University of South Bohemia České Budějovice
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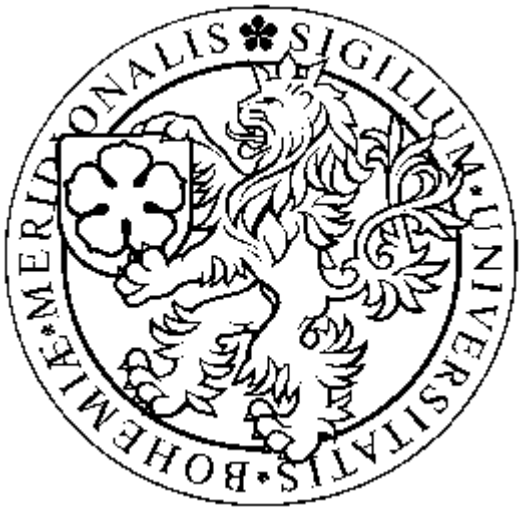

Bachelor Thesis

Detection of pilocarpine in tick saliva and its effect on the host immunity

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Annotation

This bachelor thesis deals with the determination of the pilocarpine concentration in tick saliva and its effect on immune cells. Pilocarpine is applied to the ticks' back in order to enhance salivation. The substance passes through the tick's cuticle and therefore it is also present in the secreted saliva. The concentration was determined using HPLC-MS2. The effect of pilocarpine on cytokines (TNF-α and IFN-γ) was determined using ELISA.

Tato bakalářská práce se zabývá stanovením koncentrace pilokarpinu ve slinách klíštěte a jeho vlivem na imunitní buňky. Pilokarpin byl aplikován na záda klíštěte k podpoření salivace. Tato látka proniká skrze kutikulu klíštěte a díky tomu může být detekována i ve vyloučených slinách. Koncentrace pilokarpinu byla stanovena pomocí HPLC-MS2 analýzy. Vliv pilokarpinu na cytokiny (TNF-α a IFN-γ) byl zjištěn pomocí metody ELISA.

Thema dieser Arbeit ist die Bestimmung der Pilocarpin Konzentration in Zeckenspeichel und die Wirkung dieser Substanz auf Zellen des Immunsystems. Pilocarpin wird auf den Rücken der Zecke getropft, um den Speichelfluss anzuregen. Die Substanz wird über die Haut aufgenommen und ist deshalb auch im produzierten Speichel zu finden. Die Konzentration wurde durch HPLC-MS2 bestimmt. Die Auswirkung von Pilocarpine auf Cytokine (TNF-α und IFN-γ) wurde mit Hilfe von ELISA bestimmt.

Affirmation

Hereby I declare that I did all the work, summarized in this bachelor thesis, on my own or on the basis of consultation with my supervisor.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my dissertation thesis, in full / in shortened form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Linz, 20 May 2010

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Carmen Ziebermayr

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I am grateful to Jan Kopecký, PhD, for being my supervisor and helping me in writing my bachelor thesis.
I would like to thank the members of his laboratory as well as the members of the laboratory of Analytical Biochemistry of the Institute of Entomology, BC ASCR for supporting me during my work.

Table of Contents

List of Abbreviations

Amu ..... Atomic mass unit

APS ..... Ammonium persulphate

CD8 ..... Cluster of differentiation 8

Con A ..... Concanavalin A

Da ..... Dalton

DC ..... Dendritic cell

ELISA ..... Enzyme linked immunosorbent assay

HESI ..... Heated electrospray ionisation

HPLC ..... High performance liquid chromatography

IFN-γ ..... Interferon gamma

IL-2 ..... Interleukin 2

LPS ..... Lipopolysaccharide

MeOH ..... Methanol

MHC ..... Major histocompatibility complex

MS ..... Mass spectrometry

NK cells ..... Natural killer cells

PHA ..... Phytohemagglutinin

SAT ..... Saliva activated transmission

TCR ..... T cell receptor

TNF-α ..... Tumor necrosis factor alpha

T_H ..... T helper cell

T_H1 ..... T helper cell type I

1 Introduction

1.1 Pilocarpine

Pilocarpine is a muscarinic cholinomimetic agent. It is obtained from the leaves of a tropical American shrub from the genus Pilocarpus. It is a non-selective muscarinic receptor agonist acting on the parasympathetic nervous system. It has several known effects. It contracts smooth muscle within the intestinal tract, affects blood pressure and inhibits lymphocyte function. In medicine it is used for the therapy of xerostomia and glaucoma. Pilocarpine stimulates the secretion of large amounts of saliva and sweat [1, 2, 3]. Therefore, it is used for the collection of tick saliva by applying it to the tick's back. It has an effect up to 3 hours [4]. It is known, that pilocarpine is also present in the collected tick saliva. A ten times bigger amount of pilocarpine was found in the saliva of *Ixodes scapularis* than in *Amblyoma americanum*. This might be caused by differences in the cuticle permeability [4]. The concentration of this agent in tick saliva can be measured using an HPLC-MS2 method.

1.2 Cytokines [5]

Cytokines are proteins or glycoproteins and are produced by white blood cells and other cells in response to several stimuli. They play an important role in the human body. They regulate the interactions between lymphoid cells, inflammatory cells and hematopoietic cells which are involved into the development of an effective immune response. Cytokines can be divided into the following four families:

- Hematopoetin family
- Interferon family
- Chemokine family
- Tumor necrosis factor family

Cytokines bind to specific receptors which are found on the membranes of the target cells. Binding triggers signal transduction pathways that alter the gene expression in the target cell (up or down regulation). They can act in an autocrine, paracrine or endocrine (rarely) way. They control the immune response by stimulation or inhibition of activation, proliferation and differentiation of various cells and by the regulation of the secretion of antibodies and other cytokines.

