the detection of the BAs, however they should be used as internal standards in further experiments pursuing the goal of quantification.

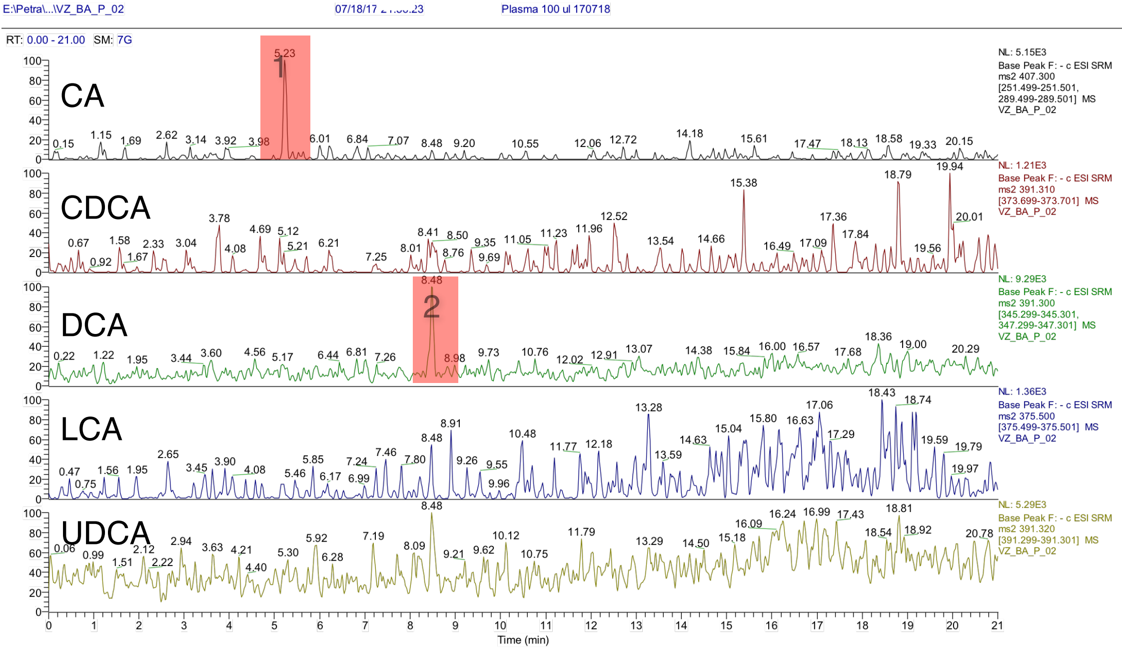


Fig. 18: Free bile acids in the plasma sample

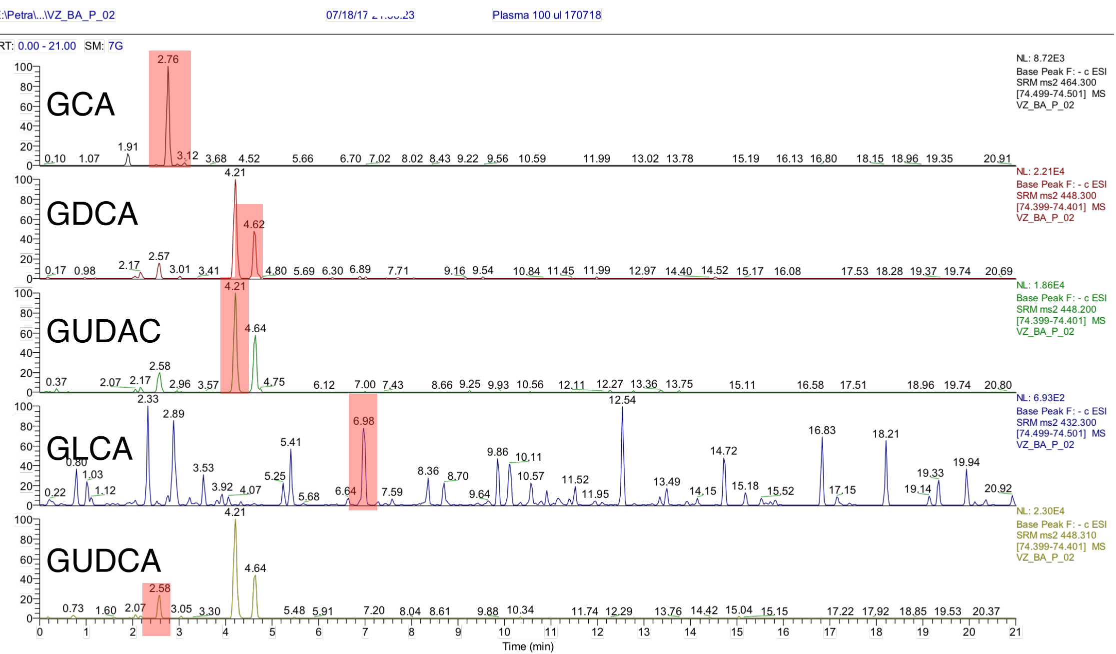


Fig. 19: Glycine conjugated bile acids in the plasma sample

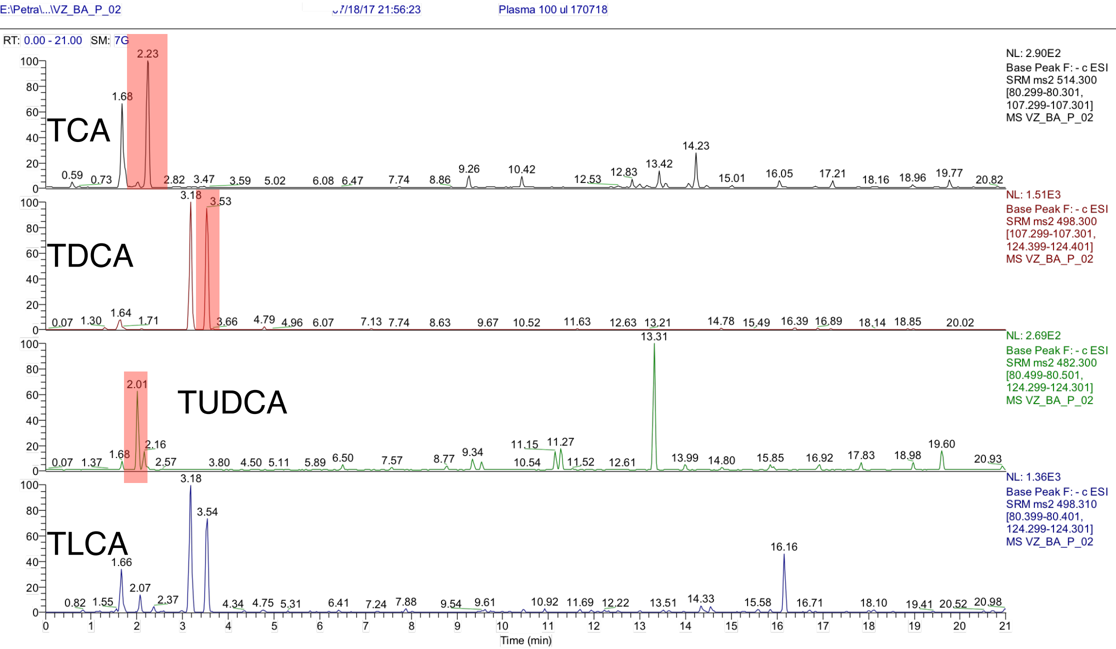


Fig. 20: taurine conjugated bile acids in the plasma sample

4.6. Evaluation of matrix effects

As stated above the peak intensity of each BA analyte measured can depend on the sample matrix. Therefore calibration curves were compared in the clean methanol and a stripped bovine serum. The comparison was made exemplary for each category of BA (free, glycine-conjugated and taurine conjugated). The results of these investigations can be seen in Fig. 21 - 23. They clearly show that the calibration curve of the standards in stripped bovine serum are shallower than in the clean methanolic standards. This indicates that serum matrix lowers the detected negative ESI analyte intensity. Consequently, the calibration data show that use of a relevant calibration in the used biological matrix is an obligatory step in the method calibration for the BA HPLC/MS analysis.

