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On-line supported liquid membrane–liquid chromatography with a phenol oxidase-based biosensor as a selective detection unit for the determination of phenols in blood plasma

J. Norberg, J. Emnéus, J.Å. Jönsson*, L. Mathiasson, E. Burestedt, M. Knutsson[1], G. Marko-Varga2

Department of Analytical Chemistry, University of Lund, Box 124, 221 00 Lund, Sweden

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Abstract

The potential of on-line combination of supported liquid membrane extraction and column liquid chromatography with a phenol oxidase-based biosensor as a selective detection unit has been investigated for the determination of phenols in human plasma. The phenols are selectively extracted into a porous PTFE (polytetraflouroethene) membrane impregnated with a water-immiscible organic solvent and further into an alkaline acceptor phase. Via an ion-exchange interface, the analytes are transferred to a reversed-phase column where they are separated and detected using the biosensor. No sample pretreatment before the extraction, except centrifugation, is made. Due to the high selectivity both in the extraction and in the detection steps and to the fact that the demands on the chromatographic separation are low, a quick separation using an eluent with a low concentration of organic modifier can be made, without affecting the biosensor response. Detection limits below the 50 μg/l level in blood plasma were obtained for the three model compounds, phenol, *p*-cresol and 4-chlorophenol. © 1997 Elsevier Science B.V.

Keywords: Phenols; Phenol oxidase; Supported liquid membrane extraction; Biosensors

1. Introduction

Traditional detection principles for liquid chromatography, such as UV-photometry, refractometry and electrochemical systems lack in selectivity. This is especially important when analyzing samples at low concentrations in complex matrixes. One way to achieve the required high selectivity and sensitivity is the application of biospecific detection systems. The selectivity of the detection is then governed by the biochemical recognition reaction which is transduced by an appropriate physical transducer. Biochemical components such as enzymes, plant cells, animal cells, tissue slices, micro-organisms, chemoreceptors, antigens or antibodies have been used in the construction of biospecific detection systems.

At our research group, a number of different biosensors have been developed for the detection of

*Corresponding author.

[1]Present address: Ferring AB, Pharmaceutical Analysis Research and Development, Box 30047, 200 61 Malmö, Sweden.

[2]Present address: ASTRA DRACO AB, Preclinical Research and Development, Department of Bioanalytical Chemistry, Box 34, 221 00 Lund, Sweden.

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phenols in environmental water [1–8]. These amperometric biosensors, modified with the enzyme tyrosinase, operate within the optimal potential range (around 0 mV) of electrochemical measurements. At this potential, the contribution to the amperometric signal from other easily oxidisable and reducible species is low, leading to low noise and background currents. A number of different electrode configurations have been studied [1–8] among which the batch-modified carbon paste electrodes (CPE) offer some advantages over traditional solid electrodes.

For the study of human exposure to aromatic hydrocarbons, there is considerable interest in determining their metabolites, mainly phenols and unsaturated carboxylic acids, in blood and in urine [9]. However, irrespectible of the detection technique, this requires a chromatographic separation and an efficient clean-up procedure of the biological matrix. One efficient technique for this is the supported liquid membrane (SLM) extraction technique. It has been used in combination with liquid chromatography (HPLC) [10–12] and capillary zone electrophoresis (CZE) [13–16] as a selective clean-up and pre-concentration step for various analytes in biological fluids. No difference is usually seen between an aqueous blank solution and an authentic blank after SLM extraction [11].

Jönsson and Mathiasson have summarized some of the SLM-applications in a review paper [17]. The technique is based on a porous PTFE membrane, impregnated with a water-immiscible organic solvent placed between two aqueous phases, the donor and the acceptor. The analytes should be kept uncharged and extractable in the donor and charged and non-extractable in the acceptor. For the extraction of phenols and other acids, the pH in the donor should be kept below the pK_a, so that the analytes are in uncharged form. Then they are extracted from the aqueous donor phase into the organic membrane and diffuse across the membrane into the acceptor, where the pH is above the pK_a-value of the phenols. Therefore these will be charged and thereby trapped in the acceptor. With a flowing donor and a stagnant acceptor, a very selective enrichment of the analytes is achieved [17,18].