Cytokines are mainly produced by T_H cells, dendritic cells, and macrophages. Their secretion is enhanced by lipopolysaccharides. They are involved into the development of the cellular and humoral immune response, induction of inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation, and the healing of wounds. They affect all cells which have the appropriate receptor.

Receptors of cytokines are divided into five groups:

- Immunoglobulin superfamily receptors
- Class I cytokine receptor family (hematopoietin receptor family)
- Class II cytokine receptor family (interferon receptor family)
- TNF receptor family
- Chemokine receptor family

With the help of specific monoclonal antibodies for each of the important cytokines, it was made possible to develop quantitative immunoassays. ELISA is one method for the quantification of cytokines. It is also used within this work to measure the amount of TNF-α and IFN-γ, produced by immune cells which were inhibited by pilocarpine.

1.2.1 TNF-α

Tumor necrosis factor alpha is secreted by macrophages. It is a cytokine of the innate immunity. In vasculature it can cause inflammation. In the liver it is responsible for the production of acute phase proteins. It is also responsible for the loss of muscle and body fat. Cell death is caused by this cytokine and it activates neutrophils.

1.2.2 IFN-γ

Interferon gamma is secreted by T_H1 cells, $CD8^+$ cells and NK cells. This cytokine activates macrophages, increases the expression of MHC class I and class II and increases also the antigen presentation.

1.3 Ticks [6, 7]

Ticks are ectoparasites (external parasites). The small animals belong to the group of Acarina, which is a taxon of arachnids. They are living by hematophagy on birds, mammals and sometimes reptiles and amphibians. Ticks are vectors of a number of diseases, like Lyme borreliosis, Tick-borne meningoencephalitis or Colorado tick fever.
Ticks comprise two families which are the *Ixodidae* (hardbodied) and the *Argasidae* (softbodied). Common types of the hardbodied ticks are *Ixodes scapularis*, *Ixodes ricinus* and *Ixodes holocyclus*.

The attachment of the tick and feeding takes several days. It involves sawing through the epidermis, inserting the mouthpart into the wound, which are then cemented in place and followed by the formation of a feeding pool which results from tick and host activities. In Figure 1 the attachment of a tick to the host's skin can be seen. Salp15 is a feeding-induced protein injected by the tick. It inhibits the T cell receptor-mediated signalling leading to a reduced cytokine production [8].

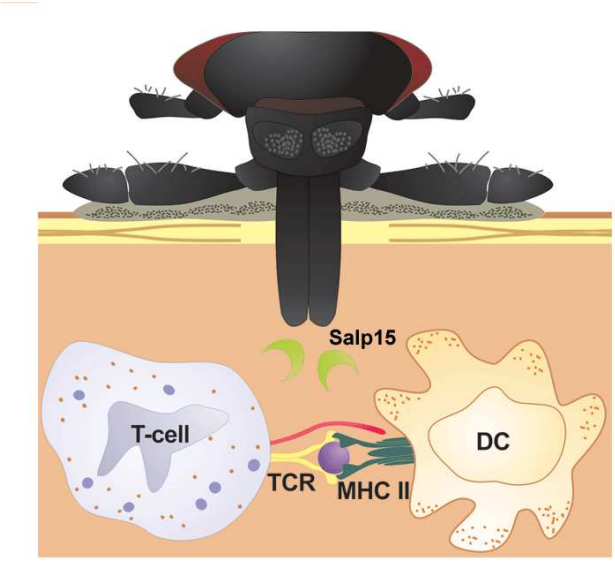

Figure 1 Tick attachment to the host [9]

This formation of a feeding pool causes haemostatic, inflammatory and immune responses. A successful bloodmeal is dependent on chemicals and substances produced from their salivary glands [10], such as anti-clotting, anti-platelet, vasodilatory, anti-complement, anti-neurophil, and several immunomodulatory substances, which are injected into the host [4]. Ticks pro-

duce saliva molecules that bind to histamine, which plays a role in the cascade of inflammatory and immune responses. Histamine up regulates the production of certain cytokines, as for example TNF-α.

While ticks are taking a bloodmeal they can transmit a variety of human and animal illnesses. During tick-host interaction, the pathogens inside the tick saliva invade the human or animal body. They exploit the binding site of the ticks. This is called saliva-activated transmission (SAT). It is the indirect promotion of tick-borne pathogen transmission via the actions of bioactive tick saliva molecules on the vertebrate host [10].

Tick saliva is used for research studies concerning the development of vaccines and for a better understanding of tick-host interactions. There are many pharmacological, biochemical and immunological active molecules present in tick saliva. If the secreted substances were blocked, a successful bloodmeal could be inhibited and probably no pathogens would be transmitted. Research tries to develop vaccines with this operating mode.

2 Aim

The incidence of tick-borne diseases has drastically increased over the past few years [11, 12], resulting in a marked increase in research on tick-host-pathogen interactions. Tick saliva is used to test for various pharmacological, biochemical and immunological activities. It is a potential source for new pharmacological agents. Salivation is induced by pilocarpine. As a consequence, pilocarpine is taken up into the tick's body fluids and therefore also present in the secreted saliva. Pilocarpine has effects on the immune system. This effect is tested on the production of cytokines TNF-α and IFN-γ. The results from this study are important for interpreting research data which were obtained from experiments using pilocarpine contaminated tick saliva.

