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Rapid isolation of angiotensin peptides from plasma by electromembrane extraction

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ABSTRACT

The present study has for the first time demonstrated the isolation of peptides from human plasma by electromembrane extraction (EME). Angiotensin 1, angiotensin 2, and angiotensin 3 migrated from 500 μL of diluted plasma, through a thin layer of 1-octanol and 8% di-(2-ethylhexyl) phosphate immobilized as a supported liquid membrane (SLM) in the pores of a porous hollow fiber, and into a 25 μL aqueous acceptor solution present inside the lumen of the fiber. The driving force for the extraction was a 15 V potential difference applied across the SLM. After only 10 min of EME, the peptides were isolated from diluted plasma (pH 3) with extraction recoveries between 25 and 43%. After optimization, the extraction system was evaluated using spiked plasma samples of angiotensin 2. The evaluation was performed by liquid chromatography electrospray mass spectrometry, showing linearity of angiotensin 2 in the range 2.5–125.0 ng/mL (r^2 = 0.989), and repeatability (RSD) between 5.6 and 11.6% (n = 6). The results demonstrate the possibility of isolating angiotensin peptides from plasma in only 10 min, using electromembrane extraction. The experimental findings are therefore promising with regard to future peptide extractions.

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1. Introduction

During the last 10 years, liquid-phase microextraction (LPME) techniques have received considerable attention as new and interesting alternatives for sample preparation [1–5]. Hollow fiber liquid-phase microextraction is based on passive diffusion of analytes from a sample solution, through a water immiscible organic solvent immobilized as a thin supported liquid membrane (SLM) in the pores in the wall of a porous hollow fiber, and into a μL volume of acceptor solution filled inside the lumen of the hollow fiber [6–8]. The acceptor solution can either be an organic solvent, providing a two-phase extraction system directly compatible with GC, or an aqueous solution, providing a three-phase extraction system compatible with HPLC or CE [9]. Since the analytes are extracted from relatively large sample volumes (typically 500–2000 μL) and into a very small volume of acceptor solution (typically 25 μL), high analyte enrichment, without the need for solvent evaporation and re-constitution, is common in most LPME applications [10,11].

Unfortunately, the extraction times in LPME are relatively long, typically in the range between 30 and 45 min for sample volumes below 2 mL [12]. In order to increase the extraction speed, a new concept was recently introduced where mass transfer across the SLM was accomplished by application of an electrical potential difference as the driving force [13]. In this concept, called electromembrane extraction (EME), the analytes were extracted by electrokinetic migration. Sufficient ionization of the target analytes in both the sample- and the acceptor solution was therefore essential. Except from two electrodes and a power supply, the equipment used for EME was exactly the same as for LPME. For basic analytes, the positive electrode was placed in an acidic sample solution, and the negative electrode was placed in an acidic acceptor solution. For acidic analytes, the potential was reversed and pH in both the sample- and the acceptor solution was increased in order to ionize the acidic analytes. With EME, the extraction time was reduced to typically 5 min per extraction [13].

In the field of analytical chemistry, electrokinetic migration is frequently used for isolation of charged chemical and biochemical substances, like in electrodialysis [14–16]. With this technique, which should not be mixed up with EME, ions are filtrated through the pores of a thin polymeric membrane, and are consequently separated due to their size. EME on the other hand is a three-phase extraction technique, where the partitioning of ions into an organic solvent is central for the extraction efficiency.

A theoretical model based on the Nernst–Planck equation was presented a few years ago to explain the extraction process in EME [17]. According to this model, the flux of analytes over the SLM is affected by the magnitude of the applied potential. Another impor-

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tant factor for successful electrokinetic cross-membrane migration was the chemical nature of the organic solvent immobilized in the SLM. The organic solvent should have a certain permittivity (ε) to support a relatively low current flowing in the system: too high electrical conductance of the SLM caused instability problems like evaporation of the acceptor solution and bubble formation from electrolysis at the electrodes, leading to unreliable results [13,18].

In the last 2 years, basic and acidic compounds have successfully been extracted by EME [18–20]. The selectivity of the extraction was dependent on the choice of organic solvent for the SLM, and most effective extractions were obtained using voltages in the range 50–300 V (with corresponding electrical field strength intensities (V/cm) of approximately 2500–15,000). Recently, low-voltage EME of basic drugs was demonstrated for the first time [21], indicating that with proper selection of the SLM, a common battery can be used for extraction. Knowledge about EME from biological samples is limited, but previous papers have shown that low molecular, non-polar basic drugs, like methadone and haloperidol, can be extracted from diluted plasma, urine and breast milk [13,21]. Recoveries of approximately 40–70% were obtained with both high- and low-voltage extractions, depending on the organic solvents chosen as the SLM. A recent paper has also demonstrated EME of the same non-polar basic drugs from untreated plasma and whole blood [22].

