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Diplomová práce

ELEKTRO MEMBRÁNOVÁ EXTRAKCE ZA NEHYBNÝCH PODMÍNEK – ULTRARYCHLÁ IZOLACE KYSELÝCH LÉČIV Z NEUPRAVENÉ LIDSKÉ PLAZMY

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Department of Analytical Chemistry

Diploma Thesis

ELECTRO MEMBRANE EXTRACTION UNDER STAGNANT CONDITIONS – ULTRA-FAST IZOLATION OF ACIDIC DRUGS FROM UNTREATED HUMAN PLASMA

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I hereby declare that this thesis is from my own work and effort, and all other sources of information used have been acknowledged.

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

ABSTRACT

The presented work describes the development of electro membrane extraction (EME) of acidic drugs from biological samples under completely stagnant conditions.
As noticeable from the term, EME across a supported liquid membrane (SLM) is a result of application of an electrical potential difference [1][2]. EME of 3 acidic drugs from untreated human plasma was performed through an organic solvent (decanol) immobilized in the pores of a polymeric hollow fibre (200 μm thickness) as a SLM, and into 1 mM NaOH as the acceptor solution. The driving force for the extractions was electrical potential difference. The influence of the extraction time, voltage and pH of both donor and acceptor solution were investigated and optimum conditions were proposed. Ketoprofen, ibuprofen and naproxen were extracted from plasma samples with recoveries ranging between 9 and 20 % after only 10 min. No agitation was applied and only low voltage (20V) was used to drive the target analytes through the SLM. The extraction system provided efficient sample pre-treatment and pre-concentration prior to separation and detection with capillary electrophoresis-ultra violet light detection (CE-UV) or high performance liquid chromatography (HPLC-UV).

Linearity was in the range between 0.5 and 30 μg/ml and repeatability (RSD) was ranging from 7.19 to 14.70 %. For EME, RSD values are acceptable up to 15 %.

ABSTRAKT

Tato práce se zabývá studiem elektro-membránové extakce (EME) léčiv kyselé povahy z biologických vzorků za naprosto stabilních podmínek; čili bez využití třepání. Jak je z názvu patrné, EME přes kapalnou membránu na nosiči (SLM) je důsledkem existence elektrického potenciálového rozdílu mezi kompartmenty oddělenými membránou [1][2]. Byla provedena extrakce tří kyselých léčiv z neupravené lidské plazmy, a to pomocí organického rozpouštědla (dekanol) imobilizovaného v pórech polymerního dutého vlákna (o tloušťce stěny 200 μm) jako SLM, do přijímacího roztoku, kterým byl roztok 1 mM NaOH. Byl studován vliv doby extrakce, napětí a hodnot pH dárcovského a přijímacího roztoku a byly navrženy optimální podmínky pro dosažení efektivity. Ketorpofen, ibuprofen a naproxen ze skupiny nesteroidních antiflogistik byly extrahovány ze vzorků plazmy s výtěžky dosahujícími hodnot 9 až 20 % za 10 minut. Systém byl během extrakce udržován v nehybném stavu a hnací silou pro přechod cílových analytů přes SLM bylo pouze napětí 20V. Metoda představuje velice efektivní způsob předúpravy a zakoncentrování před separací za použití kapilární elektroforézy (CE) nebo vysokoúčinné kapalinové chromatografie (HPLC).

Metoda vykazuje lineární odezvu signálu v rozmezí koncentrací 0.5 až 30 μg/ml a opakovatelnost (RSD) pro vzorky plazmy je mezi 7.19 až 14.70 % u všech sledovaných analytů. Pro EME jsou přípustné hodnoty relativní směrodatné odchylky do 15 %.

Contents

TABLES

FIGURES

ABBREVIATIONS

EME – Electro membrane extraction

SLM – Supported liquid membrane

LPME – Liquid-phase microextraction

CE-UV – Capillary electrophoresis – ultraviolet light

HPLC-UV – High performance liquid chromatography – ultraviolet light

GC – Gas chromatography

LC-MS – Liquid chromatography system coupled with mass spectrometer

NSAIDs – Non-steroidal anti-inflammatory drugs

1 INTRODUCTION

This work describes an extraction technique called electro membrane extraction of acidic drugs prior to CE-UV and HPLC-UV respectively. In this method model analytes were extracted from only 75 µl of plasma sample spiked with the analytes by application of a low voltage and using a very simple device.

Electro membrane extraction across a supported liquid membrane is a method that has been developed at the School of Pharmacy, University of Oslo, Norway in 2005 and it was first published in 2006. Since then many papers have been published on this topic [1][2][3][4][5][6][7][8][9].

In the following, a method is described which can be applied to determinate model NSAIDs, widely used in arthritis and other disorders, and selective and irreversible proton pump inhibitor omeprazole in biological samples to be carried out in a significantly shorter period of time. In addition to that, the equipment used can be obtained for a very little sum of money, the amount of sample necessary for a single extraction as well as the production of organic waste per extraction are extremely low compared to other common methods, such as solid phase extraction, for instance.

2 AIM OF THE WORK

Because of the need to simplify “old” techniques, this research is offering new easy alternative to common, widely-used methods, with a potential to attract more interest and enthusiasm for electro membrane extraction (EME) among researchers. In the period from 2005 until now, EME, where analytes are extracted from biological samples, through a supported liquid membrane (SLM) sustained in the pores of a polymeric membrane (hollow fibre), and into an aqueous acceptor solution by application of an electrical potential has been developed. Based on previous experience, in this work, the attention was focused on investigating extent of utilization of this concept for extraction of acidic drugs, as there was just a very little experience with them [9], whereas lots of data have been obtained for basic drugs so far.

3 THEORETICAL PART

3.1 Non-steroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs have antipyretic, analgesic and, in higher doses, anti-inflammatory effects. These agents work primarily by inhibition of the cyklooxygenase enzyme, which is responsible for converting arachidonic acid to prostaglandins, which mediate inflammation. The term "non-steroidal" is used to distinguish these drugs from steroidal anti-inflammatory drugs, which are often abused and lead to dangerous health conditions.

Most NSAIDs are chiral molecules (diclofenac is an exception) and typically only one enantiomer is pharmacologically active, however the majority are prepared in a racemic mixture. For some drugs, also isomerase enzyme exists in vivo which converts the inactive enantiomer into the active form.

Most non-steroidal anti-inflammatory drugs are weak acids, with a pKa ranging from 3 to 5. They are well absorbed from the stomach and intestinal mucosa. They are highly protein-bounded (typically > 95 %), usually to albumin (the most abundant protein in human plasma) so they may interact with other medications, especially those highly protein-bounded, resulting in increased concentration of the free fraction in plasma. Most of them are metabolized in the liver by oxidation and conjugation to inactive metabolites which are later on excreted in the urine, although some drugs are partially excreted in the bile.

NSAIDs are usually indicated for the treatment of acute or chronic conditions where pain and inflammation are present. Generally they are used for the symptoms of the following conditions: rheumatoid arthritis, osteoarthritis, dysmenorrheal, headache and migraine, postoperative pain, fever, ileus, mid-to-moderate pain due to inflammation and tissue injury [10][11][12][13].

For this diploma thesis NSAIDs classified as propionic acid derivates have been chosen (incl. ibuprofen, naproxen, gemfibrozil, ketoprofen, fenoprofen, flurbiprofen). Their chemical structures with logP and pKa values are shown in **Figure 1** [14].