3 Materials and Instrumentation

- Mass spectrometer LTQ XL (Thermo Electron, San Jose, USA)
- HPLC: Column Synergi Polar RP, 150 x 2 mm, particle size 4 µm (Phenomenex, Torrance, USA)
- Microplate spectrophotometer (Labsystems Multiskan MCC/340, Finland)
- Incubator (Jouan, France)
- Microscope (Carl Zeiss, Germany)

4 Chemicals and Solutions

4.1 HPLC/MS

- Methanol, CH_3OH
- Formic acid, HCOOH
- Pilocarpine, $C_{11}H_{16}N_2O_2$

4.2 Tick saliva collection

- 5 % pilocarpine (in ethanol), $C_{11}H_{16}N_2O_2$

4.3 Splenocytes collection

- Trypan blue
- Cell culture medium I: RPMI 1640 with L-glutamine and HEPES (PAA)
- Cell culture medium II: RPMI 1640 with 10% foetal bovine serum (PAA)
- Lipopolysaccharide from *E. coli* K-235 (Sigma)

4.4 ELISA

- Mouse TNF-α ELISA Ready-SET-Go! (eBioscience)
- Mouse IFN-γ ELISA Ready-SET-Go! (eBioscience)

5 Methods

5.1 Tick saliva collection

Tick saliva can be collected by removing feeding ticks from their hosts and injecting or applying to the tick cuticle pharmacological active agonists of salivation such as pilocarpine and dopamine. Capillary tubes are attached to the mouthpart of the ticks. The saliva is collected into these tubes. Pilocarpine is thought to act on neural ganglia stimulating the glands to produce probably dopamine. Since, isolated glands do not respond to pilocarpine but to dopamine, this might be the final effector molecule which is responsible for salivation. Dopamine gets destroyed in tick haemolymph. This is the reason, why pilocarpine is the most used agonist of tick salivation [4].

5.2 HPLC and Mass spectrometry

High performance liquid chromatography is used for the separation of pilocarpine from other substances which are present in the sample. The separation of analytes is based on different partition coefficients between the stationary and the mobile phase. The chromatography device is hyphenated with a mass spectrometer as a detection unit. A mass spectrometer consists of the following components (Figure 2): Inlet-system, ionisation source, mass separator and detector. After the ionisation the molecule is accelerated in an electrical field. An electromagnetic field is applied. The strength of this field is adapted to the mass/charge ratio of the desired analyte. This means, that only molecules with the selected mass/charge ratio reach the detector [13].

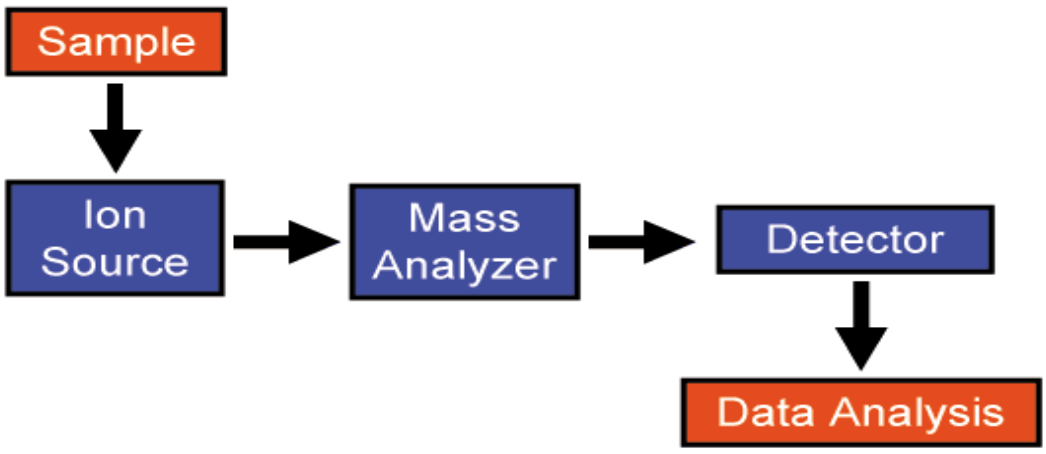

Figure 2 Mass spectrometer [14]

5.3 ELISA [15, 16]

For the determination of the antigen concentration, immunoassay can be used. This bioanalytical method is based on the interactions of antibody and antigen. Labelling of antigen or antibody can be done with isotopes (radioimmunoassay, RIA), enzymes (enzymimmunoassy, EIA) or fluorescent dyes. Immunoassays can be divided into different groups. One type involves labelled reagents and the other one involves non-labelled reagents. Those involving labelled reagents are divided into homogeneous and heterogeneous immunoassays. Enzyme linked immunosorbent assay (ELISA) belongs to the group of heterogeneous immunoassays. Two ELISA-systems are differentiated, namely competitive and non-competitive tests. Within this work, Sandwich-ELISA, a form of non-competitive assay, was used. The name "Sandwich" results from the antibody – antigen – antibody – complex which is formed during the experiment. It is conducted in the following steps:

Coating: The wells of a microtiter plate are covered with a capture antibody in a coating buffer and incubated over night (Figure 3, step 1).

Blocking: This is done to block any unspecific binding sites on the plate surface.

Sample incubation: The antigen containing samples are applied to the wells. They specifically bind to the antibodies on the surface. In this experiment, the antigens used are the cytokines TNF-α and IFN-γ (Figure 3, step 2).

Addition of detection antibody and enzyme: The detection antibodies bind specifically to the antigens. They bind to a different epitope than the coating antibodies in order to prevent steric hindrance. The enzyme can either be linked to this antibody or to a secondary detection antibody, which binds to the primary detection antibody (Figure 3, step 3 and 4).