The applicability of EME for peptides was reported for the first time in 2008 [23]. Eight different peptides migrated from an aqueous acidic sample solution (500 μL, positive electrode), through the SLM and into an aqueous acidic acceptor solution (25 μL, negative electrode), with 50 V as the driving force for extraction. The extraction recoveries (up to 56%) after 5 min of EME were found to be highly dependent on the structure of the peptides and on the composition of the SLM: a mixture containing 1-octanol with 15% of the anionic carrier di-(2-ethylhexyl) phosphate (DEHP) appeared to be optimal.

Quantitative analysis of peptides and proteins normally includes a sample preparation step, prior to capillary electrophoresis or liquid chromatography [24,25]. Due to the growing interest for determination of peptides and proteins in biological matrices, we explored for the first time in this study electromembrane extraction of peptides from human plasma. The biologically important peptides angiotensin 1, 2, and 3, which play a central role in the cardiovascular homeostasis [26,27], were selected as model analytes. EME of the angiotensins was studied and optimized in both aqueous solutions and human plasma. The analytical performance of angiotensin 2 extractions from human plasma using the optimal EME conditions was evaluated in combination with LC–MS, which is the preferred analytical technique for peptide analysis due to very selective and sensitive measurements [28]. The purpose of this study was not to develop a complete analytical method for the angiotensins, but rather to investigate the potential of EME as extraction technique for peptides in plasma for future applications.

2. Experimental

2.1. Equipment for electromembrane extraction (EME)

The equipment used for the extraction procedure is illustrated in Fig. 1. The sample compartment was a Brand Snap-Cap container of LDPE (low density polyethylene), with a volume of 0.8 mL, an internal diameter of 6 mm, and a height of 31 mm (Sigma–Aldrich, Steinheim, Germany). The porous hollow fiber used for immobilization of the SLM and for housing the acceptor solution was a PP Q3/2 polypropylene hollow fiber (Membrana, Wuppertal, Germany) with an internal diameter of 1.2 mm, wall thickness of 200 μm, and a pore size of 0.2 μm. During the experiments, the extraction unit was agitated on a Vibramax 100 agitator (Heidolph, Kelheim, Germany).

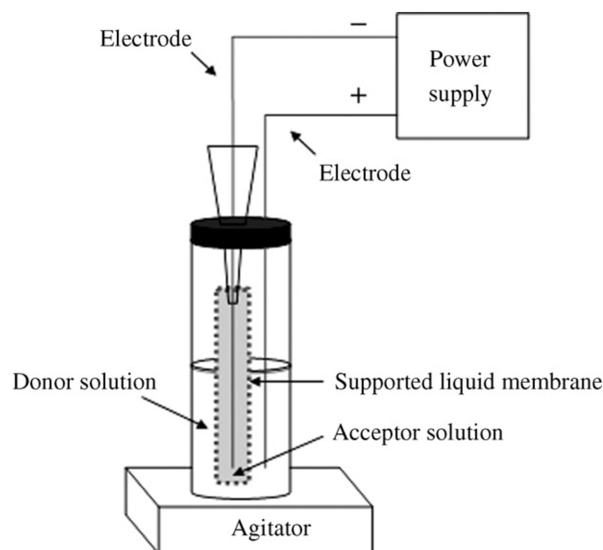

Fig. 1. Schematic illustration of the set-up for electromembrane extraction (EME).

The dc power supply used was a model ES 0300-0.45 from Delta Power Supplies (Delta Electronika, Zierikzee, The Netherlands) with programmable voltage in the range 0–300 V, and with a current output in the range 0–450 mA. Platinum wires with a diameter of 0.5 mm (K. Rasmussen, Hamar, Norway) were placed in the sample and acceptor solutions and used as electrodes. They were both connected to the power supply.

2.2. Procedure for electromembrane extraction

EME was performed according to the following procedure; 500 μL acidified sample solution (human plasma or pure water) was filled into the sample compartment. The lower end of a 2.5 cm piece of polypropylene hollow fiber was closed by mechanical pressure, whereas the upper end was connected to a 2.2 cm length pipette tip of polypropylene (Finntip 200 Ext from Thermo Electron, Vantaa, Finland) functioning as a guiding tube. The hollow fiber was dipped for 5 s in the organic solvent serving as the SLM. Excess of solvent in the SLM was then gently removed with a medical wipe. Via the guiding tube, 25 μL of acidified acceptor solution was filled into the lumen of the hollow fiber with a microsyringe. The hollow fiber and the guiding tube were inserted through the vial cap before being placed in the sample solution. Finally, the negative electrode was placed in the acceptor solution, and the positive electrode was inserted through the vial cap and into the sample solution. The electrodes were connected to the power supply, and the extraction unit was placed on a vibrator with an agitation speed of 900 rpm. Voltage was applied in the range 0–50 V (depending on the experiment), and the extraction was carried out for a predetermined time (typically 5–10 min).