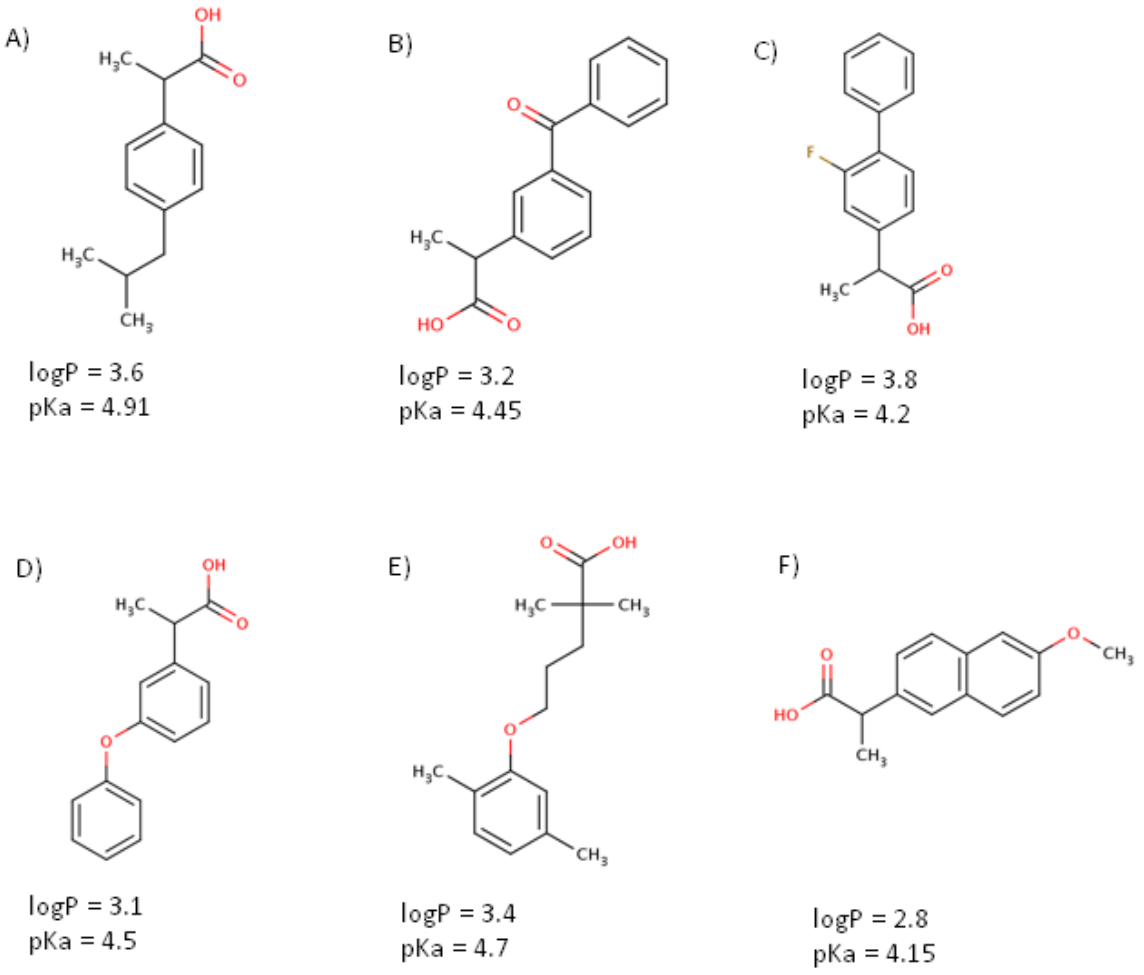

Figure 1: A) Ibuprofen, B) Ketoprofen, C) Flurbiprofen, D) Fenoprofen, E) Gemfibrozil, F) Naproxen

3.2 Electro-membrane extraction (EME)

Electro membrane extraction (EME) is an extraction technique that was first introduced by Stig Pedersen-Bjergaard and Knut Einar Rasmussen [1]. There are some similarities with LPME, but the extraction principles differ significantly. In LPME, pH gradient (optimal pH-conditions on each side of the SLM) is used to promote migration of chemical and biochemical substances and distribution into the organic liquid in the system. The type of the transport is called passive diffusion and it is based on the distribution constants between SLM (organic solvent) and the sample and acceptor solutions, respectively (**Figure 2**). Whereas in EME, the applied electrical potential over the SLM is the only driving force and it was found to be a much stronger driving force for the extraction than a pH gradient (extraction speed is approximately 6 or 7 times faster) [2].

Also couple of modifications of EME has been introduced so far. In very early experiments, EME was conducted with the use of agitation as shown on the scheme in **Figure 3**. Later on, successful extractions were accomplished also under stagnant conditions (without the use of agitation) as shown in **Figure 4**. Also miniaturized EME using flat membranes was recently introduced [3] and the schematic illustration is shown in **Figure 5**.

EME show similarities with ionthophoretic transport of drugs through skin, and models from this area were modified and used for the purpose of EME [4]. The extraction is accomplished in a three-phase system, where chemical substances cross two phase boundaries during their migration from one aqueous solution, through a thin artificial liquid membrane (≈200 μm) formed by an organic solvent immobilized in the pores of a porous hollow fibre, and into another aqueous compartment.

The experiments are carried out in the following way: the sample compartment is filled with sample solution. The lower end of the hollow fibre is closed by mechanic pressure. The porous polypropylene hollow fibre is dipped in the organic solvent for 5 seconds to impregnate the pores in the wall with the organic liquid (the optimal time necessary for dipping and width of the membrane are now under investigation). The excess is gently wiped away with a medical wipe. After immobilizing the organic liquid in the pores, the acceptor solution is introduced into the lumen of the fibre by a micro-syringe. The fibre and the electrode (polarity depends on the type of analytes of interest; for acidic analytes

it is negative) are then directed to the sample solution in the sample compartment, and the other electrode is placed into the acceptor solution. Both electrodes were coupled to the power supply. Schematic illustration of the EME device is shown in **Figure 4**. Charged analytes in the sample solution are drawn across the SLM towards the electrode of opposite charge in the acceptor solution.

This sample preparation principle is shown to be very rapid and selective method and can provide excellent sample clean-up and analyte enrichment from complicated samples.

After the extraction process, electrodes are removed, the acceptor solution is collected and subsequently analyzed by Capillary Electrophoresis (CE), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), or Liquid Chromatography system coupled with Mass Spectrometer (LC-MS).

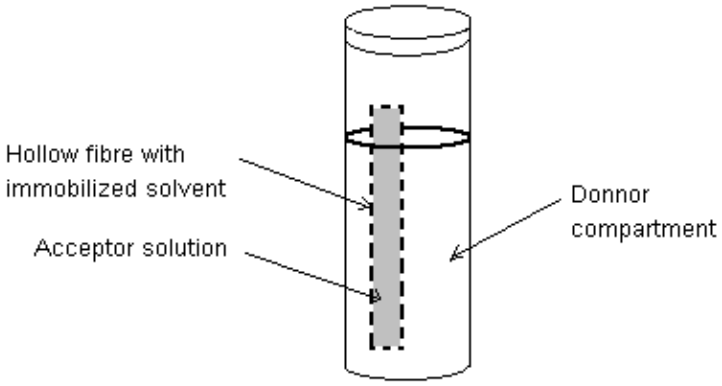

Figure 2: Schematic illustration of the set-up for liquid-phase microextraction (LPME)

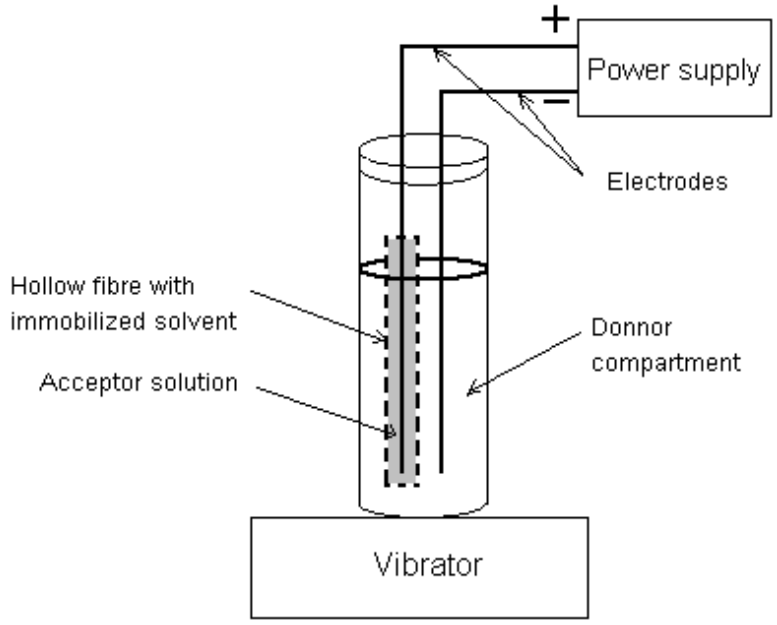

Figure 3: Schematic illustration of the set-up for EME with the use of agitation

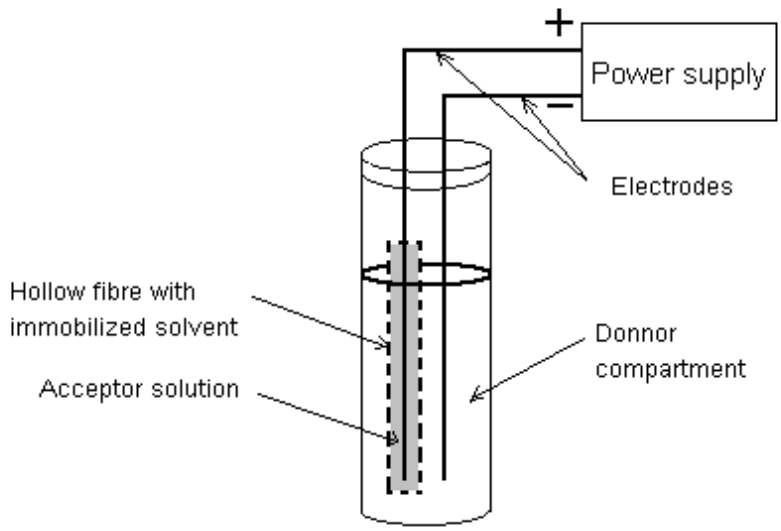

Figure 4: Basic principle of the electro membrane extraction (EME) device, extraction under completely stagnant conditions