Addition of a chromogenic substrate: A substance is applied to the wells. This substance reacts with the enzyme leading to a colour change or a fluorescent signal (Figure 3, step 5).

The colour change or fluorescence can be measured and evaluated by comparing it with the signal of known concentrations (standard curve). Between all these steps washing of the wells has to be carried out, to get rid of excessive substances (antibodies, samples, etc.).

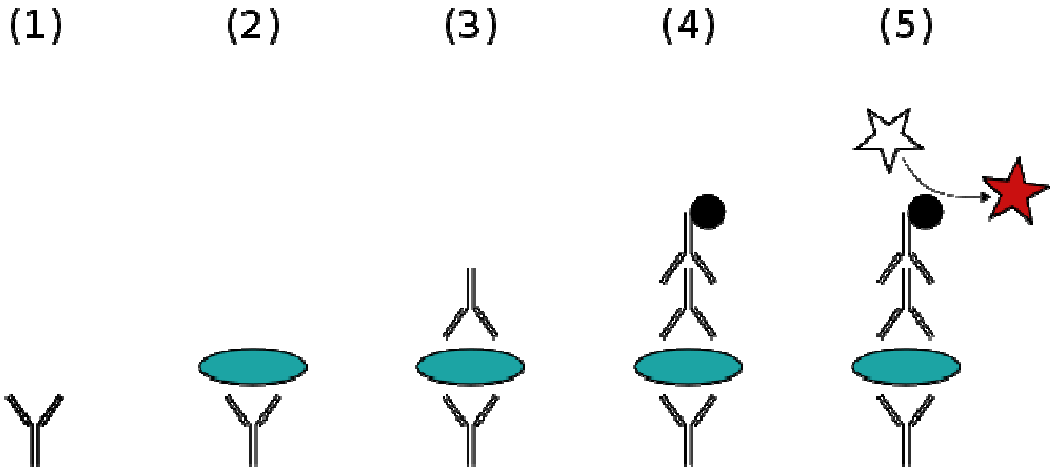

Figure 3 ELISA [17]

6 Experiment

6.1 Tick saliva collection

Tick saliva was obtained from female ticks (I. ricinus) engorging for 6-7 days on a host animal. After harvesting the animals from the host, they were fixed on a plate, to prevent their escaping. Under the microscope, glass capillary tubes (10 µl) were placed on the tick's hypostome. 2 µl of 5% pilocarpine in ethanol were applied on the back of the tick. Then they were incubated for 1-2 hours at 37°C. After incubation the saliva was collected from the capillary tubes and stored at -70°C.

6.2 Quantification of pilocarpine in tick saliva

For HPLC, Column Synergi Polar RP (Phenomenex, Torrance, USA) with a dimension of 150 x 2 mm and a particle size of 4 µm was used. The mobile phase contained formic acid and methanol. Gradient elution was carried out. For the detection of the eluted pilocarpine Mass spectrometer LTQ XL (Thermo Electron, San Jose, USA) was used. The sample was ionised using HESI. The ions were scanned at a range of 50-300 Daltons. Molecules with an m/z ratio of 209 were isolated and fractionated using collision induced dissociation at 32% of the maximum energy. The fragments were scanned again. The applied parameters are summarized in Table 1 and Table 2.

Table 1 Parameters of mass spectrometry

Method	HESI, positive ion mode
Source Voltage	3.8 kV
Capillary Voltage	40 V
Capillary Temperature	300°C
Scan	50-300 amu
m/z ratio of pilocarpine ion	209
Relative collision energy	32 %

Table 2 Parameters of HPLC

Flow rate	250 µl/min
Gradient elution	95 % A to 65 % B A: 0.1 % formic acid B: 90 % MeOH
Column size	150 x 2 mm
Particle size	4 µm

10 µl of the saliva sample were mixed with 100 µl of MeOH and centrifuged at 2000 g. The supernatant was evaporated to dryness using nitrogen. The pellet was dissolved in 50 µl of a MeOH – 0.1 % formic acid in water solution (5:95). As the first measurement showed, the sample was too concentrated. Therefore, it was diluted further 20 times with the MeOH – 0.1 % formic acid in water solution (5:95). Six standards with concentrations of 0.01, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml were prepared.

6.3 Splenocytes collection

The splenocytes were collected from the spleen of a mouse. The spleen was placed into a little amount of medium I (see section 4.3) and mashed through a sterile strainer. The suspension was transferred to a test tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded. Medium I was added and the cells were centrifuged again for 5 minutes at 1000 rpm. This step was repeated once more. The supernatant was discarded and 1 ml of medium II was added. The cells were counted under the microscope. For this, a mixture of 500 µl of Trypan blue, 500 µl of medium I and 10 µl of spleen cell suspension were used. According to the number of cells counted, three ml of sample (in medium II) with a concentration of $3x10^7$ cells/ml were prepared.

Pilocarpine was diluted serially (10-fold) in medium I starting with 40 mg/ml and ending up with 0.004 mg/ml. A microtiter plate was used. The wells were filled according to Table 3.