After EME, the acceptor solution was analyzed either by capillary electrophoresis (CE) with UV-detection or by LC–MS. For CE-analysis, 20 μL of the acceptor solution was collected with a microsyringe and transferred directly to a small vial for introduction into the capillary electrophoresis system. For LC–MS-analysis, 20 μL of the acceptor solution was collected with a microloader (0.5–20 μL, Eppendorf AG, Hamburg, Germany) and diluted with 40 μL mobile phase A in a small vial. Fifty microliters of this mixture was injected onto the HPLC column.

2.3. Stability of angiotensin peptides during electromembrane extraction

A standard solution of angiotensins (50 μg/mL in 1 mM HCl) was analyzed for EME stability in the following way: 500 μL standard solution was filled into the sample compartment. Two electrodes were placed in the solution and connected to a power supply. After the application of voltage for 5 min, 100 μL of the solution was analyzed by CE. The electropherogram obtained from this solution was compared to an electropherogram after 0 min of electrolysis. The EME stability was tested at three different voltages (0.9, 4.9, and 7.5 V). The current flowing in the system was measured during the experiments.

2.4. Capillary electrophoresis conditions

The CE analyses were performed on an Agilent Capillary Electrophoresis System with UV-detection (Agilent Technologies, Santa Clara, CA, USA). Data acquisition was performed using ChemStation (Agilent Technologies). The separations were accomplished in a 75 μm I.D. (360 μm O.D.) fused-silica capillary with an effective length of 50 cm, and a total length of 58.5 cm (Polymicro Technologies, Phoenix, AZ, USA). The background electrolyte solution was 50 mM sodium dihydrogen phosphate buffer adjusted to pH 3.0 with *ortho*-phosphoric acid. The instrument was operated at 18–20 kV, which generated a current level of approximately 55–60 μA. Samples were introduced by hydrodynamic injection at 50 mbar for 4 s. Detection was accomplished at 200 nm.

2.5. LC–MS conditions

The chromatographic system consisted of a Shimadzu SIL-10ADvp auto injector, two Shimadzu LC-10ADvp gradient pumps, a Shimadzu DGU-14A degasser, a Shimadzu SCL-10Avp system controller and a Shimadzu LCMS-2010A single-quadrupole MS detector. Data acquisition and processing were carried out using Shimadzu LCMS Solution software Version 2.04-H3 (all Shimadzu Scientific Instruments, Kyoto, Japan).

Chromatographic separation was carried out on a 50 mm × 1 mm I.D. Biobasic-C8 column (Thermo Fisher Scientific, Waltham, MA, USA) with average pore size of 300 Å, and particle diameter of 5 μm.

The mobile phases consisted of A: 20 mM formic acid and methanol (95:5, v/v) and B: 20 mM formic acid and methanol (5:95, v/v). Flow rate was set to 50 μL/min. The injection volume was 50 μL. The first 4 min of each run were not analyzed by MS to prevent contamination.

A linear gradient was run up to 70% mobile phase B in 20 min using 100% mobile phase A/0% mobile phase B as starting point. After these 20 min the mobile phase composition was kept constant for 2 min. Subsequently, the column was regenerated for at least 10 column volumes using starting conditions.

An electrospray ionization (ESI) source operated in the positive ionization mode was used to interface the HPLC and the MS. Analyses were performed with selected ion monitoring (SIM), where m/z values 349.7^{3+} and 524.0^{2+} were used for angiotensin 2. Quantification of angiotensin 2 was based on the sum of m/z values 349.7^{3+} and 524.0^{2+}.

The MS operating conditions were as follows: drying gas between 10 and 20 L/min, nebulizer gas of 1.5 L/min, curved desolvatation line (CDL) temperature of 200 °C, block temperature of 200 °C and probe voltage of +4.5 kV.

2.6. Chemicals

Angiotensin 1 (Ang 1), angiotensin 2 (Ang 2), and angiotensin 3 (Ang 3) were purchased via Sigma–Aldrich (St. Louis, MO, USA). Di-(2-ethylhexyl) phosphate (DEHP), 2-nitrophenyl octyl ether (NPOE), and 4-nitro-m-xylene of analytical grade were obtained from Fluka (Buchs, Switzerland). The supplier of analytical grade 1-octanol and captopril was Sigma (St. Louis, MO, USA). Di-isobutyl ketone of analytical grade was from Aldrich (Steinheim, Germany). HPLC grade methanol and all inorganic chemicals were purchased from Merck (Darmstadt, Germany). Deionized water was obtained using an EASYpure UV system (Barnstead, Dubuque, IA, USA).