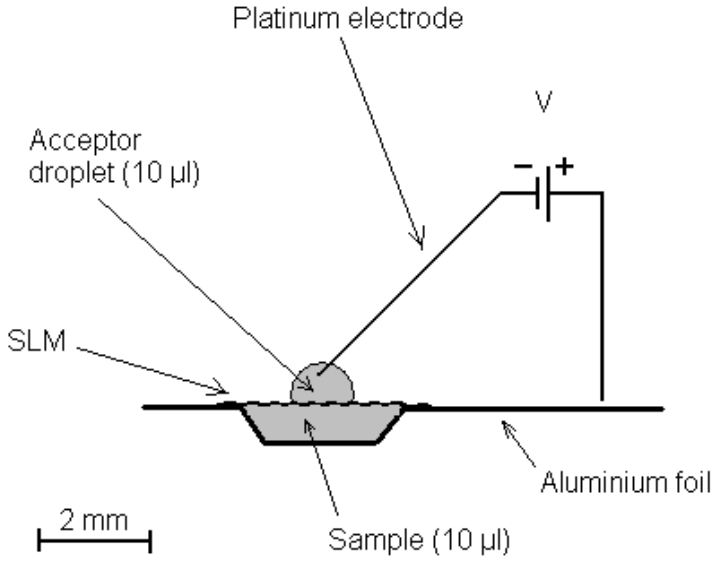

Figure 5: Schematic illustration of the set-up for drop-to-drop EME

4 EXPERIMENTAL PART

4.1 Materials and methods

4.1.1 Chemicals

- Ibuprofen, ketoprofen, omeprazole, gemfibrozil, naproxen, fenoprofen calcium salt hydrate, flurbiprofen, n-decyl alcohol,1-octanol, 1-nonanol, 1-heptanol, 1-undecanol, acetonitrile, tetraethylamonium bromide (Sigma-Aldrich, Germany)
- NaOH, methanol, benzene (Merck, Germany)
- 1-hexanol, cyklopentanol, 1-dodecanol, 2-nitrophenyl octyl-ether (NPOE), silicone oil AR20, 3-(N,N-Dimethylmyristyl-ammonio)propansulfonate, hexadecyltrimethyl-ammonium bromide, trioctylphosphine oxide, 18-crown-6, 15-crown-5, dibenzo-24-crown-8, butyl-trifenyl-phosphonium bromide, 3-chlorpentan and trioctylphosphine oxide (TOPO) (Fluka, Buchs, Switzerland)
- Cyklohexane (Ridel-de Haën, Germany)
- sodium hydroxide (VWR International LTD, Poole, England)
- Peppermint oil (local pharmacy, Oslo, Norway)
- soy-bean oil (local grocery, Oslo, Norway)
- Alamine 300, Aliquat 336 (Gognis, Cincinati OH, USA)
- Propylene glycol (I.T. Baker Chemicals, Holland)
- L-Phenylalanine (Koch-Light Laboratories LtD, Colnbrook Bucks England)
- Carvacrol, anethol, 2,4,6-trimethylacetophenon, 2-hydroxy-5-methyl-1-acetophenon, 4-ome-acetophenon, 4-benzyloxy-2-hydroxyaceton, 3,4,5-trimethoxy-benzoic acid-methylester (a gift from Karl Malterud, School of Pharmacy, University of Oslo, Norway)
- Human plasma (Ulleval hospital, Oslo, Norway)
- Ultrapure water

All drug substances, chemicals, and solvents were of analytical grade.

4.1.2 Membranes

5 cm piece of Q3/3 Accurel KM polypropylene hollow fibre (Membrana, Wuppertal, Germany) with inner diameter of the fibre of 600 μm, the thickness of the wall of 200 μm, and the pore size of 0.2 μm.

Supported liquid membranes (SLM)

Supported liquid membranes (SLMs) are used in analytical chemistry for extraction and pre-concentration of chemical substances (acidic or basic) before high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE). With the SLM extraction substances can be extracted from aqueous sample (donor solution), through a SLM (the mechanism of mass transfer is passive diffusion), into another aqueous solution on the other side of the SLM (acceptor solution). The membrane support may be either a flat sheet or a hollow fibre, resulting in two different technical configurations with the second one known as liquid phase micro-extraction (LPME). [5]

The supported liquid membrane in EME (made exactly in the same way as in LPME) is formed by dipping the hollow fibre (closed at one end by mechanic pressure) into the organic solvent which penetrates the pores of the hollow fibre and is bound by capillary forces to the polypropylene network comprising the fibre wall (creating support). In this system, the mechanism of mass transfer is electro-kinetic migration. Model for electro-kinetic transport in a SLM is shown in **Figure 6**. Equipment used for creating SLM for experiments discussed further in this work is shown in **Figure 7** and **Figure 8**.

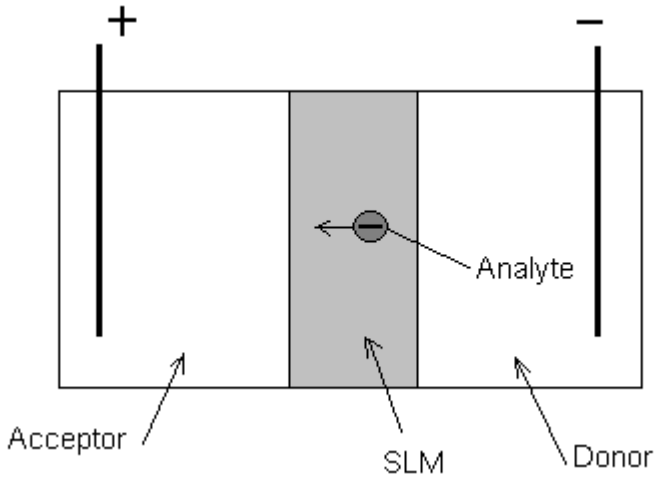

Figure 6: Model for electro-kinetic transport in a SLM

Figure 7: Equipment used for creating SLM

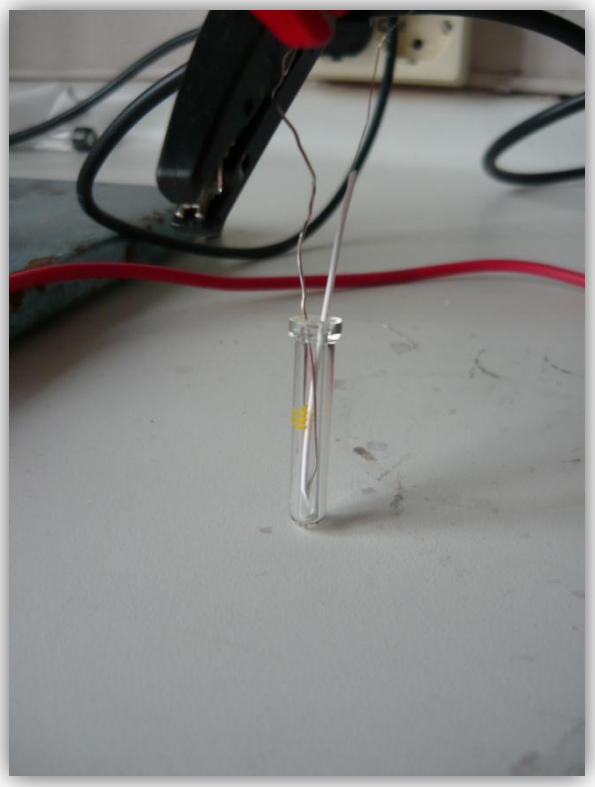

Figure 8: Very simple EME device formed by SLM, two electrodes of oposite polarity connected to power suply and a sample compartment

4.1.3 Standard solutions and samples

A stock solution containing 1 mg/ml of each of the model analytes was prepared in ethanol and were stored at fridge (approx 8°C) protected from light. Sample solutions were prepared by dilution of these stock solutions by 10 mM NaOH to concentration of 10 µg/ml for the EME experiments. This stock solution was also utilized to spike human plasma samples containing 10 µg/ml of the analytes of interest for CE. A stock solution containing 10 mg/ml of each of model analytes was used to spike human plasma providing a spiked plasma samples for validation on HPLC.