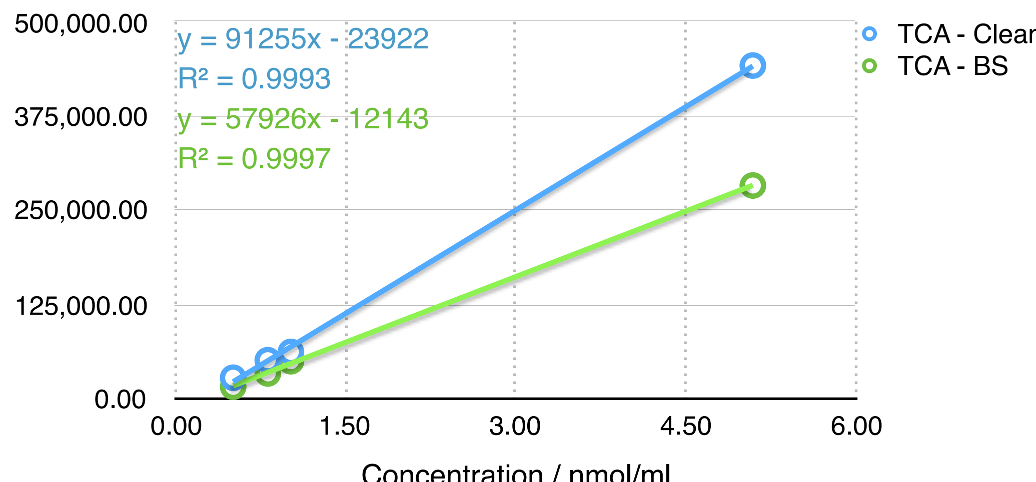


Fig. 21: Comparison of TCA in methanol and in BS

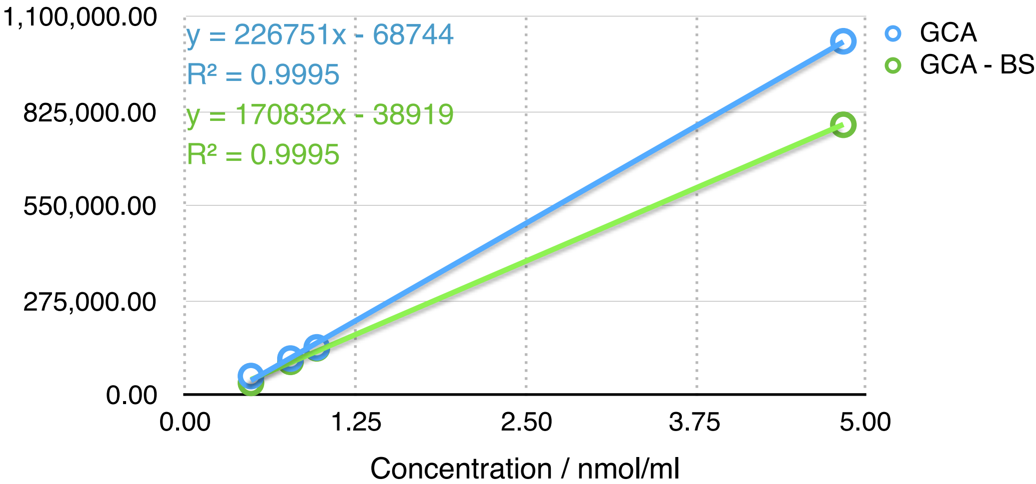


Fig. 22: Comparison of GCA in methanol and in BS

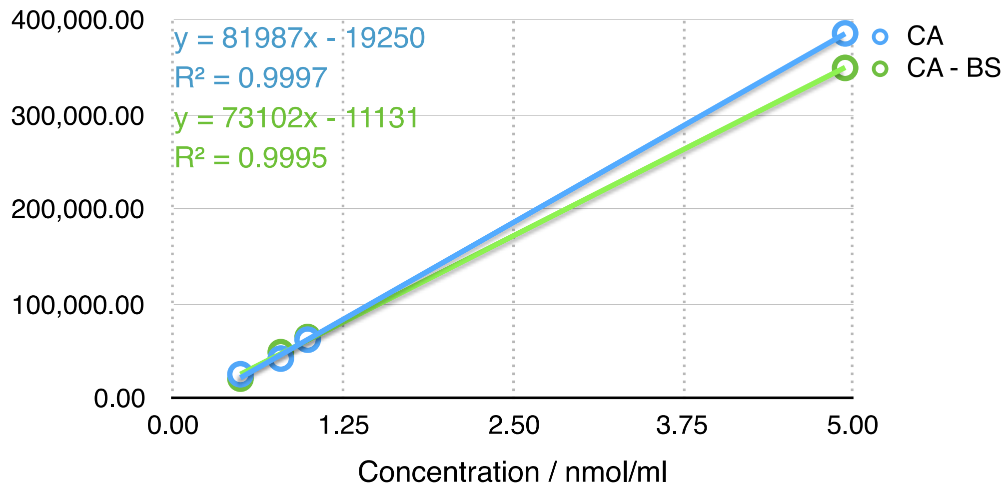


Fig. 23: Comparison of CA in methanol and in BS

4.7. BA measurements with a UHPLC-HRMS (Orbitrap)

For comparison the same measurements for linearity, matrix effects and real samples were conducted on the Thermo Scientific Orbitrap Instrument. The

monoisotopic masses used for BA analysis are shown in Table 7. The orbitrap experiments confirmed on the one side that the identification and separation of the particular bile acids worked perfectly fine and showed that the BA levels in urine and serum of healthy humans are only detected in traces.

Table 7: Monoisotopic masses and

	Formula [M]	mono isotopic mass	[MM-H]+
Ursodeoxycholic acid-d5	C24H35D5O4	397.3240434	396.31677