2.7. Biological matrixes

Drug-free human plasma was obtained from Oslo University Hospital (Ullevål, Oslo Norway), and was stored at −32 °C protected from light and thawed before extraction.

2.8. Standard solutions and samples

Stock solutions containing 1 mg/mL of each peptide were prepared in water and stored at −32 °C protected from light. Standard solutions were prepared daily by dilution of the stock solutions. Plasma and aqueous sample solutions were prepared daily before extraction.

2.8.1. Initial method optimization experiments

The angiotensin-converting enzyme (ACE)-inhibitor captopril was added to plasma and water to selectively inhibit the conversion of Ang 1 to Ang 2 [27]. For plasma samples, 400 μL captopril (50 mg/mL in water) was added to 4 mL of plasma (to ensure ACE-inhibition before the addition of peptides) and stirred rapidly. Six hundred microliters standard solution with peptides (variable concentrations), and 5 mL 100 mM HCl were finally added to give a dilution factor for plasma of 2.5, and pH 3 (no protein precipitation was observed). For aqueous sample solutions, 400 μL captopril (50 mg/mL in water) and 100 μL standard solution with peptides (variable concentrations) were added to 9.5 mL 1 mM HCl and stirred rapidly. The final pH of the aqueous samples was 3.

2.8.2. Method evaluation experiments

Plasma was spiked with Ang 2 to concentrations of 2.5, 12.5, 25.0, 62.5, and 125.0 ng/mL, and stirred rapidly. The total volume was 2 mL (maximum 25 μL of spike solution). Prior to extraction, 3 mL 100 mM HCl was added to give a dilution factor for plasma of 2.5, and pH 3 (no protein precipitation was observed).

2.9. Calculation of recovery

Recovery (R) after EME was calculated according to the following equation for each peptide:

$$R = \frac{n_{a,final}}{n_{d,initial}} \times 100\% = \left(\frac{V_a}{V_d}\right)\left(\frac{C_{a,final}}{C_{d,initial}}\right) \times 100\% \quad (1)$$

where $n_{d,initial}$ and $n_{a,final}$ are the substance amount of analyte originally present in the donor solution and the substance amount of analyte finally collected in the acceptor solution, respectively. V_a is the volume of acceptor solution, V_d is the volume of donor solution, $C_{a,final}$ is the final concentration of analyte in the acceptor solution, and $C_{d,initial}$ is the initial analyte concentration within the donor solution.

3. Results and discussion

3.1. Extraction from pure water; variation in the SLM composition (organic solvent)

The organic solvents 1-octanol, DEHP, NPOE, 4-nitro-m-xylene and di-isobutyl ketone were mixed at different levels to pro-

Table 1
Extraction recovery of Ang 1, Ang 2, and Ang 3 with different organic solvents in the SLM.

	Recovery (%)[a]		
	Ang 1	Ang 2	Ang 3
4-Nitro-m-xylene:di-isobutyl ketone (60:40)	nd	nd	nd
1-Octanol:DEHP:4-nitro-m-xylene:di-isobutyl ketone (40:10:25:25)	61	77	78
1-Octanol:DEHP:4-nitro-m-xylene (55:10:35)	32	49	44
1-Octanol:DEHP:di-isobutyl ketone (55:10:35)	46	61	61
DEHP:4-nitro-m-xylene:di-isobutyl ketone (10:50:40)	52	58	73
1-Octanol:DEHP:NPOE (55:10:35)	43	58	59
1-Octanol:DEHP (85:15)	34	57	46

Electrical potential: 50 V; extraction time: 5 min; agitation: 900 rpm; 1 mM HCl in donor solution; 50 mM HCl in acceptor solution. Each peptide was present at 1 μg/mL in the sample.
[a] $n = 3$.

vide six new organic phases as compared to earlier work [23]. These phases were tested as more optimal SLMs for the extraction of angiotensins: 4-nitro-m-xylene and di-isobutyl ketone were selected as new organic solvents based on their structure and on former successful experiments with SLM extractions [13,29]. The results from the extractions performed with these phases as well as their composition (w/w) are shown in Table 1, together with results of extractions performed with 1-octanol and 15% DEHP as the SLM (SLM used for peptides in former work [23]). As illustrated in the table, high recoveries were obtained with all the new phases, with exception of phase 4-nitro-m-xylene:di-isobutyl ketone (60:40) where no analyte transport was observed. These results indicated that the presence of the anionic carrier DEHP is crucial for the extraction of angiotensins.

