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Determination of a basic drug, bambuterol, in human plasma by capillary electrophoresis using double stacking for large volume injection and supported liquid membranes for sample pretreatment

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Abstract

In this work we show the potential of using a double stacking procedure based on field enhancement as a means to increase the concentration sensitivity in CZE analysis of human plasma extracted by the supported liquid membrane (SLM) technique. A basic drug, bambuterol, was used as a model substance. The low ionic strength of the SLM extract makes this pretreatment technique compatible with the double stacking sequence. No significant loss of separation performance was observed when 3 μl of SLM extract was concentrated by the CZE double stacking sequence. Almost no visible difference was seen between the electropherograms after enrichment of a plasma blank and an aqueous blank. Good performance of the whole procedure was demonstrated and detection limits in the low n*M* range were obtained in spite of the relatively weak UV absorbance of bambuterol. The developed procedure was evaluated for both achiral and chiral separation. In the latter approach chiral selectivity was obtained by adding cyclodextrin to the separation electrolyte.

Keywords: Bambuterol

1. Introduction

The high resolving power, possibility to reach short analysis times and ease of operation makes capillary zone electrophoresis (CZE) an attractive separation technique. CZE is now accepted as an important analytical tool for the determination of substances with widely varying properties. Biological samples are however difficult to analyze by CZE due to their complexity, capillary adsorption problems, relatively high salt concentrations and often low concentrations of the analytes.

The small optical path length involved using on-column UV detection imposes an inherent detection problem. The detection limit of CZE can be improved either by using more sensitive detection methods such as laser-based fluorescence detection [1], electrochemical detection [2] or mass spectrometry [3] or by performing an on-line preconcentration step. In previous work [4] we introduced double stacking as an on-capillary preconcentration step for cationic analytes. This procedure allowed for filling the whole CZE capillary with sample (about 3 μl) without significant loss of separation perform-

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ance. In this way the detection limit for the model substance could be lowered about 400 times compared to conventional 7-nl injection.

Although some work has been published recently [5] on direct injection of biosamples for CE analysis this has a limited use. In most cases only a few nl are injected, leading to high detection limits when using UV detection. More often when biological samples are analyzed. a clean-up procedure is necessary for elimination of interfering matrix components before the CE determination. This is especially important if some kind of sample stacking is applied to increase concentration sensitivity. This demands clean extracts with low ion content for good separation performance.

The most frequently used pretreatment methods for biological samples are liquid–liquid solvent extraction, solid-phase extraction (SPE) or protein precipitation [6]. Liquid–liquid solvent extraction often gives a good clean-up from the matrix but is laborious and difficult to automate and the use of large amounts of organic solvents should also be avoided for environmental and health reasons. Another approach based on the efficient clean-up of liquid–liquid extraction, utilizing minimal amounts of organic liquid, is the use of supported liquid membranes (SLM). The SLM technique [7] offers very selective extraction of analytes from complex biological matrices such as plasma and urine [8,9]. Both basic and acidic compounds can be feasibly enriched. The pH of the sample is adjusted so that the analytes become uncharged. This permits them to pass through an organic liquid immobilized in a porous membrane into an aqueous solution having a pH such that the analyte molecules become ionized and are therefore prevented from re-entering the membrane.

In a recent publication [10] we showed that it is possible to use SLM as a clean-up and enrichment step before CZE determination of bambuterol in human plasma. Bambuterol is a prodrug of terbutaline, a drug commonly used in the treatment of asthma. In that application 120 nl of the SLM pretreated plasma could be successfully injected using stacking without too much band broadening.

In this work we evaluate the possibility of using double stacking (3 μl injection) [4] in the CZE analysis of SLM treated human plasma in order to decrease the detection limit of bambuterol towards low nmol/l concentrations which are encountered in clinical use of bambuterol. The developed procedure was evaluated for both achiral and chiral separation. In the latter approach, chiral selectivity was obtained by adding cyclodextrin to the separation electrolyte.