4.1.4 Buffer preparation

The running buffer for the capillary electrophoretic analysis was 50 mM borate buffer, providing a natural pH of 8.85. It was prepared in this way:
0.47 g of di-Natriumtetraborat-Decahydrat were weighed and dissolved in 100 ml of ultrapure water.

4.1.5 Equipment

Capillary Electrophoresis (CE)

Analytes were determined by capillary electrophoresis using a MDQ instrument (Beckman, Fullerton, CA, USA) equipped with a UV-detector operated at 200nm. Separations were performed in 75 µm-I.D. fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an effective length 19 cm (Beckman). The running buffer was (50 mM borate with pH 8.85). The instrument was operated at 20 kV, which generated a current level of approximately 30 µA. The sample was injected (from the anodic side) by a pressure of 0.5 psi for 5 sec. The temperature was kept constant at 22°C, while the sample storage was kept at 5°C. Detection was accomplished at 200 nm utilizing a 100 µm x 800 µm slit.

Before each work the capillary was rinsed for 15 minutes with 0.5 M NaOH, 5 minutes with pure water and 15 minutes with borate buffer. At the end of each day it was rinsed for 5 minutes with pure water and 2 minutes with air.

At the beginning of each run there was a pre-conditioning of the capillary (2 minutes with borate buffer) to clean remains and avoid carry over from the previous sample.

High Performance Liquid Chromatography (HPLC)

HPLC system (Agilent Technologies, CA, USA) was used for the validation of this method. Other equipments used coupled with the HPLC-UV were an autosampler, a degasser, and a system controller (Agilent Technologies, CA, USA). The sample was separated with a Gemini-C18 column (Phenomenex, CA, USA) and the stationary phase with a length of 150 mm, an internal diameter of 2 mm and a particle diameter of 5 µm. The flow (0.200 µl/min) was provided by pump containing mobile phase (40 % acetonitrile / 60 % 25 mM phosphate buffer with pH 2.70). The injection volume was 20 µl. The temperature of the column was kept constant at 20°C, while the sample storage was kept at 7°C. UV-detector was coupled to HPLC instrument and detection was accomplished at 200 nm. Each run lasted 35 minutes.

At the beginning of each day the column was rinsed for 5 minutes with a mix of acetonitrile and water (90:10), 5 minutes with a mix of water and acetonitrile (90:10),

another 5 minutes with a mix of acetonitrile and water (90:10) and 5 minutes with mobile phase in order to rinse remains from the column.

4.1.6 Electro membrane extraction (EME)

Analysis by capillary electrophoresis

A 5 cm piece of polypropylene hollow fibre was dipped into the organic phase which was immediately immobilized in the pores of the fibre through capillary forces and created a supported liquid membrane. Excess of solvent was then removed by a medical wipe.

Subsequently, 75 µl of the sample solution containing 10 µg/ml of the target analytes dissolved in 10 mM NaOH or in plasma (donor solution) were injected in the sample compartment (Chromacol, 01-CVG) with a syringe (Hamilton 805 RNW 50 µl, 0.508 mm OD) and for plasma samples at the same time 10 µl of 1 M NaOH was added to the donor phase. 7.5 µl of aqueous solution of 10 mM NaOH (acceptor solution) were carefully injected in the lumen of the fibre with a (Hamilton 702 RN 25 µl, 0.180 mm OD, length 70 mm, PS3 – flat) so the liquid was evenly distributed from the bottom towards the upper end and subsequently the fibre was placed in the sample compartment with sample solution. The negative platinum electrode (cathode; 0.50 mm OD) was then placed in the compartment with donor solution and the positive platinum electrode (anode; 0.20 mm OD) into the fibre so it just reached the bottom.

EME procedure was utilized to determine model NSAIDs in biological samples. Based on the EME experiments, 15 V and 20 V were applied to the system to perform extractions from water and plasma samples respectively. The acidic drugs were extracted from the sample solution into the organic phase, and further into the acceptor solution forced by electrical potential difference. The extraction process lasted 10 minutes. After that, the acceptor solution was collected with a syringe and transferred to a micro insert and was directly subjected to analysis by capillary electrophoresis.

Analysis by HPLC

EME procedure prior to analysis by HPLC was carried out in the same manner as prior to CE. After immobilizing the organic solvent in the pores of the fibre 7.5 µl of acceptor solution (10 mM NaOH) was injected in the lumen of the fibre and it was placed in the sample compartment with 75µl of the sample solution containing 10 µg/ml of the target analytes dissolved in 10 mM NaOH. Afterwards, electrodes were carefully inserted in the sample compartment and inside the fibre and voltage (20 V) was applied to the system.

After 10 minutes the acceptor phase was collected and diluted 5 times by mobile phase (60 % of 25 mM phosphate buffer of pH 2.70 / 40 % of acetonitrile) before it was subjected to analysis by HPLC.

4.2 Results and discussion

Different acidic drugs were selected as model analytes, and their chemical structures are shown in **Figure 1**. The model analytes were relatively hydrophilic as shown by their logP values (1-octanol/water partition coefficients) typically ranging from 3 to 5.

Earlier experiments have shown that the potential difference and the composition of the organic solvent are important factors for successful extractions [1][2][3][4]. Suitable extraction conditions were optimized for both water samples and plasma samples with slightly different results. For the optimization of water samples omeprazol, gemfibrozil, ibuprofen and naproxen were chosen as model analytes. In order to gain experience with more different drugs ibuprofen, naproxen and ketoprofen were then picked for the subsequent experiments with plasma samples. The separation and detection was accomplished on CE-UV.

4.2.1 Optimization of the organic phase

In a first experiment to study the fundamentals of EME for acidic drugs, attention was focused on the selection of an organic solvent for pre-treatment (immobilization) of the porous polypropylene hollow fibre because this is one of the critical steps in EME. The organic solvent served to separate the aqueous acceptor solution inside the hollow fibre from the aqueous donor solution (sample) in the sample compartment, and should therefore be immiscible with water. As a second criterium, the solubility of the analytes should be higher in the organic phase than in the donor phase in order to promote extraction. Third, the solubility of the analyte in the organic phase should be lower than it's solubility in the acceptor solution, in order to achieve a high degree of analyte pre-concentration. As a fourth criterium, in order to maintain a stable layer of organic solvent, it should be easily immobilized in the pores of the fibre and should be nonvolatile [8].

Based on these criteria and on earlier experience, many organic solvents were tested as a liquid forming SLM for extraction, as showed in **Table 1**. Experiments were repeated three times with each organic phase. It was discovered that long chain aliphatic alcohols were providing best recoveries by improving partitioning to the organic liquids and decanol was chosen as immobilization solvent for SLM for the rest of this study as it was found to provide the highest recoveries of the three NSAIDs in compared to other solvents and with lowest RSD values (ranging from 2.20 to 10.38 % for water samples and from 7.16 to 12.26 % for plasma samples). With this solvent, no leakage was observed, both to the donor and acceptor phases. Also octanol enabled extraction of the acidic model substances with comparable performance but other solvents of similar chemical structure were tested and no other long-chain alcohol appeared to be a suitable solvent as they did not improve the recoveries and also due to high RSD values (ranging from 21.09 to 38.43 %). In further experiments, many substances (**Table 2**) were also tested as additives to decanol in order to modify the chemistry of the membrane but no improvement was observed.