The electrical current flowing in the system was measured during all the extractions. The reason for this was that earlier work has shown that the cross-membrane transport of analytes was dependent on the electrical resistance of the SLM. With a decreasing electrical resistance, the extraction recoveries increased, but there was also an unfavorable increase in current flowing in the system. A high current may generate electrolysis (O_2 and H_2) at the two electrodes, and this bubble formation in combination with an increase in temperature, would give an unstable EME system [13]. When extractions were performed from pure water samples, all the new SLMs provided stable EME systems with no observed bubble formation.

3.2. Extraction from diluted plasma

In initial experiments, extraction of peptides from untreated plasma appeared to be difficult. After approximately 1 min of EME, the electrical current flowing in the system increased, and bubble formation due to electrolysis at the electrodes and vaporization of the acceptor solution were observed. The sudden increase in current was probably caused by a puncture in the SLM. Plasma is a very complex matrix with a high content of proteins, ionic compounds, and emulsifying agents, and most probably, the puncture was caused by partial dissolution of the SLM in plasma. This observation is consistent with former research on EME from untreated plasma [22].

Derived from these experiences, plasma was diluted to pH 3 with HCl (2.5 times dilution, see experimental section) prior to EME. This pH was chosen based on experience from earlier research [23]. Three different organic phases were chosen from Table 1 as possible SLMs for the extraction of angiotensins from diluted plasma. 1-Octanol:DEHP:4-nitro-m-xylene:di-isobutyl ketone (40:10:25:25), and DEHP:4-nitro-m-xylene:di-isobutyl ketone (10:50:40) were selected based on their

Table 2
Composition of three new organic phases.

SLM nr.	1-Octanol (%)	DEHP (%)	4-Nitro-m-xylene (%)	Di-isobutyl ketone (%)
1	42	8	25	25
2	–	8	52	40
3	92	8	–	–

high extraction recoveries, while 1-octanol:DEHP (85:15) had been central in the former experiments on EME of peptides and was therefore also included in the experiment. Before extraction with the three phases, some precautions were taken. In order to reduce the current flowing in the system, the voltage was reduced to 15 V, and the concentration of DEHP in the organic phase was reduced to 8% (Table 2). The reduction in voltage and DEHP-concentration resulted in lower current, but also lower extraction recoveries. To circumvent this limitation, the extraction time was increased from 5 to 10 min. The results from EME of angiotensins from diluted plasma were compared to EME from pure water. As shown in Table 3, the SLM with 1-octanol and 8% DEHP (SLM nr. 3) gave the highest recoveries from diluted plasma (25–43%). Although the recoveries obtained from diluted plasma were significantly lower than from pure water samples, the first results on EME of angiotensins from plasma were highly promising. Further experiments were required to optimize the system with regard to voltage and extraction time, and 1-octanol and 8% DEHP (SLM nr. 3) was selected as the SLM for the rest of the study.

During extractions from plasma, a number of endogenous enzymes, like angiotensin-converting enzyme (ACE), may alter the concentration of Ang 1 and its metabolites [30,31]. The addition of the specific ACE-inhibitor captopril to plasma will block the cleavage of Ang 1 to Ang 2. The importance of ACE-inhibition during EME of Ang 1, Ang 2, and Ang 3 from diluted plasma was investigated. The extraction recovery of the angiotensins from plasma containing captopril was compared with the extraction recovery from plasma lacking captopril. No significant difference in the extraction recoveries was seen (data not shown). During further EME evaluation, captopril was not added to plasma.

3.3. Effect of voltage and time

In order to find the optimal potential, the voltage applied across the SLM was varied between 5 and 20 V. For all the three angiotensins the recovery increased up to 15 V (18–30%). No gain in recovery was observed when the voltage was increased to 20 V, hence 15 V was used for the rest of the study.

In a next experiment, the extraction recovery from plasma samples was studied as a function of time with 15 V applied across the SLM. As shown in Fig. 2, the recoveries increased rapidly as a function of time for the first 600 s (up to 20–40% depending on the peptide). With extraction times above 600 s, the recoveries decreased. The possible cause for this is probably the instability of the electrical current in the system. Fig. 3 shows the current plotted against the extraction time for EME of angiotensins in plasma (trace

Table 3
Extraction recovery of Ang 1, Ang 2, and Ang 3 after 10 min of EME.

SLM nr.	Sample	Recovery (%)[a]			Sample	Recovery (%)[a]		
		Ang 1	Ang 2	Ang 3		Ang 1	Ang 2	Ang 3
1	Water	70	52	70	Plasma	13	11	16
2	Water	33	13	50	Plasma	8	4	13
3	Water	65	55	67	Plasma	36	25	43

Electrical potential: 15 V; extraction time: 10 min; agitation: 900 rpm; 50 mM HCl in acceptor solution. Each peptide was present at 10 μg/mL in the sample.
[a] $n = 3$.