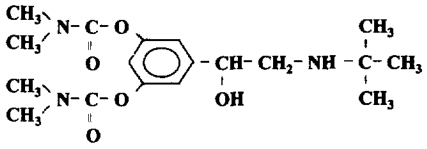
2. Experimental

2.1. Chemicals

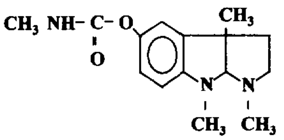
The membrane liquid 6-undecanone was obtained from Janssen Chimica (Geel, Belgium) and the porous PTFE (polytetrafluoroethene) membrane (Model TE35) from Schleicher and Schuell (Dassel, Germany). Racemic bambuterol (hydrochloride) was obtained from Astra Draco (Lund, Sweden) and the esterase inhibitor physostigmine from Sigma (St. Louis, MO, USA). Their structures are shown in Fig. 1. 2,6-Dimethyl-β-cyclodextrin was obtained from Sigma. All other chemicals were of analytical reagent grade from Merck (Darmstadt, Germany). All water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Buffers and standard solutions

Phosphate buffers were prepared from H_3PO_4 and the sodium salts of $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} by



Bambuterol



Physostigmine

Fig. 1. Structures of the model compound and inhibitor used in this work.

mixing appropriate concentrations to give the right pH value. Daily preparation of the low concentration phosphate buffers was necessary to avoid changes in pH values.

Standard solutions of bambuterol were prepared in water and were stable for at least one month when kept at room temperature protected from light. A 1 m*M* standard solution of the esterase inhibitor physostigmine was prepared in 0.9% NaCl solution. The solution was stable for at least two weeks when kept in a refrigerator and protected from light.

2.3. Plasma samples

Blank plasma was obtained from blood that was collected into Na-heparinized Venoject tubes (10 ml). The tubes were turned upside down seven times and then immediately centrifuged for 10 min at 1400 *g*. All samples were stored, frozen (−18°C). Before analysis, plasma samples were thawed and centrifuged for 10 min at 2000 *g*.

2.4. SLM extraction

The SLM set-up is shown in Fig. 2. The membrane unit was connected to the valve of a Model 233 XL sample processor with a Model 401 syringe pump (Gilson Medical Electronics, Villiers-le-Bel, France) used to process the plasma samples. The membrane unit was machined from blocks of PTFE by cutting two grooves in a U-shaped fashion. The membrane was clamped between the blocks, forming one channel on each side of the membrane. The channels were 1.5 mm wide, 0.15 mm deep and 160 mm long, giving a nominal volume of 36 μl. The porous membrane was cut to fit the membrane unit, immersed for 15 min in the organic solvent and mounted in the membrane unit. Before use, water was passed through both channels to wash out excess solvent from the membrane surface.

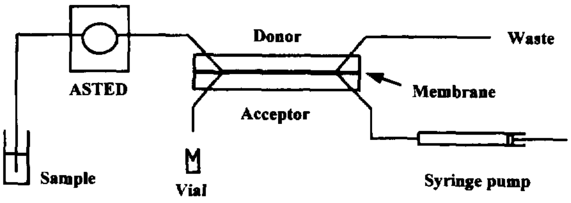


Fig. 2. Set-up for supported liquid membrane extraction.

To prevent hydrolysis of bambuterol, 5 μl of the esterase inhibitor physostigmine was automatically added to 500 μl blank plasma before spiking it with 100 μl of appropriate bambuterol standard solutions. Finally 395 μl of 0.3 *M* phosphate buffer of pH 11.1 were added to give pH 10.7 in the plasma mixture. After mixing, 900 μl of the plasma mixture was pumped with the sample processor syringe pump at a rate of 18 μl/min through the donor channel of the membrane unit. Bambuterol is a moderately polar secondary amine [11] with a pK_a value of 9.6 and is degraded in strong alkaline solutions. At pH 10.7, the degradation rate is still slow and ca. 90% of the molecules are uncharged. The uncharged bambuterol molecules diffuse through the membrane liquid into the stagnant acidic acceptor phase, where they are protonated and trapped. The acceptor phase consisted of 10% methanol and 1 m*M* H_3PO_4 giving a pH of 3.1.

After enrichment the acceptor phase was pumped by means of a CMA/100 microinjection pump (CMA/Microdialysis, Stockholm, Sweden) into a vial fitting the autosampler of the capillary zone electrophoresis instrument used for the final determinations. Totally 50 μl were pumped, i.e. the nominal volume of the acceptor channel plus an additional 14-μl aliquot to ensure complete transfer of the extract to the vial and 50 μl methanol were then added to the vial to make the sample more compatible with the double stacking procedure [4].

After each enrichment the needle and the sampling loop of the sample processor instrument were washed with 8 ml water, the donor channel of the membrane unit was washed with 10 channel volumes of water and the acceptor channel with 10 channel volumes of acceptor phase.