Hexanol
Heptanol
Octanol
Nonanol
Decanol
Undecanol
Decanol - mixture of isomers
Cyklopentylalkohol
Pepermint oil
Soja oil
Silicone oil
Octanol + 10% TOPO
Anethol
3-chlorpentane
Carvacrol

Table 1: Organic solvents tested as a liquid forming SLM for extraction of acidic drugs

18-crown-6
trioctylphosphine oxide
aliquat 336
propylene glykol
15-crown-5
butyl-trifenyl-phosphonium bromide
tetraethylamonium bromide
2,4,6-trimethylacetophenon
3,4,5-trimethoxy-benzoic acid-methylester
2-hydroxy-5-methyl-1-acetophenon
4-ome-acetophenon
4-benzyloxy-2-hydroxyaceton

Table 2: Aditives to decanol to improve the characteristics of the SLM in order to get more effective EME system

Organic phase	Recovery (%)			
	Omeprazol	Gemfibrozil	Ibuprofen	Naproxene
Decanol	9.3	20.2	19.3	11.1
Nonanol	0	11.9	18.1	11.1
Octanol	1.2	4.1	6.4	2.7
Decanol+benzene (25:1)	7.5	18.7	21	16.3
3,4,5-trimethoxy-benzoic acid-methylester	8.3	21.1	27.5	19.1
4-benzyloxy-2-hydroxyaceton	6.5	16.9	19.2	10.3
Dibenzo-24-crown-8	2.8	16.7	20.9	16.9
Decanol+Alamine 300 (39:1)	1.2	14.9	16.9	14.1

Table 3: Recovery with different artificial liquid membranes for water samples

Organic phase	Recovery (%)		
	Ketoprofen	Ibuprofen	Naproxen
Decanol	19.1	9.4	2.8
Octanol	20.1	8.4	3.2
Decanol + alamine 300, 39:1	6.0	2.1	1.2
3,4,5-trimethoxy-benzoic acid-methylester	18.1	10.5	2.5

Table 4: Recovery with different artificial liquid membranes for plasma samples

4.2.2 Optimization of the extraction voltage

In a second experiment, recovery of the different analytes was studied as a function of applied electrical potential difference. Each experiment was repeated six times and the results are demonstrated in **Table 5, Figure 9, Table 6** and **Figure 10**. In general, recoveries increased with increasing voltage up to 20 V, and then increasing voltage above this level resulted in decreasing of the recoveries. Increasing the voltage led to production of bubbles both in the donor and acceptor solution due to Joule effect and resultant partial evaporation, while low voltage did not appear to give the analytes sufficient strength for migration through the SLM. Experiments were also operated with higher voltage then showed in the table (>30 V), but in this case the system suffered from massive bubble formation at the electrodes in both donor and acceptor solution and corresponding instability problems. The highest recoveries were obtained with 30 V with water samples (RSD values were in the range from 4.90 to 15.82 %), whereas 20 V were found to be appropriate for plasma samples, and were used during the rest of this work (RSD values were ranging from 9.98 to 13.81 %).

Voltage (V)	Recovery (%)			
	Omeprazol	Gemfibrozil	Ibuprofen	Naproxen
10	7.7	16.0	15.6	8.5
15	7.8	15.7	17	11.4
30	9.2	21.1	25.9	19.4
40	4.4	12.3	19.3	11.4

Table 5: Recoveries of the target analytes after EME from water samples related to the increase of the voltage

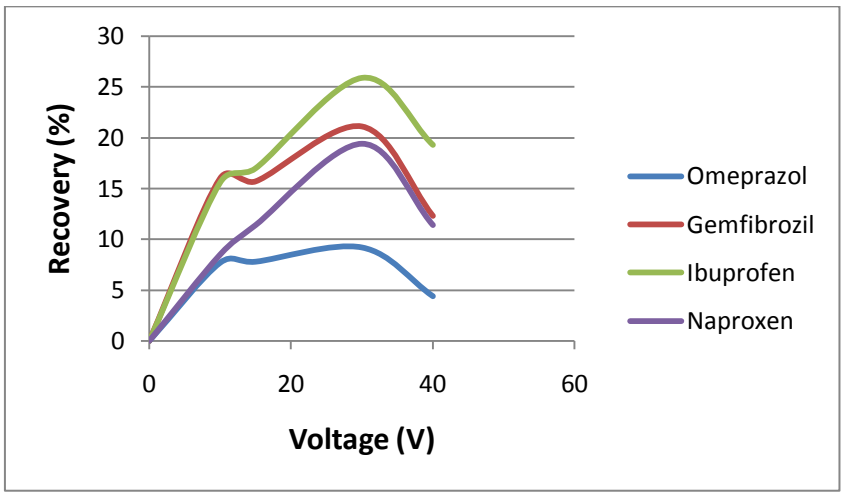

Figure 9: Recoveries of the target analytes after EME from water samples related to the increase of the voltage

Voltage	Recovery (%)		
	Ketoprofen	Ibuprofen	Naproxen
2V	0	0.7	0
5V	0.6	1.7	0.3
10V	4.0	8.4	1.9
15V	7.9	12.1	3.2
20V	12.8	15.8	3.6
25V	10.8	10.2	2.3
30V	12.5	8.4	2.4

Table 6: Recoveries of the target analytes after EME from plasma samples related to the increase of the voltage

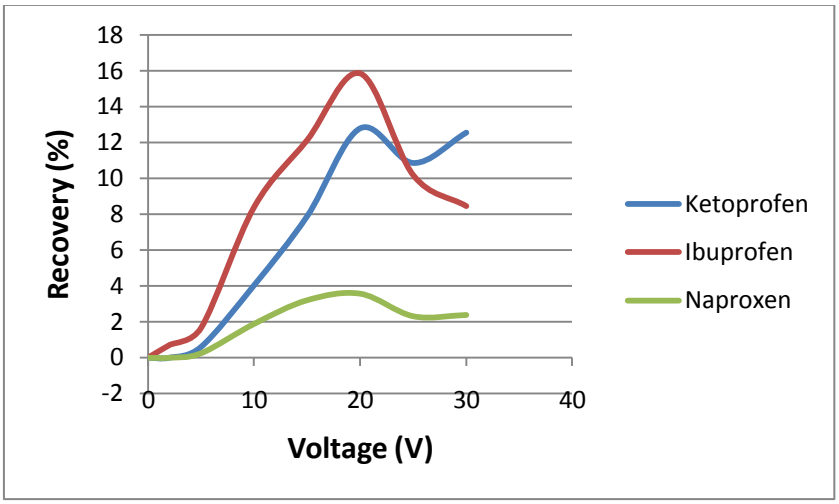

Figure 10: Recoveries of the target analytes after EME from plasma samples related to the increase of the voltage

4.2.3 Optimization of the extraction time

An experiment on recovery versus extraction time was conducted with applying the electrical potential difference as chosen previously and the results are displayed in **Table 7**, **Figure 11**, **Table 8** and **Figure 12.** Increasing the extraction time led to increasing the recoveries and after a certain period of time the analyte recovery approached its maximum level and no further gain in recovery was observed as a function of time. The reason for this is currently not fully understood, but may be due to mass transfer resistance and built-up of a boundary layer of ions NaOH at the interfaces at both sides of the artificial liquid membrane. As a most appropriate EME time, 5 min were chosen for next experiments with water samples, whereas 10 min were optimal for plasma samples.

Extraction time	Recovery (%)			
	Omeprazol	Gemfibrozil	Ibuprofen	Naproxen
1 min	0.4	1.6	2.8	1.4
2 min	1.8	4.8	6.3	4.3
5 min	9.2	21.1	25.9	19.4
6 min	3.2	15	24.6	13.8
7 min	2.1	9.4	16.4	8.1

Table 7: Recovery versus extraction time from water samples changing the extraction time

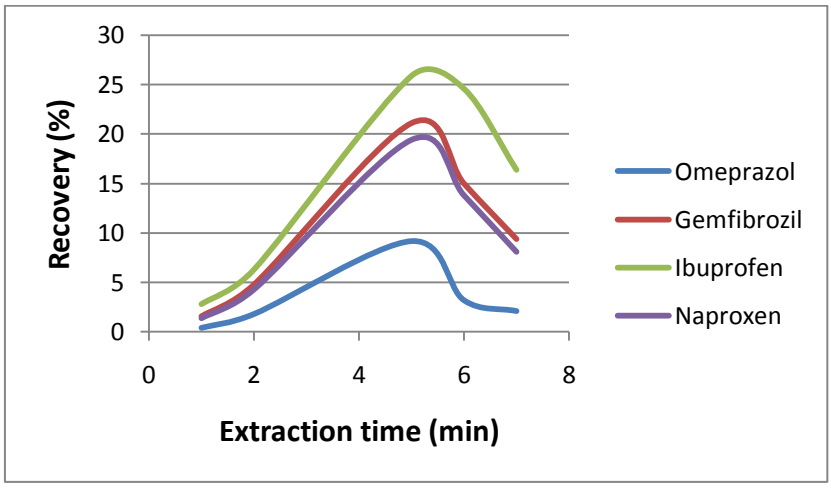

Figure 11: Recovery versus extraction time from water samples changing the extraction time