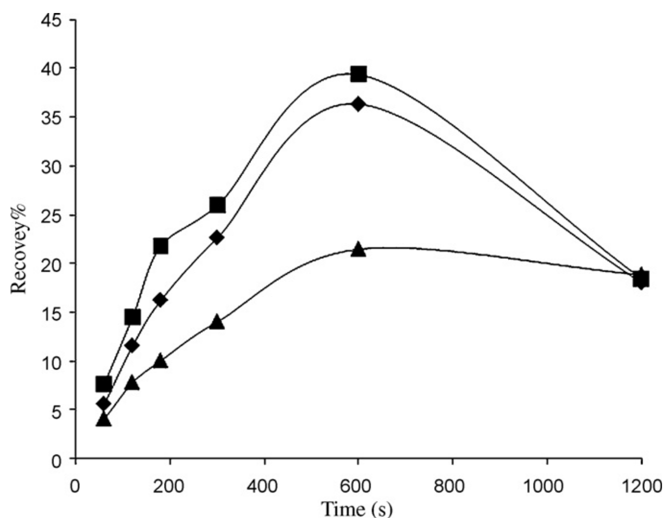

Fig. 2. Effect of time on EME recovery of Ang 1 (■), Ang 2 (▲), and Ang 3 (♦). Electrical potential: 15 V; agitation: 900 rpm; 50 mM HCl in acceptor solution. Organic solvent: 1-octanol + 8% DEHP. Each peptide was present at 1 μg/mL in a diluted plasma sample.

A) and water (trace B). As seen in Fig. 3, for plasma samples there was a sudden increase in current after approximately 800 s of EME. The increased current resulted in an unstable EME system, with bubble formation and partial evaporation of the acceptor solution. In contrast, EME from a pure water sample showed stable current for the whole extraction time (1200 s/20 min). This indicated that when EME was conducted from diluted plasma, as in contrast to from pure water, the SLM did not remain stable for extraction times above 800 s. The reason for this was probably due to the difference in matrix. Although literature describes that it is possible to overcome the membrane instability problems caused by matrix composition [22], more research is needed before the same procedure can be conducted on EME of peptides. For the rest of this study, 10 min (600 s) was chosen as an optimal extraction time. During all the extractions from diluted plasma, the SLM remained stable for at least 10 min, with a current output in the range 150–250 μA.

3.4. Stability of angiotensin peptides during electromembrane extraction

It is important that the peptides are stable during EME and that they are not degraded when there is a high electrical current in the system. Any degradation will have a negative influence on the extraction recovery. The EME stability was tested at three different voltages (in the absence of a SLM, see Section 2.2). The voltages were chosen to give a high diversity in the current output of the system. When the lowest voltage (0.1 V) was applied, there was no bubble formation tendency, and only a very low current was observed (5 μA). When the voltage was increased to 4.9 V, some bubble formation was observed together with an increase in the current (200 μA) flowing in the system. At the highest voltage applied (7.5 V), the current increased up to 450 μA, and there was extensive bubble formation. The results from CE-analysis showed no peptide degradation in any of these experiments (data not shown). Thus, the angiotensins appeared to be stable under the electrochemical conditions used during EME.

The stability of angiotensins in the low pH range has been tested before [32]. Based on these results, it was assumed that the peptides were stable under the experimental conditions used in this study as well.

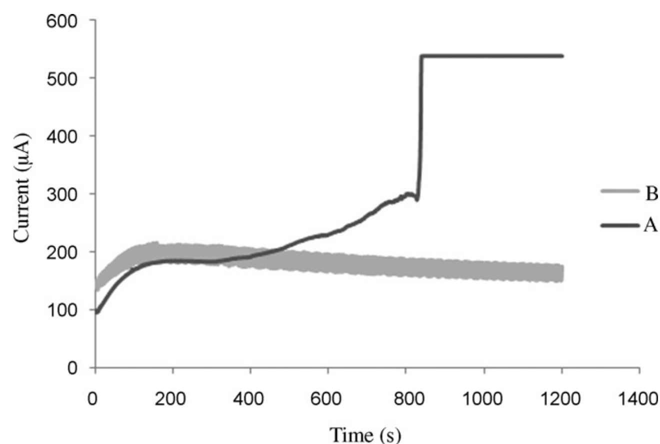

Fig. 3. The current as a function of extraction time obtained with EME from diluted human plasma (A) and from an aqueous sample (B). Electrical potential: 15 V; agitation: 900 rpm; 50 mM HCl in acceptor solution. Organic solvent: 1-octanol + 8% DEHP. Each peptide was present at 1 μg/mL.