2.5. CZE double stacking procedure

CZE was performed using a Prince programmable injector for capillary electrophoresis supplied with autosampler and high-voltage power supply (Prince Technologies BV, Emmen, Netherlands) using a Spectra 100 UV-Vis detector (Spectra-Physics, Mountain View, CA, USA) for on-capillary UV detection at 205 nm. CZE was performed in 70 cm effective length (78 cm total length) untreated fused-silica capillaries, 75 μm I.D. and 375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA). The solutions in the autosampler were kept at 20°C but no cooling was applied to the capillary. Data analysis and collection were accomplished with a Compaq Deskpro 4/33i computer, using System Gold software (Beckman, Palo Alto, CA, USA), version 712.

Before each run the capillary was washed, with four column volumes of 0.1 *M* NaOH, water, methanol and electrolyte solution respectively. The capillary was stored filled with methanol between working days but dried with air if the system was not used for longer periods.

During all stacking and separation steps a positive voltage was applied at the capillary inlet and the analyte cations migrated towards the cathode because of the net effect of the electrophoretic and electroosmotic mobilities.

The Prince system is equipped with a facility which allows for applying a back-pressure which is used in the different steps to either balance the electroosmotic flow or to pump the stacked band to the inlet part of the capillary.

The double stacking principle is described in detail in Ref. [4]. In the beginning of the procedure the CZE capillary was filled with 5 m*M* phosphate buffer pH 7.5. Next the capillary was filled with the SLM extracted plasma sample (200 mbar for 2.85 min) almost to the detection window (3 μl). The first stacking procedure was then carried out by applying a voltage of 30 kV for 10 min, while at the same time a back-pressure of 68 mbar was applied. The back-pressure was used to oppose the electroosmotic flow and thus to prevent the stacking analyte from moving too fast towards the outlet of the capillary and allowing some of the first bulk electrolyte solution to remain in the capillary end. At the end of this step all cationic sample components have been collected in a short band near the detection window. The stacked peak was now forced back to the injection end of the capillary by introducing 0.1 *M* phosphate buffer pH 2.5 into the outlet end of the capillary. This was done by applying a back-pressure of 180 mbar for 1.7 min to the system after turning off the high voltage. This resulted in more than half of the original sample volume being pushed out of the capillary. The second stacking step is now carried out, with the 0.1 *M* phosphate buffer pH 2.5 now present at both the outlet end and inlet vial, by applying 30 kV over the capillary. The positive species will now stack up at the concentration boundary between the 0.1 *M* buffer and the 5 m*M* buffer (mixed with methanol solution) and the band broadening obtained after transportation of the previously stacked sample will be compensated for. While the stacking is in progress, a back pressure of 150 mbar is used to push the methanol containing band out of the capillary. After 2 min the separation voltage is lowered to 23 kV to prevent too much Joule heating in the system. When the current indicates that most of the high-resistant solution has been pushed out of the capillary (80 μA), the back-pressure was switched off. The final separation of the stacked compounds is now carried out. If a chiral separation of the enantiomers was performed 3.9 m*M* dimethyl-β-CD was added to the 0.1 *M* phosphate buffer of pH 2.5.

3. Results and discussion

3.1. Performance of the SLM-CZE double stacking system

The ionic strength of the sample solution is the most critical factor when applying double stacking successfully. However, a higher methanol concentration in the sample plug allows for analysis of samples having higher ionic strength [4]. The possibility of getting a clean extract from the SLM treatment with low ionic strength makes this sample pretreatment technique compatible with the double stacking procedure.

Electropherograms resulting from SLM extraction followed by CZE analysis utilizing the double stacking procedure are shown in Fig. 3. As can be seen, there is very little difference between plasma samples and aqueous samples. This signifies a high degree of clean-up of the plasma samples which have been concentrated more than thousand times (ca. 400 times by the double stacking procedure and ca. three times by the SLM treatment). Thus, the extraction is highly selective for bambuterol. The bambuterol peak (B) is clearly seen and can be used to estimate a detection limit of about 4 n*M*. The peak labeled A was also found in blank aqueous solution and was concluded to be a contamination in the Milli-Q water. Peak C is the esterase inhibitor physostigmine.