Extraction time	Recovery (%)		
	Ketoprofen	Ibuprofen	Naproxen
2 min	2.1	2.2	0.3
3 min	7.2	6.2	1.7
5 min	12.8	8.4	3.2
8 min	13.9	8.1	2
10 min	20.1	10.1	2
15 min	15.3	5.8	1.1
20 min	4.8	1.8	0.2

Table 8: Recovery versus extraction time from plasma samples changing the extraction time

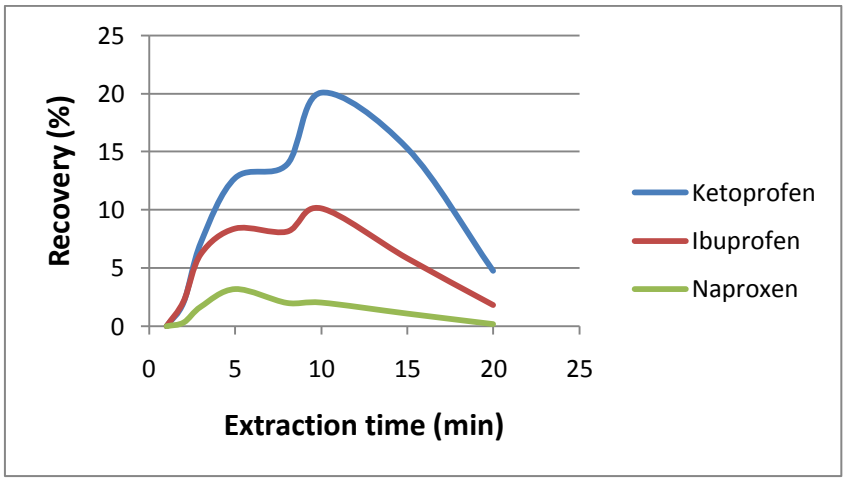

Figure 12: Recovery versus extraction time from plasma samples changing the extraction time

4.2.4 Optimization of the composition of the donor and acceptor phase

In a fourth experiment, the composition of the donor phase was optimized. Basically, the pH should be adjusted into the alkaline (usually 2 units below or above the pKa values) in order to fully ionize the analytes of interest. Therefore in experiments with plasma samples, alkaline solutions were tested as an additive to the sample compartment (1 mM, 10 mM and 50 mM NaOH, 10 mM NH_3). It was found that 10 µl of 1 mM NaOH added to 75 µl of plasma sample improved recoveries of two of the model compounds significantly (ketoprofen, ibuprofen) while no improvement in recovery was observed for naproxen. Therefore it was used for further experiments. For comparison, electroforegrams are displayed in **Figure 13** and **Figure 14**.

The effect of zwiter ions (molecules with both positive and negative charges, but with a net charge of zero) was investigated. For these experminets, phenylalanine was chosen. The reason for assuming they might have had a positive effect on extraction process was their chemical behavior - when the pH level of the solution is equal to the isoelectric point they are both positively and negatively charged and might serve as a transport carrier for the analytes from the aqueous compartment, through the organic phase, and to the aqueous acceptor solution compartment, but no significant effect was observed.

In order to improve solubility of the analytes to the organic solvent, and then to the acceptor solution, an organic solvent (methanol) was also added to both donor and acceptor water compartment but without any improvement of the recoveries.

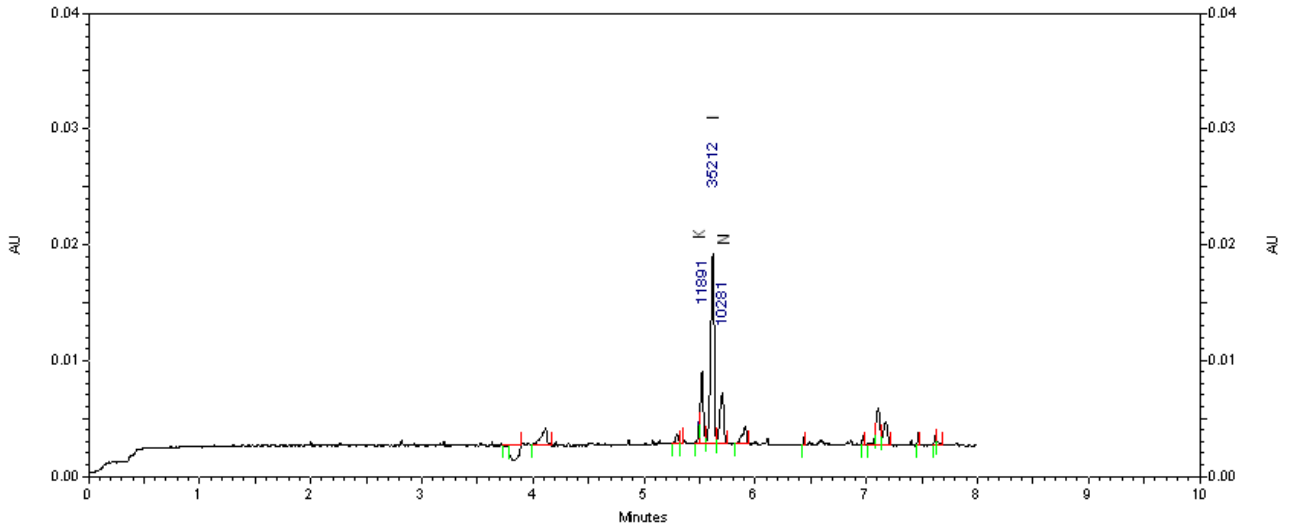

Figure 13: Electroforegram showing the separation after EME from plasma sample (K – ketoprofen, I – ibuprofen, N – naproxen)

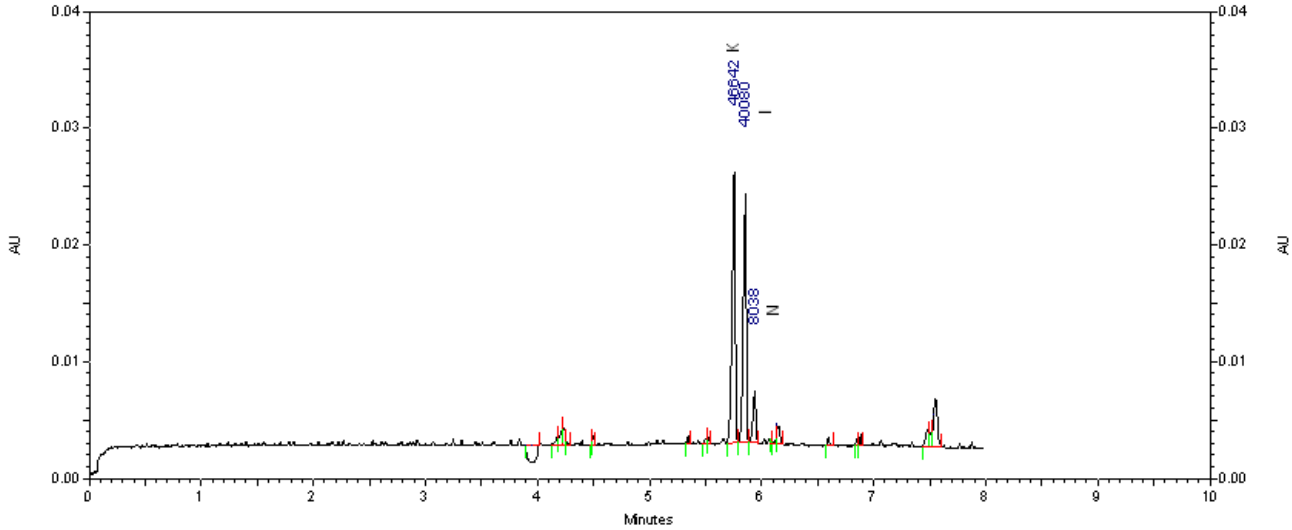

Figure 14: Electroforegram showing the separation after EME from plasma sample with 1 mM NaOH added to the sample (K – ketoprofen, I – Ibuprofen, N – naproxen)

4.2.5 LPME-like system

In order to find another way how to improve the migration, LPME- like system was created.