Table 4
Recovery and repeatability of EME of Ang 2 from plasma.

Plasma concentration (ng/mL (*n*))	Recovery (%)	Repeatability (RSD)
2.5 (6)	23	5.6
12.5 (3)	26	
25.0 (6)	24	11.6
62.5 (3)	24	
125.0 (6)	30	9.4

Electrical potential: 15 V; extraction time: 10 min; agitation: 900 rpm; 50 mM HCl in acceptor solution. Organic solvent: 1-octanol + 8% DEHP.

3.5. Evaluation of EME of angiotensin 2 from plasma after optimization

The results from the optimization experiments discussed above showed that the optimal conditions for EME of angiotensins from plasma were obtained utilizing 1-octanol containing 8% DEHP as the SLM, 50 mM HCl in the acceptor solution, a sample solution containing plasma diluted 2.5 times with HCl to pH 3, 15 V potential difference, and 10 min extraction time.

In order to evaluate the extraction of angiotensins from human plasma by EME, Ang 2 was spiked to plasma in concentrations ranging from 2.5 to 125.0 ng/mL. The extraction system was evaluated with regard to recovery, repeatability, and linearity. As shown in Table 4, Ang 2 migrated into the acceptor solution with recoveries in the range 23–30%. The recoveries were comparable to the extraction recoveries of Ang 2 obtained in the optimization experiments (Table 3). It should be stressed that experiments in Table 4 were carried out at much lower concentrations (2.5–125.0 ng/mL) compared to those used in Table 3 (10 μg/mL). This indicated that the extraction recovery of Ang 2 was not influenced by high concentration.

To evaluate the performance of EME of Ang 2 from spiked plasma, linearity was investigated in the range 2.5–125.0 ng/mL. As shown in Table 5, linearity was obtained with r^2-value 0.989. The repeatability ($n = 6$) was determined at three different concentration levels (2.5, 25.0, and 125.0 ng/mL). As demonstrated in Table 4, the relative standard deviations (RSD) were between 5.6 and 11.6%.

Table 5
Linearity of Ang 2 after 10 min of EME from plasma.

Linearity	Range (ng/mL) r^2
2.5–125.0	0.989

Electrical potential: 15 V; extraction time: 10 min; agitation: 900 rpm; 50 mM HCl in acceptor solution. Organic solvent: 1-octanol + 8% DEHP.

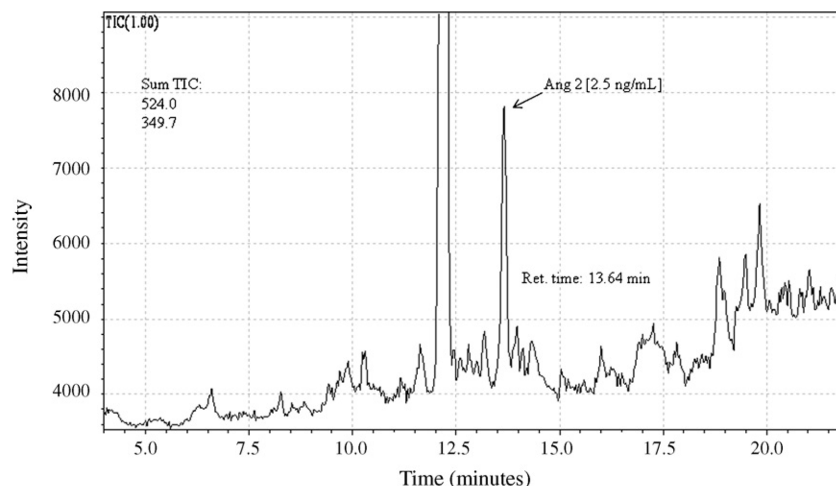

Fig. 4. LC–MS chromatogram of Ang 2 after electromembrane extraction from spiked plasma. Extraction time 10 min.

Compared to values reported for miniaturized analytical extraction procedures [6] and earlier evaluation data on EME [13,22], the repeatability was satisfactory. Taking into consideration that the experiments were performed with home-built equipment, both the linearity and RSD-values were acceptable.

In Fig. 4, the LC–MS chromatogram after 10 min of EME of Ang 2 from spiked plasma is illustrated. Considering that the experiments were performed with single MS and selected ion monitoring (SIM), the acceptor solutions after EME were relatively clean. The estimated detection limit (S/N ratio of 3:1) for Ang 2 was determined to be 240 pg/mL, and the estimated quantification limit (S/N ratio of 10:1) was determined to be 800 pg/mL with the current EME-LC–ESI-MS system. Although these values were in the pg/mL-range, they were still above the endogenous concentration of 10–60 pg/mL [33]. Further research on EME, in combination with a more sensitive detector, for example a triple quadrupole (LC–MS/MS), will probably enable extraction and detection (and quantification) of endogenous Ang 2.