In the present work, a tyrosinase CPE biosensor was coupled on-line following extraction using the supported liquid membrane (SLM) technique and reversed-phase liquid chromatographic separation. This principle was applied to the determination of three model phenolic compounds (phenol, *p*-cresol and 4-chlorophenol) in human plasma. The major challenge was the incompatibility of the three different techniques. The high pH necessary to trap the phenols in the membrane acceptor phase and the organic modifier necessary for the chromatographic separation are incompatible with the optimum pH and stability of the biosensor. This was solved by the use of an interface based on the use of a small anion-exchange column for the retention of hydroxide ions and rendering the phenols in a neutral state before introduction into the chromatographic separation column. Additionally, a short separation column made it possible to use only a low concentration of organic modifier in the mobile phase, still obtaining sufficient separation and short retention times.

2. Experimental

2.1. Preparation of the biosensor–carbon paste electrode (CPE)

Unmodified carbon paste was prepared by thoroughly mixing 100 mg of graphite powder (Fluka, Buchs, Switzerland) and 40 μl of paraffin oil (Fluka) to a homogeneous paste in an agate mortar. It was packed into the tip of a 1-ml plastic syringe (tip I.D. 0.85 mm, ONCE, ASKI, Rødby, Denmark). The outer 3–4 mm of the syringe tip was left empty to be filled with tyrosinase modified carbon paste. This was prepared by mixing 4 mg of tyrosinase (3900 U/mg, lyophilised powder, Sigma Chemical, St. Louis, MO, USA) with 100 mg of graphite powder in an agate mortar for 5 min. To this mixture was then added 50 mg of octadecane (Fluka) and put into an oven at 37°C for 10 min. After the melting of the octadecane, the mixture was thoroughly mixed in the agate mortar for another 15 min until a homogeneous paste was formed. The syringe tip was thereafter filled with the modified paste and the surface of the syringe was polished on a glass surface and then gently rubbed on a paper to obtain a smooth surface. When not in use, the electrodes were stored at 4°C in dry state.

2.2. Apparatus

For flow injection (FI) measurements the biosensor was inserted into a confined wall jet flow through amperometric cell as was described earlier [3].

For SLM extraction, a modified Model 231 sample preparation processor (Gilson Medical Electronics, Villiers-le-Bel, France) with two Gilson 401 dilutors and a six port injection valve (Model 7010, Rheodyne, Cotati, CA, USA) was used (see Fig. 1). A manipulation in the program setup of the sample preparation processor, setting the volume of the syringe to 10 ml instead of 1 ml, makes it possible to vary the donor flow-rate between 0.018 and 9.60 ml/min. A 20×2.1 mm I.D. precolumn was slurry-packed in the laboratory with Dowex 2 X-8 (Dow, Chemical, Midland, MI, USA) ion-exchanger.

The holder for the supported liquid membrane, shown in Fig. 2, was made by the faculty work-shop. This consisted of two titanium blocks with identical machined grooves having the dimension 0.15×2.0×40 mm^3, forming channels with a nominal volume of 12 μl each. A porous PTFE membrane (Model TE 35, pore size 0.2 μm, Schleicher and Schuell, Dassel, Germany) was soaked in *n*-hexyl ether (Sigma) for at least 15 min before it was placed between the two blocks, which were tightly clamped together with six bolts. Before use, a few ml of the donor and acceptor solutions were passed through the channels in order to wash out excess solvent from the membrane surface. The acceptor solution, 0.1 *M* sodium hydroxide, was prepared from sodium hydroxide (p.a) (Eka Nobel, Bohus, Sweden) and reagent water, purified with a Milli-Q-RO4 system (Millipore, Bedford, USA) which also was used for the preparation of all other aqueous reagents and as the donor solution.

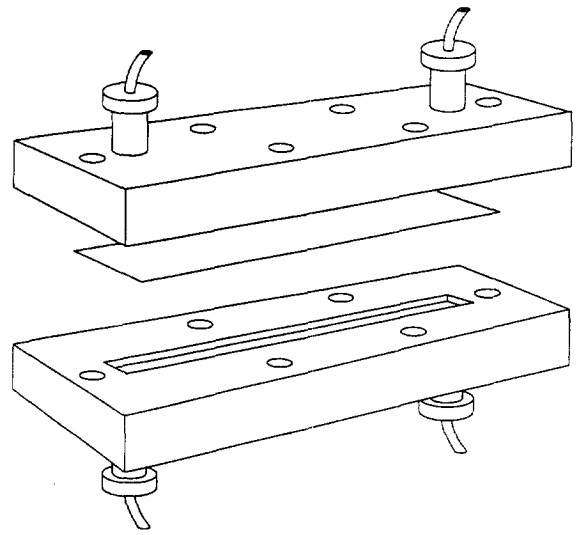

Fig. 2. Membrane unit. The PTFE-membrane is placed between the two blocks made in titanium. The two channels (donor and acceptor) that are formed have a nominal volume of 12 μl (from Ref. [10] with permission).