Deoxy cholic acid-d4	C24D4H36O4	396.3177666	395.31049
Tauro cholic acid-d4	C26H41D4NO7S	519.3167802	518.3095
Glyco chenodeoxycholic acid	C26H43NO5	449.3141233	448.30685
Glyco deoxycholic acid	C26H43NO5	449.3141233	448.30685
Glyco ursodeoxycholic acid	C26H43NO5	449.3141233	448.30685
Chenodeoxycholic acid	C24H40O4	392.2926596	391.28538
Deoxycholic acid	C24H40O4	392.2926596	391.28538
Ursodeoxycholic acid	C24H40O4	392.2926596	391.28538
Tauro lithocholic acid	C26H45NO5S	483.3018441	482.29457
Cholic acid	C24H40O5	408.2875742	407.2803
Tauro deoxycholic acid	C26H45NO6S	499.2967587	498.28948
Tauro ursodeoxycholic acid	C26H45NO6S	499.2967587	498.28948
Lithocholic acid	C24H40O3	376.297745	375.29047
Tauro cholic acid	C26H45NO7S	515.2916733	514.2844
Glyco cholic acid	C26H43NO6	465.3090379	464.30176
Glyco lithocholic acid	C26H43NO4	433.3192087	432.31193
Glyco ursodeoxycholic acid-d4	C26H39NO5D4	453.3392303	452.33195