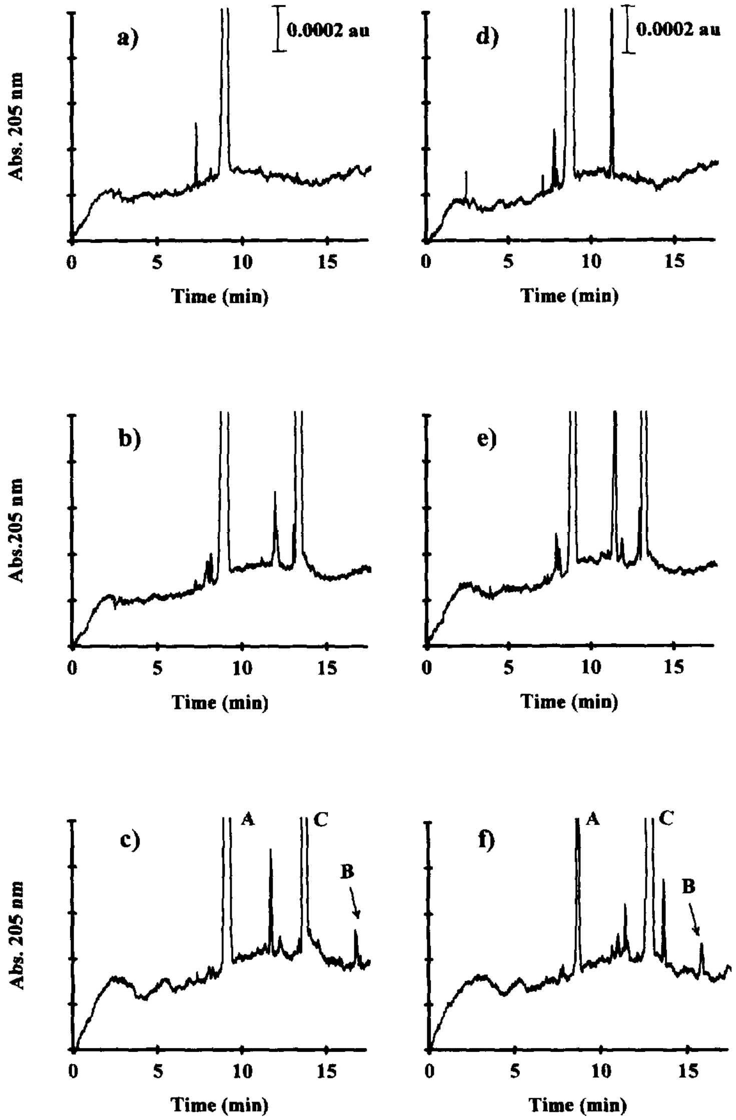


Fig. 3. Electropherograms of aqueous samples (a,b,c) and plasma samples (d,e,f) after enrichment with SLM technique. The electropherograms show blank sample (a,d), sample containing 10 μ*M* physostigmine (b,e) and sample containing physostigmine and 8 n*M* racemic bambuterol (c,f). Peak C is the physostigmine inhibitor and peak B is bambuterol. The membrane extraction conditions were the following: flow-rate at donor side, 18 μl/min; donor pH=10.7, acceptor pH=3.1. In all cases, the 50-μl membrane extract was diluted with 50 μl methanol before CZE analysis using double stacking. The double stacking procedure used is described in Section 2.

The inhibitor is unstable and gives rise to several extra peaks in the electropherogram.

Fig. 4 illustrates electropherograms of the same samples as in Fig. 3 using dimethyl-β-cyclodextrin as chiral selector in the final separation step. The bambuterol enantiomer peaks labeled B are clearly seen. The concentration level of 4 n*M* of each enantiomer is close to the detection limit. The cyclodextrin concentration had a great effect on the migration time of physostigmine and its degradation products. Careful adjustment of the cyclodextrin concentration was thus necessary to avoid peak overlapping (see Fig. 4). The physostigmine peak is obviously strongly complexed with the cyclodextrin