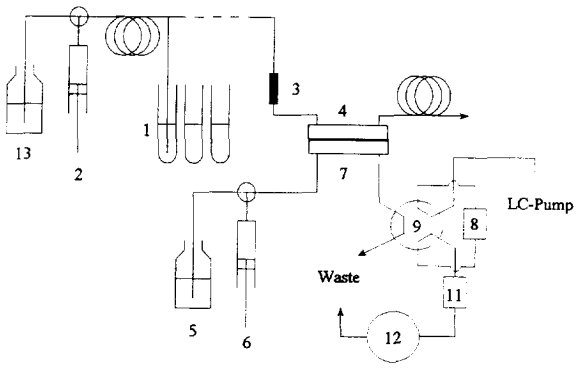

Fig. 1. Setup of the analytical system, consisting of a rack of vials (1); a syringe pump for delivery of sample and donor solution (2); an injection port (3); a membrane unit with a donor (4) and an acceptor channel (7); a plastic flask contaning the acceptor solution (5) with corresponding syringe pump (6). A precolumn packed with strong anionic-exchange resin (8) is placed on a high-pressure valve (9). By valve switching the sample is introduced to the analytical column (11). After chromatography, the analytes are detected by the biosensor (12). After completed extraction the donor channel is washed with donor solution (13) (=reagent grade water).

The LC system consisted of a pump (Model 2150, LKB, Bromma, Sweden), and a short 2 cm×1 mm I.D. analytical column slurry-packed in the laboratory with LiChrosorb C-18 RP-100 (5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of a mixture of a 50 m*M* phosphate buffer, pH 6, with an addition of sodium chloride (Merck) and methanol (gradient grade, Merck).

The detector was the biosensor in a wall jet flow through amperometric cell, described previously [3].

In some experiments, where stated, this device was replaced with a UV detector (Waters Model 440, Milford, MA, USA).

2.3. Analytes and samples

The analytes studied were phenol, *p*-cresol and 4-chlorophenol (all obtained from Merck), at least 98% pure. Stock solutions, 1000 mg l^{-1}, were made in methanol. From the stock solutions, a 10 mg l^{-1} mixture was made and used for preparing aqueous solutions and for plasma spiking. The solutions were stored, well sealed, in darkness at 4°C. Human plasma, obtained from the University Hospital in Lund, was weighed in centrifugation tubes before storage in the deep freezer at −20°C. It was never stored more than one month. Before analysis, the plasma was thawed over night in a refrigerator at 4°C, and then centrifuged. No other sample pretreatment, except spiking, was made before the samples were transferred into the vials of the rack of the sample processor.

2.4. Analytical procedure

Using a 108 positions rack, programming the robot system gives the opportunity of a fully automated analysis of the same number of vials each containing 1 ml of sample. In Fig. 1, the operation of the system can be seen. The robot arm is programmed to choose one vial (1) and 1.0 ml of sample is sucked into the needle by one of the syringe pumps (2). Then the needle is moved to the injection port (3), where the sample is pressed into the donor channel (4) of the membrane unit. The uncharged phenols are extracted into the liquid membrane and subsequentially re-extracted into the acceptor channel, where they are deprotonated by a stagnant 0.1 *M* NaOH solution, and thus irreversiblly trapped. When this step is completed, 20 μl of the acceptor solution (5) is pressed with the other syringe pump (6), out of the acceptor channel (7) and onto an ion-exchanger column (8), placed on a high-pressure valve (9). The precolumn is then switched into the eluent stream of the LC-system, the analytes are separated on the analytical column (11) and finally, selectively detected with the biosensor (12). During the chromatographic step, both channels of the membrane unit are flushed with acceptor (5) and donor solution (13) respectively, and a new sample preparation cycle begins. The system has been set up with equal operation times for chromatography and sample preparation (including flushing, robot-arm movement, valve switching etc.), so that one sample is chromatographed while the next one is extracted. All parts of the system are fully automated and controlled by the sample preparation processor software package (Gilson).