5. Conclusion

The bile acids are very important parts of the human and mammalian digestive system and several other functions and disease related properties in the human body have yet to be discovered. In this thesis, physico-chemical properties and biochemistry of BAs (metabolism, enterohepatic cycle, BA regulation in the body, abundance in urine and serum) were briefly reviewed. In the next part, sample preparation methods for HPLC-MS analysis were presented and discussed. In the experimental part, chromatographic separation and mass spectrometric properties of examined BAs, the essential prerequisites for the HPLC/MS/MS analysis by a triple quadrupole and HRMS Orbitrap MS, were investigated. Optimized separation and detection conditions are reported for 14 BAs in 26 min and is two times faster than those reported in the earlier report of WEGNER ET. AL. [10]. Also the obtained peak shape was very good. The method was tested for a calibration range between 0.5 and 100 nmol/ml, however the observed BA levels in urine and serum were as described in literature (0.25 - 0.0015 nmol/ml) [5, 6] well below our calibrated range. One of the first real obstacles was the ionization and fragmentation in the triple quadrupole MS. The conjugated BA showed very good ionization in the negative ion mode. They simply formed $[M-H]^-$ Ions and in the CID they fragmented the conjugated group(sulphate, glycine and taurine). Also the free BA showed good ionization in the negative ion mode but the fragmentation is difficult. The LCA showed no fragmentation at all and was therefore determined in SIM mode. The same was also the case for UDCA but it showed better sensitivity for the UDCA. Positive Ion mode operation would also be possible due to ammonium adduct formation, but in this particular method the negative was evaluated as more favorable. In the Orbitrap this problems were not as severe due to the Full-Scan and high-resolution ability of the system. The calibration curve produced by the TSQ triple quadrupole and also the orbitrap (data not shown) where satisfactory and showed very good linearity between concentrations of 0.5 to 100 nmol/ml.
The developed HPLC/MS was applied to a model urine and serum sample. However, the BA abundance was lower than the calibrated range of the method.
So overall it can be said that BAs were detected in urine and serum but could not be quantified. To quantify them with the developed method a pre-concentration step has to be considered. In the literature solid phase extractions are fairly common [7, 10, 11]. Another factor for reliable quantification of BAs is, that the sample matrix had

been proven to influence the observed ESI MS response. Further research and method development would be necessary in this area. For a the final method also the usage of the described deuterated (internal) standards should be considered, they were only used for the SRM-experiments and the fundamental method developed but were not yet utilized for the calibration and real sample measurements.
Unfortunately it was not possible to resolve and investigate all of the mentioned problem areas because it would exceed the scope of this thesis.
However overall the separation and detection of bile acids with this method can be successfully used, although the topics of pre-concentration, extraction and quantification need to be addressed before this can be implemented as reliable, versatile laboratory method.

6. Bibliography

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12. Meng, M., Liquid chromatography-high-resolution mass spectrometry for regulated bioanalysis: bile acid and oligonucleotide quantitation as a reference. Bioanalysis, 2016. **8**(24): p. 2519-2521.

7. List of figures

Fig. 1 Boghog2 - Own work, Public Domain, viewed on 13 February 2017, <https://commons.wikimedia.org/w/index.php?curid=5613812>

Fig. 2 Monte M. J., Marin J. JG., Antelo A., Vazquez-Tato J. (2009), Bile acids: *Chemistry, physiology, and pathophysiology*, World J Gastroenterol 2009; 15(7)

Fig. 3 BorisTM - own work (ISIS/Draw 2.5 --> MS Paint --> Infan View); Public Domain; viewed on 15 February 2017 <https://commons.wikimedia.org/w/index.php?curid=645994>

NEUROtiker - own work; public domain; viewed on 11 May 2018 <https://commons.wikimedia.org/wiki/File:Cholsäure.svg>

Fig. 4 Chiang J.Y.L.; *Bile Acid Metabolism and Signaling*; Compr Physiol. July 2013, 3(3); HHS Public Access; 32

Fig. 5 Carey MC, Cahalane MJ; Enterohepatic circulation; In: Arias IM, Jakoby WB, Popper H, et al; The Liver: Biology and Pathology. 2nd ed. New York, NY: Raven Press; 1988; 591;

Fig. 6 viewed on 15 February 2017, <http://codigovisual.com.ar/wp-content/enterohepatic-circulation-of-bile-salts-501.jpg>

Fig. 7 viewed on 28 March 2018, https://www.shimadzu.com/an/hplc/support/lib/lctalk/47/47intro.html

Fig. 8 private photo - “UHPLC-Orbitrap System used in this study“

Fig. 9 private photo - “UHPLC-Triple Quadrupole System used in this study“

Fig. 10 generated graphic - “First attempt for a mobile phase HPLC-program“

Fig. 11 generated graphic - “Final optimized HPLC mobile phase gradient program“

Fig. 12 Chromatogram - “Chromatogram of the improved separation gradient - Part 1“

Fig. 13 Chromatogram - “Chromatogram of the improved separation gradient - Part 2“