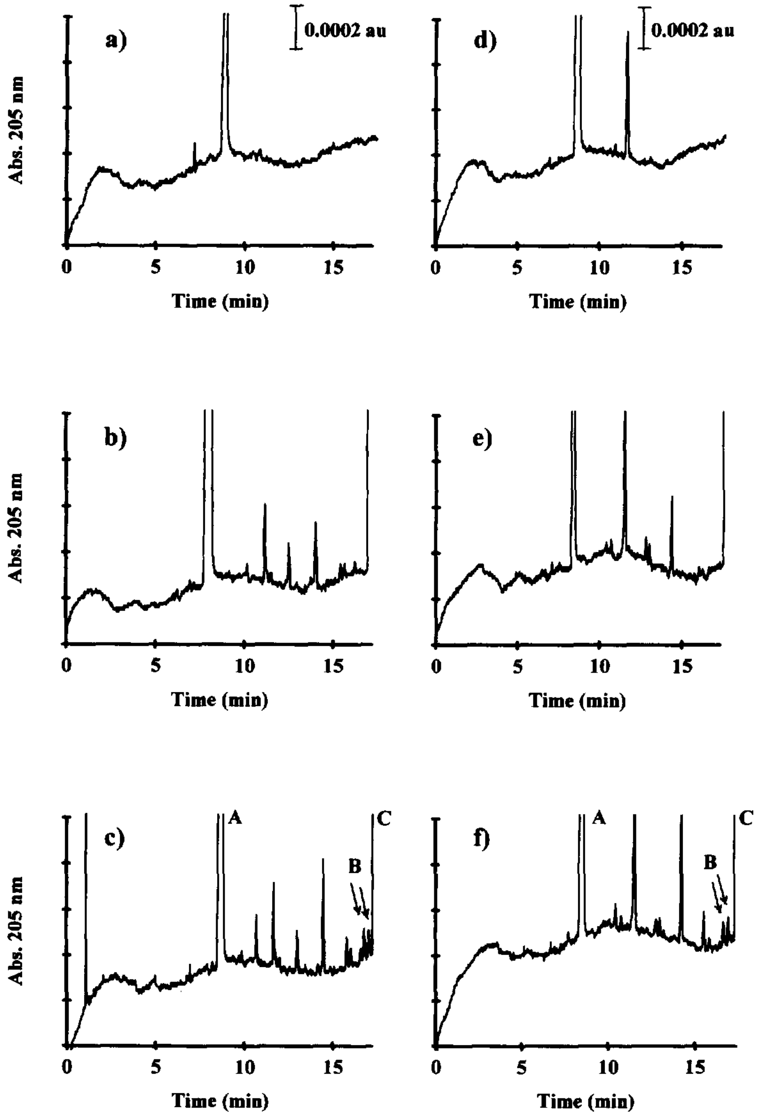


Fig. 4. Electropherograms of aqueous samples (a,b,c) and plasma samples (d,e,f) after enrichment with SLM technique. The electropherograms show blank sample (a,d), sample containing 10 μ*M* physostigmine (b,e) and sample containing physostigmine and 8 n*M* racemic bambuterol (c,f). Conditions as in Fig. 3 except for the stacking electrolyte 2 and separation electrolyte which now also contained 3.9 m*M* dimethyl-β-cyclodextrin to obtain a chiral separation of the bambuterol enantiomers. Peak C is the physostigmine enantiomer and peaks labeled B are the bambuterol enantiomers.

molecule. As shown in a previous study [12], the cyclodextrin concentration is an important factor for tuning the selectivity of the separation. Comparison of dimethy-β-cyclodextrins from different manufacturers revealed a large difference in selectivity at the same concentrations. Different selectivity was also obtained between batches of the same cyclodextrin. This is clearly a complication when using cyclodextrins in routine analysis and became a very important factor in this study. Baseline resolution of the bambuterol enantiomers was not accomplished at the same cyclodextrin concentration for the three different batches tried and a new optimization of the cyclodextrin concentration had to be performed to avoid peak overlapping.

3.2. Linearity and reproducibility

Calibration curves in plasma based on peak area and double injections at six different concentrations were made for both achiral and chiral separation of racemic bambuterol (concentration range, 4–128 n*M*). All curves showed good linearity and the intercepts did not differ significantly from zero. For the achiral separation the correlation coefficient (*r*) was 0.9984 and the confidence interval at the 95% level was −28±138 for the intercept and 29±2 for the slope (arbitrary units). For the chiral separation the correlation coefficient was 0.9999 for both enantiomers and for enantiomer a and enantiomer b, the confidence intervals at the 95% level were −11±18 and 1±12 for the intercept and 30±1 and 30.0±0.4 for the slope, respectively.

The repeatability for the achiral separation based on peak area of five samples was, calculated as relative standard deviation, ca. 10% at 8 n*M* concentration of bambuterol and ca. 5% at 32 n*M* and 64 n*M* concentration. For the chiral separation, the repeatability for five samples was ca. 10% at 4 n*M* concentration and ca. 5% at 32 n*M* concentration for both enantiomers. A study of the reproducibility was carried out on three different days with a new membrane installed in the membrane holder each day. For 15 samples (5 each day) at 32 n*M* concentration of bambuterol the relative standard deviation was ca. 8% for the achiral determination.