4. Conclusions

In the current paper, extraction of peptides from human plasma by electromembrane extraction (EME) has been demonstrated for the first time. The peptides were extracted as net cationic species from a plasma sample, across a supported liquid membrane (SLM) sustained in the pores of a porous hollow fiber, and into an acceptor solution placed in the lumen of the hollow fiber by application of 15 V across the SLM. Using plasma as sample matrix, the stability of the system was found to be an important issue, and dilution of the samples was required in order to avoid puncture of the SLM. Also, proper selection of the SLM as well as of the applied potential was crucial to avoid excessive current in the system, which again resulted in electrolysis and bubble formation in the aqueous compartments. Taking these precautions, EME provided efficient extractions within 10 min. Excellent sample clean-up was obtained, and the method evaluation indicated that the system provided reliable results. The promising initial results presented in this paper suggest that further research should be directed towards EME of peptides in the future.

References

[1] J.Å. Jönsson, L. Mathiasson, Trends Anal. Chem. 11 (1992) 106.
[2] J.Å. Jönsson, L. Mathiasson, Trends Anal. Chem. 18 (1999) 318.
[3] J.Å. Jönsson, L. Mathiasson, Trends Anal. Chem. 18 (1999) 325.
[4] J.Å. Jönsson, L. Mathiasson, J. Chromatogr. A 902 (2000) 205.
[5] J.Å. Jönsson, L. Mathiasson, J. Sep. Sci. 24 (2001) 495.
[6] S. Pedersen-Bjergaard, K.E. Rasmussen, Anal. Chem. 71 (1999) 2650.
[7] K.E. Rasmussen, S. Pedersen-Biergaard, Trends Anal. Chem. 23 (2004) 1.
[8] K.E. Rasmussen, S. Pedersen-Bjergaard, M. Krogh, H.G. Ugland, T. Gronhaug, J. Chromatogr. A 873 (2000) 3.
[9] S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. B 817 (2005) 3.
[10] T.S. Ho, S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 963 (2002) 3.
[11] T.S. Ho, T. Vasskog, T. Anderssen, E. Jensen, K.E. Rasmussen, S. Pedersen-Bjergaard, Anal. Chim. Acta 592 (2007) 1.
[12] T. Grønhaug Halvorsen, S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. B 760 (2001) 219.
[13] S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 1109 (2006) 183.
[14] B.A.P. Buscher, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 788 (1997) 165.
[15] L. Firdaous, P. Dhulster, J. Amiot, A. Gaudreau, D. Lecouturier, R. Kapel, F. Lutin, L.P. Vezina, L. Bazinet, J. Membr. Sci. 329 (2009) 60.
[16] F. Steiner, P. Bohn, Chromatographia 58 (2003) 59.
[17] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1174 (2007) 104.
[18] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1124 (2006) 29.
[19] M. Balchen, A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1152 (2007) 220.
[20] T.M. Middelthon-Bruer, A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Sep. Sci. 31 (2008) 753.
[21] I.J.O. Kjelsen, A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1180 (2008) 1.
[22] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, Anal. Bioanal. Chem. 393 (2009) 921.
[23] M. Balchen, L. Reubsaet, S. Pedersen-Bjergaard, J. Chromatogr. A 1194 (2008) 143.
[24] N. Visser, H. Lingeman, H. Irth, Anal. Bioanal. Chem. 382 (2005) 535.
[25] V. Kasicka, Electrophoresis 29 (2008) 179.
[26] K.J.M. Abesundara, M. Higuchi, T. Matsui, K. Matsumoto, Anal. Sci. 18 (2002) 467.
[27] M.K. Sim, X.S. Qui, Regul. Pept. 111 (2003) 179.
[28] I. van den Broek, R.W. Sparidans, J.H.M. Schellens, J.H. Beijnen, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 872 (2008) 1.
[29] C.-Y. Lin, S.-D. Huang, Anal. Chim. Acta 612 (2008) 37.
[30] V.F. Fredline, E.M. Kovacs, P.J. Taylor, A.G. Johnson, Clin. Chem. 45 (1999) 659.
[31] J.R. Voelker, S.L. Cobb, R.R. Bowsher, Clin. Chem. 40 (1994) 1537.
[32] J.L.E. Reubsaet, H. Loftheim, A. Gjelstad, J. Sep. Sci. 28 (2005) 1204.
[33] N.W. Tietz, Clinical Guide to Laboratory Tests, W.B. Saunders Co., Philadelphia, 1995.