Fig. 14 generated graphic - “TCA/GCA/CA at different ESI temperatures"

Fig. 15 generated graphic - “Calibration curve of the gylcine conjugated bile acids“

Fig. 16 generated graphic - “Calibration curve of the taurine conjugated bile acids“

Fig. 17 generated graphic - “Calibration curve of the unconjugated bile acids“

Fig. 18 Chromatogram - “Free bile acids in the plasma sample“

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Fig. 21 generated graphic - “Comparison of TCA in methanol and in BS“

Fig. 22 generated graphic - “Comparison of GCA in methanol and in BS“

Fig. 23 generated graphic - “Comparison of CA in methanol and in BS“

8. Appendix

External Calibration Taurine Conjugates

	TUDCA		TCA		TDCA		TLCA	
theoretical Concentration / nmol*ml^{-1}	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**
0.5	0.52	51,141	0.51	27,932	0.48	43,186	0.51	43,369
0.8	0.84	79,401	0.81	51,128	0.77	86,110	0.81	78,133
1	1.05	108,418	1.02	62,125	0.96	122,798	1.01	118,224
5	5.25	696,479	5.09	441,279	4.81	873,324	5.06	823,109
25	26.23	2,764,188	25.45	1,719,431	24.03	3,519,540	25.30	4,054,784
50	52.45	5,005,111	50.90	3,088,568	48.05	6,415,965	50.60	6,969,276
100	104.90	9,078,326	101.80	5,474,238	96.10	11,444,759	101.20	12,700,808

External Calibration Glycine Conjugates

	GUDCA		GCA		GCDCA		GDCA		GLCA	
theoretical Concentration / nmol*ml^{-1}	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**	**real Concentration / nmol*ml^{-1}**	**Peak Area**
0.5	0.59	100,357	0.48	54,064	0.51	64,894	0.51	81,539	0.47	100,185
0.8	0.94	178,621	0.77	104,882	0.82	142,646	0.82	162,163	0.74	173,205
1	1.17	235,918	0.97	137,582	1.02	188,552	1.03	216,165	0.93	233,895
5	5.87	1,727,95	4.84	1,029,15	5.12	1,352,88	5.14	1,680,21	4.66	1,734,56
25	29.33	8,677,31	24.18	4,942,16	25.60	6,481,64	25.70	8,835,64	23.28	9,322,11
50	58.65	16,132,0	48.35	8,638,23	51.20	12,086,6	51.40	15,612,4	46.55	17,638,0
100	117.30	28,127,7	96.70	14,964,8	102.40	22,106,1	102.80	27,420,2	93.10	32,755,3

External Calibration Unconjugated

	UDCA		CA		CDCA		DCA		LCA	
theoretical Concentration / nmol* ml^{-1}	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area
0.5	0.50	10501	0.49	25006	0.48	11463	0.56	63698	0.52	0
0.8	0.80	15876	0.79	41627	0.77	15597	0.89	116925	0.84	0
1	1.00	17181	0.99	61651	0.96	19397	1.11	137415	1.04	1008
5	5.00	126576	4.94	386038	4.82	118502	5.57	1058851	5.22	10885
25	24.98	708626	24.70	1572270	24.08	741565	27.83	5367991	26.10	51600
50	49.95	1740782	49.40	2697898	48.15	1789268	55.65	11341218	52.20	169037
100	99.90	4083168	98.80	4767080	96.30	4413407	111.30	24200665	104.40	566996

Comparison of Clean vs BS Standards

	TCA - Clean		TCA -BS		GCA		GCA - BS		CA		CA - BS	
	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area	real Concentration / nmol* ml^{-1}	Peak Area
0.5	0.51	27,932	0.51	16,024	0.48	54,064	0.48	34,058	0.49	25,006	0.49	20,392
0.8	0.81	51,128	0.81	33,621	0.77	104,882	0.77	95,473	0.79	41,627	0.79	48,390
1	1.02	62,125	1.02	49,790	0.97	137,582	0.97	134,751	0.99	61,651	0.99	64,428
5	5.09	441,279	5.09	282,465	4.84	1,029,15	4.84	785,965	4.94	386,038	4.94	349,506