Table 3 Microtiter plate: Final pilocarpine concentrations [mg/ml]

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	10	1	0.1	0.01	0.001	C+	C-					
C	10	1	0.1	0.01	0.001	C+	C-					
D	10	1	0.1	0.01	0.001	C+	C-					
E												
F												
G												
H												

100 µl of cell suspension were pipetted into 3x7 wells. 50 µl of serially diluted pilocarpine were added into the first five wells of each of the three rows. 50 µl of LPS in a concentration of 4 µg/ml were added into the first six wells of each row. Instead of pilocarpine and LPS, 50 µl of medium II were added into the fifth well and 100 µl of medium II were added into the last well of each row instead of pilocarpine and LPS. This lead to a final concentration of 10, 1, 0.1, 0.01, 0.001 mg/ml of pilocarpine as it is shown in Table 3. The positive and negative control wells did not contain pilocarpine. The negative control wells did not contain LPS. The cells were incubated over night at 37°C and 5% CO_2 atmosphere. 150 µl of sample were taken from each well, transferred to a new microtiter plate and stored at -70°C.

6.4 ELISA

ELISA was carried out for the detection of the cytokines TNF-α and IFN-γ, which should be produced by splenocytes stimulated by LPS. The reagent sets *Mouse TNF-α ELISA Ready-SET-Go!* and *Mouse IFN-γ ELISA Ready-SET-Go!* from eBioscience were used. The experiment was conducted according to the experimental procedure of these products, except step number 5 which was modified a little bit. Instead of 100 µl, only 50 µl of standard were added to the wells.

7 Results

7.1 Pilocarpine concentration in tick saliva

The concentration of pilocarpine in tick saliva was measured using HPLC-MS2 as it was described in section 6.2. The resulting chromatograms (Figure 4 and Figure 5) show the elution of the molecules and its fragments. The retention time lies between 6.07 and 6.41 minutes.

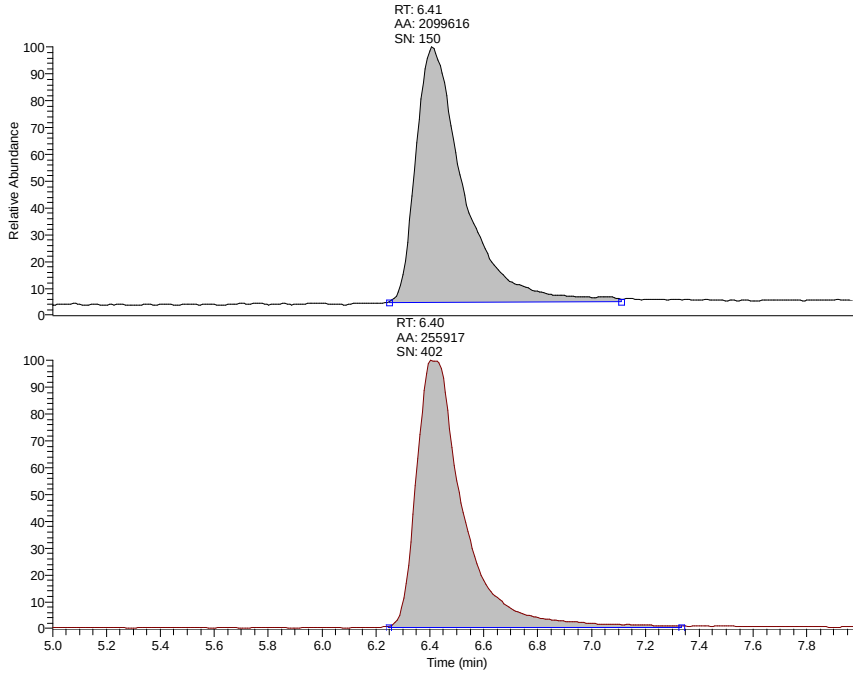

Figure 4 HPLC Chromatogram of standard 1µg/ml

The upper part of Figure 4 and Figure 5 shows the resulting peak of the detected pilocarpine molecules of m/z between 208.7 and 209.7. The lower part shows the peak area after fragmentation (detected m/z ratios: 94.5 – 95.5, 162.5 – 163.5 and 208.6 – 209.6).

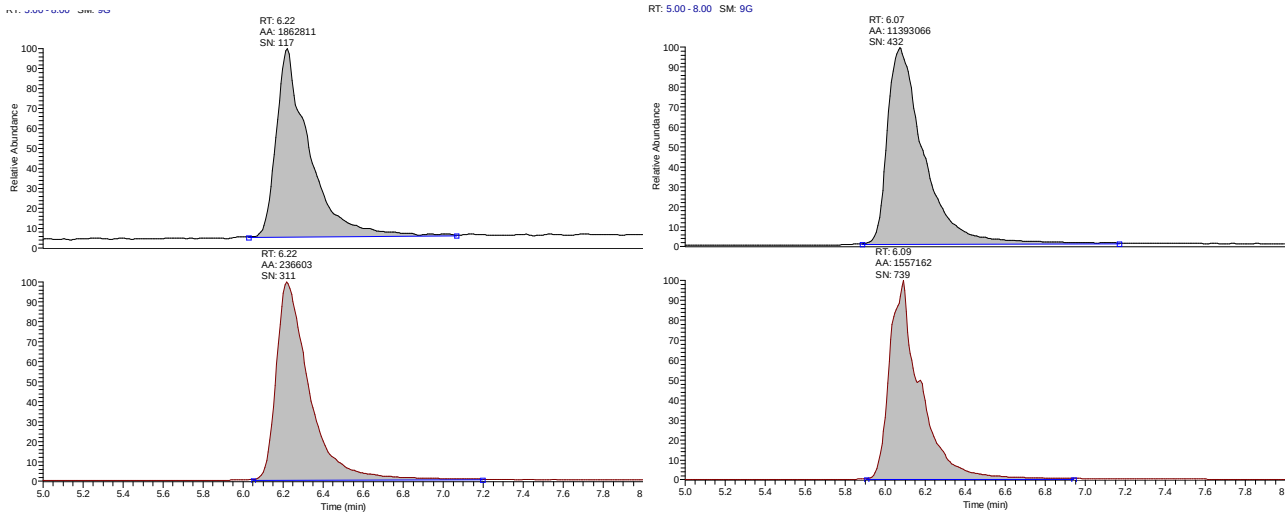

Figure 5 HPLC Chromatogram of sample 1 and 2

Figure 6 illustrates the mass spectrum of the parent ions (pilocarpine) which have an m/z ratio of 209.3 and Figure 7 shows the mass spectrum after fragmentation, with the main fragment of m/z 163.1. As it was expected, the spectrum of the samples showed the same fragmentation patterns as the standard.