Sample solution was prepared in 10 mM HCl instead of 10 mM NaOH so that the acidic compounds were deionized. The idea was that they'll get through the organic membrane easier because it was proved by experiments that most of the anayles stay in the sample solution after the extraction, even when voltage is applied in the system.

So the system, where the driving force was both passive diffusion and electrical potential difference, was created.

Nevertheless this system did not prove to be efficient to achieve improvement in extraction and no better recoveries were obtained.

4.2.6 Validation

Recovery, calibration curve, limit of detection (LOD) and repeatability were evaluated analyzing the extracts of the target analytes from EME by HPLC.

Also limit of quantification (LOQ) and precision inter-day should be measured to complete the package of experiments; however they could not be completed due to lack of time.

Recovery

Plasma samples spiked with 10 µg/ml of the target analytes were treated with EME procedure and analyzed by capillary electrophoresis. The corresponding peak areas were compared with peak areas obtained by analyzing standard solutions of the model analytes at the same concentration.

Recovery (R) during the EME was calculated according to the following equation for each analyte:

$$R = \frac{n_a, final}{n_s, initial} \times 100\% = \left(\frac{V_a}{V_s}\right)\left(\frac{C_a, final}{C_s, initial}\right) \times 100\%$$

where n_s, initial and n_a, final are number of moles of analyte originally present in the sample and the number of moles of analyte collected in the acceptor solution. V_a is the volume of acceptor phase, V_s is the volume of sample, C_a, final is the final concentration of analyte in the acceptor phase and C_s, initial analyte concentration within the sample.

Recoveries from water samples ranged between 9 to 26 %, while those from plasma samples were lower and ranged from 9 to 20% due to protein-binding of the target drugs in plasma, or higher viscosity in compared to water samples.

Calibration curve

The calibration curves of the analytes were obtained by analyzing plasma spiked with 6 different concentrations of the analytes (0.5, 1, 10, 30, 75, 100 µl/ml) pre-treated with EME. Each HPLC analysis was repeated 3 times. The calibration curves are shown in **Figure 15.** Correlation coefficient was >0.99 for all the model analytes which refers to a good linearity of HPLC-signal to concentration and RSD values were in the rage:

- from 7.19 to 17.66 % for 0.5 µl/ml,
- from 8.74 to 15.52 % for 1 µl/ml,
- from 7.25 to 13.29 % for 10 µl/ml,
- from 1.02 to 5.32 % for 30 µl/ml,
- from 3.96 to 13.65 % for 75 µl/ml,
- from 9.38 to 14.70 % for 100 µl/ml.

Although the experiments were performed in the range of concentrations from 0.5 to 100 µl/ml, after 30 µl/ml there were deviations in linearity.
There might be several reasons for this phenomenon, such as overloading the system, for instance.

Analyte	Equation of the curve	Correlation coefficient (R2)
Ketoprofen	y=136.42x-50.028	0.9985
Fenoprofen	y=65.414x-57.356	0.9919
Flurbiprofen	y=56.937x/11.648	0.9997
Ibuprofen	y=32.473x+7.6177	0.9997

Table 9: Equations of the calibration curves of the target analytes and their respective correlation coefficients

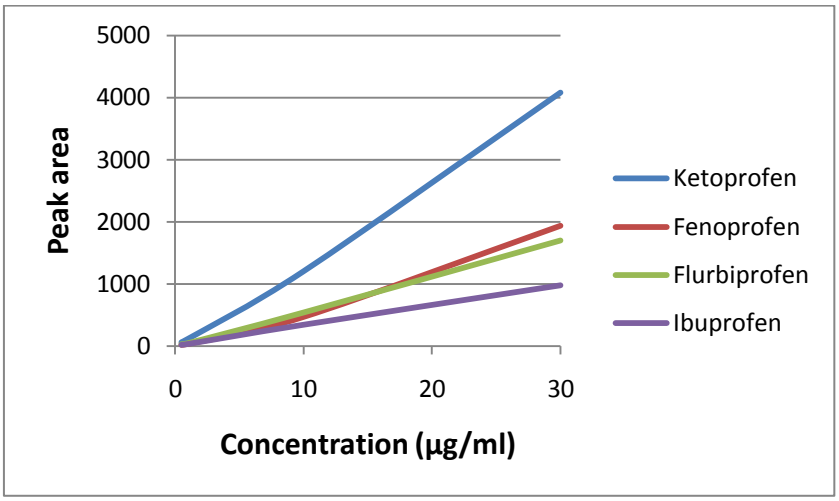

Figure 15: Calibration curves of the target analytes. Peak areas versus plasma concentration after EME process.

Repeatability

The repeatability of the method was evaluated by analyzing plasma samples at three different concentrations (corresponding to 0.5-10-100 µg/ml) after a pre-treatment with EME by HPLC. Each analysis was repeated 6 times within the same day to calculate the repeatability expressed as relative standard deviation percentage (RSD%). The results are shown in **Table 10**.

To avoid an instrumental variability standard mixture containing 10 µg/ml of the target analytes was injected and ibuprofen was used as an internal standard to calculate the different RSD% values.

Repeatability		
Analyte	**Concentration (µg/ml)**	**Repeatability (RSD %)**
Ketoprofen	0.5	7.19
	10	7.25
	100	14.70
Fenoprofen	0.5	12.90
	10	13.29
	100	12.67
Flurbiprofen	0.5	14.16
	10	10.67
	100	9.38

Table 10: Repeatability intra-day of the target analytes (n=6)

5 CONCLUSIONS

This project offers a fast, reliable and reproducible method for quantitative analysis of acidic drugs from biological samples. So far, only one paper has been published on this topic and there are only very few data considering using electro membrane extraction under completely stagnant condition as a method for pre-treatment and pre-concentration of biological samples prior to separation using CE, LS-MS or HPLC. Commercially available hollow fibre of different parameters was used than until now which makes the method widely utilizable. Although the outcomes were not as good as with previously used hollow fibre, and with electro membrane extraction of basic drugs, these data are an important part of the research concerning the development of electro membrane extraction. Numerous tests were performed to get better understanding of EME of acidic drugs and new approaches and modifications of the system presented in this work are currently under further investigation.

6 SHRNUTÍ

Tlak na zdokonalování analytických metod a zjednodušování jejich aplikace se stále zvyšuje. Nároky na jejich spolehlivost, přesnost, finanční a časovou nenáročnost, především kvůli potřebě obrovského množství stanovení biologicky aktivních látek, vedou ke snahám nalézt jednodušší alternativní techniky. Cíle tohoto výzkumu byly zaměřeny na extrakční krok se snahou takovou techniku nabídnout a zvýšit zájem výzkumníků o elektro membránovou extrakci (EME).

Elektro membránová extrakce byla vyvinuta v roce 2005 na Farmaceutické fakultě Univerzity v Oslu v Norsku a za jejím vznikem stojí především profesoři Stig Pedersen-Bjergaard a Knut Einar Rasmussen [1]. Od jejího vzniku byla publikována řada článků [1][2][3][4][5][6][7][8][9]. Intenzivně se pracuje se na jejím zefektivnění, přičemž potenciál jejího uplatnění je široký; ve všech laboratořích zpracovávajících biologické tekutiny, například toxikologické laboratoře, nemocnice, antidopingové laboratoře, ale take v kontrole životního prostředí.

Elektro membránová extrakce je metoda umožňující extrakci analytů z biologických vzorků přes kapalnou membránu na nosiči (SLM), zadrženou v pórech dutého vlákna, do vodného přijímacího prostředí, přičemž jedinou hnací silou vlastního transportu je elektrický potenciálový rozdíl mezi dárcovským a přijímacím kompartmentem. Tento potenciálový rozdíl v EME se také ukázal být mnohem silnější hnací silou pro migraci analytů než pH gradient u mikroextrakce kapalnou fází (LPME).

V tomto výzkumu byla pozornost zaměřena na rozsah využití tohoto konceptu pro extrakci kyselých léčiv, se kterými je dosud jen velmi málo zkušeností . O extrakcích bazických léčiv bylo již pořízeno množství dat a ze zkušeností s nimi se rovněž vycházelo. Jako modelová léčiva byli do experimentů zahrnuti zástupci nesteroidních antiflogistik, jmenovitě ibuprofen, naproxen, gemfibrozil, ketoprofen, fenoprofen a flurbiprofen, pro jejich antipyretické, analgetické a ve vyšších dávkách teké antiflogistické účinky široce využívané pro zvládání mnoha symptom a chorob, a v počátečních experimentech také selektivní ireverzibilní inhibitor protonové pumpy – omeprazol. Jejich struktury nesteroidních antiflogistik a hodnoty pKa a logP jsou uvedeny v obrázku (**Figure 1**). Extrakce trvají pouze 5-10 minut, potřebné zařízení je jednoduché a levné, množství vzorku a činidel potřebných

pro extrakci je velmi malé a tedy i produkce organického odpadu je velmi nízká v porovnání s jinými metodami, jako například extrakce na pevné fázi.