The high degree of clean-up from the plasma matrix by the SLM extraction resulted in samples that did not cause any capillary adsorption problems in the CZE capillary. The same capillary could be used for weeks without problems using the washing steps described above. The main complication in the CZE step was the unstable surface conditions of the capillary wall resulting in variation in the electroosmotic flow velocity. Especially if the capillary had been unused for some time it took some effort to equilibrate the capillary surface. This was usually done by running the system with the washing procedure in-between for 1–2 h. Occasionally it was even necessary to slightly adjust the back-pressure used to oppose the difference in the electroosmotic flow. The most stable conditions were definitely obtained when running the system without interruption over a long period of time.

The pH and ionic strength of the sample obtained from the SLM extraction was of great importance with regard to successful double stacking. Slight changes in the pH of the extracted sample affected the electroosmotic flow in the system which however could be adjusted for by changing the back-pressure in the first stacking step.

A part of the variation between runs probably originates from differences in sample composition after the SLM treatment. The addition of methanol to the treated sample had to be done with care to decrease any risk of evaporation.

3.3. Extraction efficiency and extraction time of the SLM treatment

Using the conditions described above with a flow-rate of 18 μl/min, the extraction efficiency of the SLM treatment was around 65% and a 900-μl sample (450 μl plasma) was processed within 50 min. As short analysis times are always of interest we studied the effect of flow-rate and the effect of dilution of the plasma sample on the extraction efficiency. Fig. 5 shows the effect on extraction efficiency when the flow-rate was increased in steps from 18 μl/min to 58 μl/min. Line (a) represents a plasma sample that has been diluted as described above. Line (b) represents a less diluted plasma

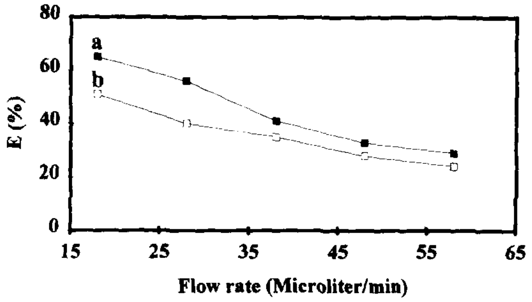


Fig. 5. The dependency of the extraction efficiency (E) on donor flow-rate. Line (a) represents a plasma sample diluted 1:1 with donor solution and line (b) represents a plasma sample diluted 1:0.26 with donor solution. Other conditions as presented in Fig. 3.

sample with 5 μl physostigmine, 50 μl bambuterol standard and 75 μl 0.3 *M* phosphate buffer of pH 12.3 mixed with 500 μl plasma. At a flow-rate of 18 μl/min it takes ca. 31 min to enrich a sample containing 450 μl plasma.

As can be seen from the figure, the extraction time could be shortened to little more than 30 min, still obtaining more than 50% extraction efficiency, either by extracting the less diluted plasma at a flow-rate of 18 μl/min or by extracting the more diluted plasma at a flow-rate of 28 μl/min. The limiting step in the system will then be the double stacking CZE analysis which takes about 40 min. The time for this analysis can however be decreased down to the same level as the SLM procedure by shortening the double stacking procedure taking into account that more than 60% of the analyte [4] is recovered in the first minute of the first stacking step which was carried out for 10 min. Another way to shorten the CZE analysis is to use a shorter capillary which would shorten all steps in the double stacking sequence at the expense of higher detection limits.

4. Conclusion

The results presented in this work demonstrate that the supported liquid membrane technique can provide clean low ionic strength plasma extracts compatible with the double stacking CZE procedure. Good performance of the whole procedure was demonstrated and detection limits in the low n*M* range were obtained for the basic drug bambuterol in spite of its relatively weak UV absorbance.

Further work will focus on the on-line combination of the SLM technique with the double stacking CZE analysis either directly or via a micro-CLC interface. Work will also be focused on improving the overall productivity to make the approach more suitable for routine work. The direct combination of the SLM technique with the CZE system is facilitated by the recent development of a miniaturized membrane holder in our laboratory [13]. These approaches will allow for the injection of the entire extracted sample into the CZE capillary.

This work thus demonstrates that by combining the SLM technique for sample preparation with double stacking for enhancement of sensitivity, conventional CZE equipment can be used in bioanalysis at low concentrations.

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