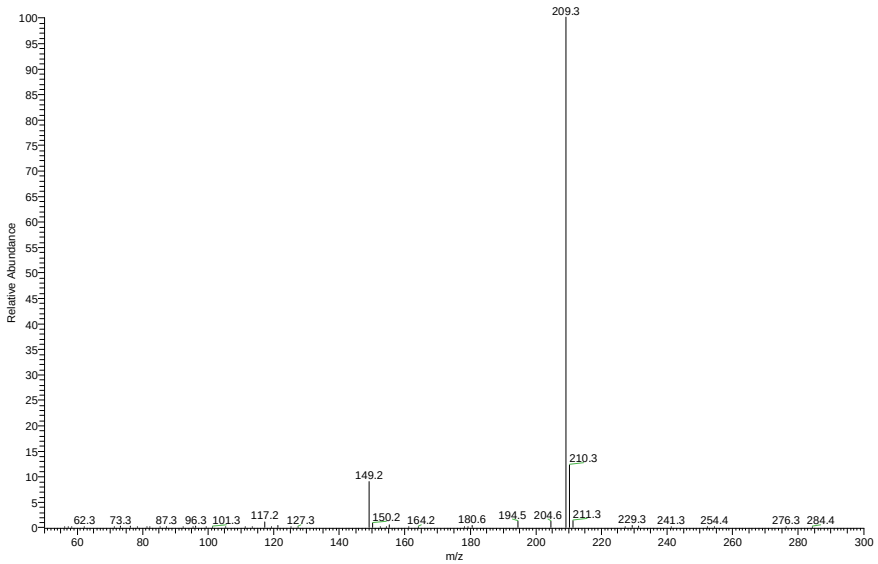

Figure 6 Mass spectrum of parent ion

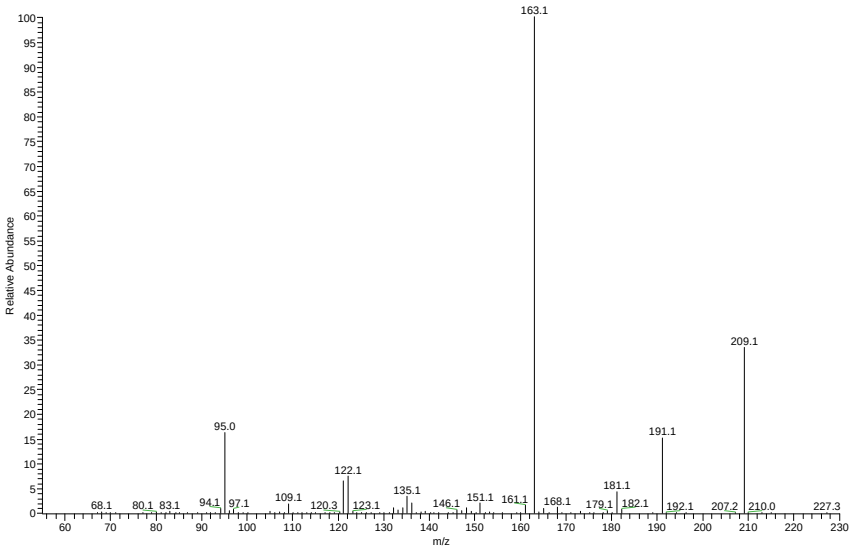

Figure 7 Mass spectrum after fragmentation

The sample concentration can be calculated from the peak area resulting from HPLC-MS2. Therefore a standard curve in the range of 10 ng/ml to 10 µg/ml was determined (Figure 8).

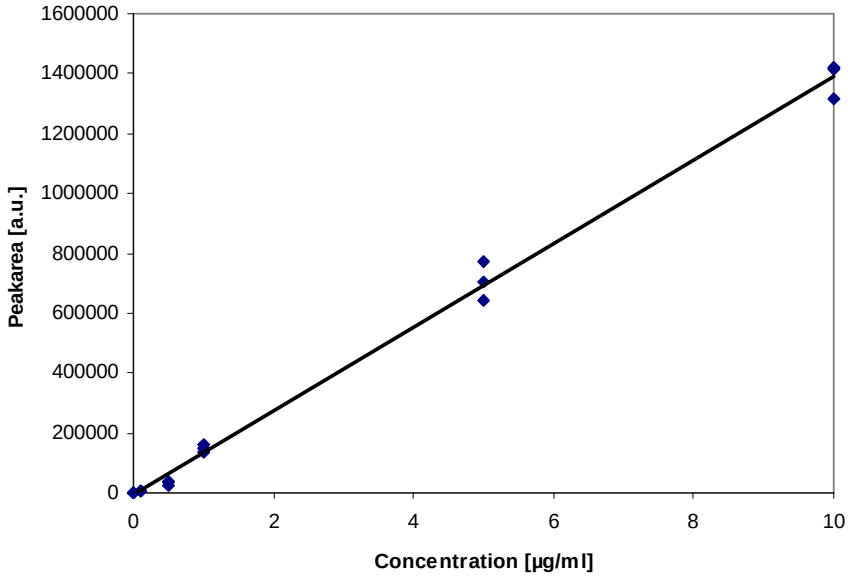

Figure 8 Standard curve of pilocarpine

Two saliva samples were analysed. The measurements gave a concentration of **0.22 mg/ml** and **1.46 mg/ml**.