Vlastní extrakce se provádí v třífázovém systému, kde chemické substance přecházejí přes dvě fázová rozhraní. Sledované analyty migrují z prvního vodného roztoku (i biologického vzorku) přes tenkou uměle vytvořenou tekutou membránu (SLM; ≈200 μm), tvořenou organickým rozpouštědlem imobilizovaným v pórech porózního dutého vlákna (parametry v kapitole 4.1.2), do druhé vodné části. SLM vynikne ponořením dutého vlákna (na jednom z konců uzavřeného mechanickým tlakem) do organického rozpouštědla, které proniká do pórů a je kapilárními silami vázané do polypropylenové sítě tvořené stěnou vlákna (podpora). Po imobilizaci organického rozpouštědla v pórech je vodný přijímací roztok injekcí vpraven do lumen vlákna, které je umístěno do kompartmentu obsahujícího vodný dárcovský roztok (vzorek). Poté podle chemické povahy analytů; bazické, kyselé) je jedna platinová elektroda umístena do lumen vlákna s přijímacím roztokem a druhá do kompartmentu se vzorkem. Schématicky je celé zařízení znázorněno v obrázku (**Figure 4**), znázornění transportu přes SLM v obrázku (**Figure 6**), vybavení použité při přípravě SLM je ukázáno v obrázku (**Figure 7**) a vlastní sestava, ve které extrakce probíhá je na obrázku (**Figure 8**). Po skončení extrakčního procesu jsou elektrody vyjmuty a přijímací roztok je injekční jehlou sebrán a ihned podroben separaci za použití kapilární (CE-UV), vysokoúčinné kapalinové chromatografie (HPLC-UV), kapalinové chomatografie s hmotnostně spektrometrickou detekcí (LC-MS).

Podmínky byly optimalizovány pro vodné vzorky i vzorky plazmy, připravené naspikováním lidské plazmy připravenými roztoky analytů (vždy s koncentrací všech obsažených analytů 10 μg/ml), s poněkud rozdílnými výsledky. Tento jev může být způsoben obtížnější extrakcí ze vzorků plazmy kvůli její vyšší viskozitě v porovnání s vodným vzorkem a vysokou vazebností nesteroidních antiflogistik na plazmatické bílkoviny (většinou > 95 %). Směsi analytů ve vzorcích byly během práce měněny, aby byly získány zkušenosti s více léčivy.

Cíle prvních experimentů byly zaměřeny na optimalizaci organické fáze (rozpouštědla imobilizovaného v pórech dutého vlákna), protože se jedná o jeden z kritických bodů při EME. Ideální rozpouštědlo by mělo být nemísitelné s vodou, aby mohlo sloužit jako membrána oddělující od sebe dvě vodná prostředí a druhým kritériem je rozpustnost analytů v tomto rozpouštědle, jež by měla být větší, než jejich rozpustnost ve dárcovském roztok

a současně menší než rozpustnost v přijímacím roztoku, aby snadno přecházely přes organickou fázi dále. Byla vyzkoušena řada organických rozpouštědel (**Table 1**) a také přísad k dekanolu (**Table 2**) a různých směsí s cílem vhodně nastavit chemické složení membrány. Jako nejlepší rozpouštědlo byl zvolen dekanol, jelikož s ním bylo dosaženo nejvyšších výtěžků a rovněž kvůli nejnižším hodnotám relativní směrodatné odchylky (RSD), které se pohybovaly od 2.20 do 10.38 % u vodných vzorků a od 7.16 do 12.26 % u vzorků plazmy. Výtěžnost extrakcí při použití vybraných rozpouštědel pro vodné vzorky a vzorky plazmy je uvedena v tabulkách (**Table 3**, **Table 4**).

Ve druhé fázi se studovala korelace velikosti napětí s extrakčními výtěžky. Výsledky jsou znázorněny pro vodné vzorky v tabulce a obrázku (**Table 5**, **Figure 9**) a pro vzorky plazmy v tabulce (**Table 6**, **Figure 10**). Jelikož právě napětí je hnací silou pro migraci analytů, jeho zvyšování vedlo ke zvýšení výtěžnosti, avšak jen do 30 V u vodných vzorků (RSD od 4.90 do 15.82 %) a do 20 V u vzorků plazmy (RSD od 9.98 do 13.81 %). Další zvyšování napětí vedlo k nežádoucímu vytváření bublin v obou vodných kompartmentech a z toho vyplývající částečné evaporaci.

Zkoumala se také závislost výtěžnosti na extrakčním čase a výsledky jsou znázorněny v tabulce a obrázku (**Table 7**, **Figure 11**) pro vodné vzorky a (**Table 8**, **Figure 12**) pro vzorky plazmy. Prodlužování doby extrakce vedlo ke zvyšování výtěžků a jako optimální čas bylo zvoleno 5 minut pro vodné vzorky a 10 minut pro vzorky plazmy. Další prodlužování doby extrakce již výtěžnost nezlepšovalo. Příčina zatím není přesně známa, nicméně jev by pravděpodobně mohl být způsoben omezenou kapacitou pro průchod analytů a vytvoření vrstvy iontů NaOH z obou stran membrány na jejím rozhraní s vodnými kompartmenty.

Dále byl studován vliv složení vodných fází. V zásadě by pH dárcovského prostředí mělo být alkalické, aby bylo dosaženo úplné ionizace analytů. Pozitivní vliv na výtěžky extrakce proto měla alkalizace vzorků plazmy přidáním 10 µl 1 mM NaOH (znázorněno v grafech **Figure 13**, **Figure 14**).

Byl studován efekt fenylalaninu jako zwitter ion, který by díky svým chemickým vlastnostem (při pH roztoku rovnému izoelektrickému bodu má kladný i záporný náboj) mohl sloužit jako přenašeč modelových analytů přes SLM. Rovněž byla snaha zlepšit migraci analytů přidáním metanolu postupně do obou vodných kompartmentů – dárcovského i přijímacího prostředí a tím zvýšit solubilitu analytů v organické fázi, avšak nebylo pozorováno žádné zlepšení. Také byla vytvořena modifikace, spojující mechanismy

transportu EME a LPME. V tomto systému byla hnací silou prostá difúze (jako je tomu u LPME) i potenciálový rozdíl (charakteristika EME). Na základě předpokladu, že neionizované analyty budou snáze procházet přes organickou membránu, jelikož bylo experimenty dokázáno, že většina zůstává po skončení extrakce stále ve vzorku, byl přípraven roztok vzorku v HCl, namísto NaOH, čímž nedošlo k ionizaci analytů. Nicméně systém lepší efektivitu neprokázal.

Pro vodné vzorky a vzorky plazmy byly dle výsledků z optimalizace vyhodnoceny nejvhodnější podmínky pro EME, přičemž je možné pozorovat drobné rozdíly; především v optimální extrakční době a napětí.

Výtěžnost se pohybovala mezi 9 a 26 % u vodných vzorků, zatímco u vzorků z plazmy byly zjištěny výtěžky mezi 9 a 20 %, pravděpodobně kvůli vazbě analytů na plazmatické proteiny nebo vyšší viskozitě v porovnání s vodnými vzorky.

Korelační koeficienty pro všechny analyty přesahují hodnotu 0.99, což ukazuje na dobrou lineární závislost signálu z HPLC na koncentraci analytů ve vzorku. Graf lineární závislosti a rovnice kalibračních křivek jsou znázorněny na obrázku a v tabulce (**Figure 15**, **Table 9**).

Opakovatelnost vyjádřená relativní směrodatnou odchylkou se pohybuje mezi 7.20 až 14.70 %, což v případě EME metody vyhovuje požadavkům (tolerují se hodnoty RSD do 15 %).

Aby byla validace metody kompletní, bylo by potřeba doplnit limit kvantifikace a přesnost. V tomto případě ovšem pro nedostatek času experimenty nemohly být dokončeny.

7 ACKNOWLEDGEMENTS

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