7.2 The effect of pilocarpine on the cytokine production

For the determination of the standard curve, the absorbance was plotted against the cytokine concentration (Figure 9 and Figure 10).

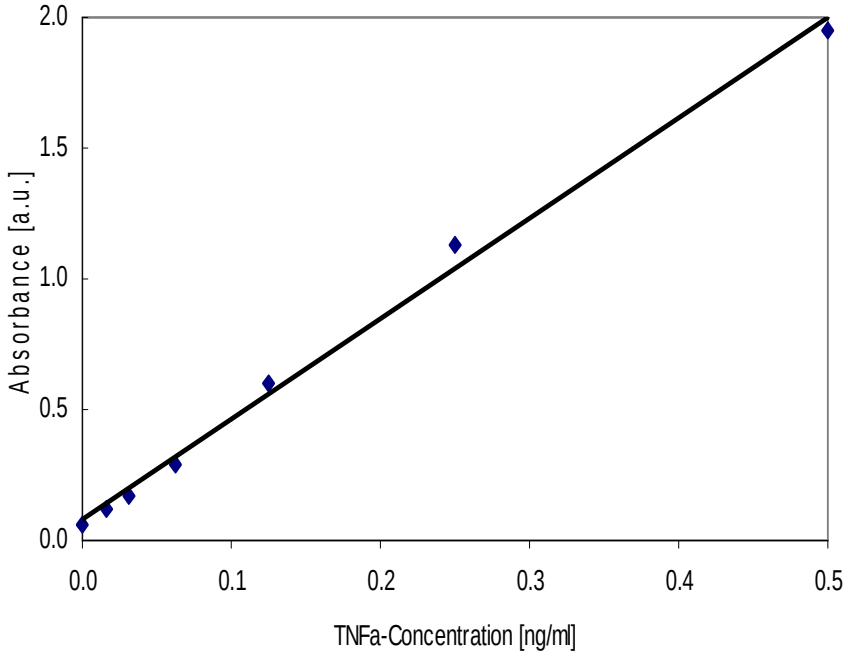

Figure 9 TNF-α standard curve

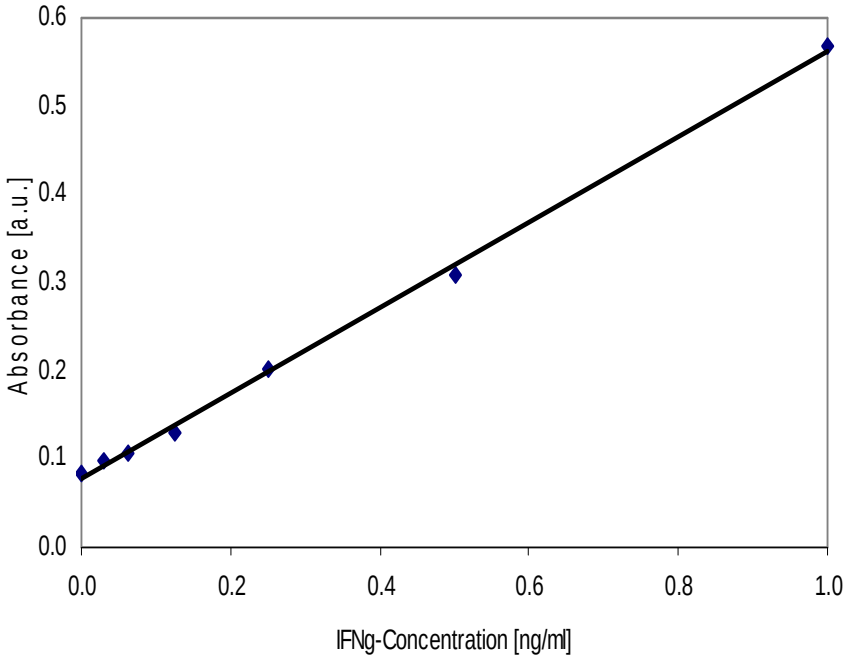

Figure 10 IFN-γ standard curve

In the following two figures, the effect of the pilocarpine concentration on the cytokine production can be seen. It shows that pilocarpine has an inhibitory effect on the production of TNF-α and IFN-γ.

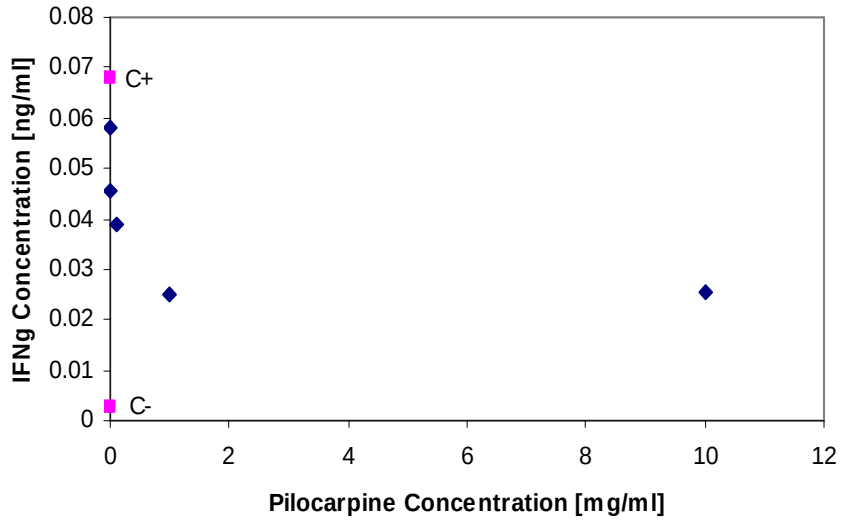

Figure 11 Effect of pilocarpine on IFN-γ production

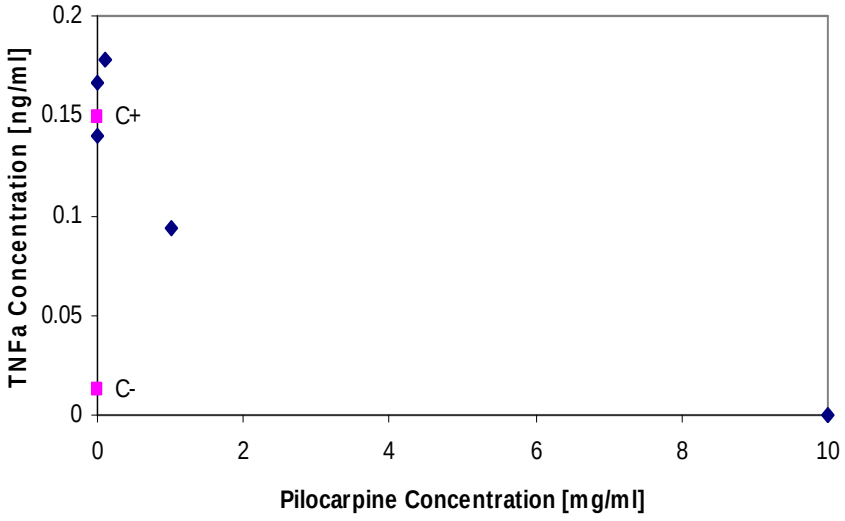

Figure 12 Effect of pilocarpine on TNF-α production

The production of TNF-α was completely inhibited at a pilocarpine concentration of 10 mg/ml. A concentration of 1 mg/ml already inhibited the production apparently. At a con-

centration of 0.1 mg/ml and lower the inhibition of TNF-α is hardly observable. The cytokine concentration was approximately the same as in the positive control sample (C+), where no pilocarpine was added and therefore no inhibition occurred.

Pilocarpine has a similar effect on the production of IFN-γ. At a concentration of 10 mg/ml the inhibition of IFN-γ can be seen obviously, but in contrast to TNF-α, it is not inhibited completely. A pilocarpine concentration of 1 mg/ml still inhibits the production a lot. At a concentration of 0.1 mg/ml and lower the cytokine production increases again rapidly. The cytokine concentration can be compared with the negative (C-) and positive (C+) control sample. The negative one lies rather below the inhibited samples and the positive one quite above.

Since the concentrations of pilocarpine measured in the tick saliva samples are 0.22 mg/ml and 1.48 mg/ml, the contamination will affect the results of studies using pilocarpine induced tick saliva.

8 Discussion

The ranges for the pilocarpine concentration in tick saliva are 3.0-19.3 mg/ml for *I. scapularis* and 0.40-1.92 mg/ml for *A. americanum* [4]. The concentration difference between the two tick species may indicate a difference in the permeability of the tick's cuticle. Our samples with concentrations between 0.22 mg/ml (1.1 mM) and 1.48 mg/ml (7.1 mM) are quite well within this range.

ELISA showed that pilocarpine has an inhibitory effect on the production of TNF-α and IFN-γ. Therefore, the contamination of tick saliva with this agonist has to be taken into account when doing immunological research using pilocarpine induced tick saliva. The results could be falsified. Pilocarpine might also have an effect on other cytokines and immunocompetent cells. This contamination could be problematic in studies examining saliva effects on tick-borne microbes or in other in vitro bioassays.

There are several alternatives to using pilocarpine induced tick saliva. Dopamine is also a trigger of salivation. Unfortunately it is quite fast degraded in biological fluids such as tick haemolymph and is therefore not very often used. Another possibility is to use salivary gland extract. The problem here is that these extracts contain additional components which are not present in saliva and may not contain products which are normally synthesized during the process of saliva secretion [4].

The antidote atropine could be added to the saliva to inhibit the activity of pilocarpine. Alternatively the pilocarpine concentration could be reduced using size exclusion chromatography and other chromatographic methods or microdialysis. The disadvantage of these methods is that important salivary components may be lost from the sample [4].

9 Summary

The incidence of tick-borne diseases has drastically increased over the past few years, resulting in a marked increase in research on tick-host-pathogen interactions. Tick saliva is used for research studies concerning the development of vaccines and for a better understanding of tick-host interactions. There are many pharmacological, biochemical and immunological active molecules present in tick saliva. Salivation is introduced by applying pilocarpine on the tick's cuticle. It is known, that pilocarpine is then also present in the collected saliva. The concentration of this agent in the saliva was measured using HPLC-MS2. The measurement gave a result of 0.22 mg/ml and 1.48 mg/ml pilocarpine in tick saliva. The effect of these concentrations on the production of cytokines was tested using ELISA. The test showed that pilocarpine has an inhibitory effect on the production of TNF-α and IFN-γ. Therefore, this effect has to be taken into account when doing research studies with pilocarpine-induced tick saliva.

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