3. Results and discussion

3.1. Optimization of the biosensor

The optimum pH of the previously developed tyrosinase-modified biosensors were all in the range 6–6.5 [1–8], which is similar to the optimum value when the enzyme is dissolved in solution [19]. Based on these findings the optimum pH was not further investigated and pH 6.0 was used for all experiments.

The response of the biosensor decreases with flow-rate, as previously shown for other tyrosinase biosensors [4]. This reflects the actions of both the enzymatic step and the electrochemical step where the enzymatic step is rate limiting. By decreasing the flow-rate, the mass transport of the substrate to the electrode surface is slower. However, this makes it possible for the enzyme to react with the substrate during a longer period of time, resulting in higher overall response currents.

3.2. Stability of biosensor response

In the current work, a slightly modified electrode configuration was developed by using a harder carbon paste material, resulting in better stability of the biosensor in comparison with earlier designs [3,4], which was also shown by Petit and Kauffmann [20]. After 100 injections of catechol, the response of the tyrosinase biosensor decreased about 25%, which took approximately 3.5 h in flow injection analysis. This indicates that when the biosensor is fully integrated in the sequential SLM–LC–biosensor setup, the stability should be sufficient.

For organic–aqueous mixtures up to 10% (v/v) of

methanol, a stable biosensor response was obtained for the first 3 h. However, there was a pronounced decrease in response with time when the methanol content was increased to 15% (v/v). The organic modifier may affect the enzyme in a detrimental way resulting in slower enzyme kinetics.

3.3. Optimization of the membrane extraction

The pH in the donor and acceptor phases are important parameters. To obtain complete extraction, the pH of the acceptor must be well above the pK_a of the analytes. The pK_a of the phenols used in this work ranges between 9.38 and 10.26 [21]. Therefore, as acceptor solution, 0.1 *M* NaOH, i.e. pH 13, was used. The analytes showed sufficient stability over an 8-h period in this rather extreme pH.

The donor pH is not very critical, but it should be less than the pK_a to insure that a large portion of the analytes are protonated and thereby uncharged and possible to extract into the organic membrane liquid. Since blood plasma is naturally buffered to a pH of 7.4 [22] no pH adjustment is needed.

The influence of different donor flow-rates on the extraction efficiency (E), was investigated by preconcentrating a 500 μg/l phenol mixture in reagent water. Since a long term stability is demanded for those experiments, the biosensor was replaced by UV detection at 280 nm. The extraction efficiencies for the three analytes decreased with donor flow-rate and followed the familiar pattern of flow dependence well documented in earlier publications [17,18].

3.4. Interface

The high pH needed to irreversibly trap the analytes in the acceptor is not compatible with either reversed-phase chromatography or biosensor detection. To solve this problem, the analytes were first loaded onto an anion-exchange precolumn from which they were eluted with the mobile phase in their neutral state.

The performance of two different anion-exchange packing materials packed in two different precolumn bodies, 20×1 mm I.D. and 20×2.1 mm I.D. were investigated. Parameters of interest were capacity and band broadening. When using 20–50 mesh packing material, sufficient capacity was obtained only for the larger precolumn. The band broadening was acceptable. With the 200–400 mesh packing material the smaller precolumn was never tested since it gave rise to a high pressure incompatiable with the syringe pump available. The capacity of the larger precolumn was sufficient, but a retention of the analytes in the column caused unacceptable band broadening. Consequently, for the final system the 2 mm I.D. precolumn was used, packed with 20–50 mesh packing material.

3.5. Intergrated SLM–LC–interface–biosensor system

The fully intergrated system is depicted in Fig. 1. Separation was carried out by reversed-phase LC using a mobile phase consisting of methanol and a 10 m*M* sodium phosphate buffer in a 0.9% (w/v) sodium chloride solution. The pH was 6.0 to obtain a high response from the biosensor (see Section 3.1). Since the flow-rate influences the response of the biosensor, the use of a low flow-rate was preferred. To combine a fast separation with a low flow-rate, a miniaturized analytical column was used. The separation at a flow-rate of 0.1 ml/min on a 20×1 mm I.D. column was investigated using different methanol contents in the mobile phase (0–10%). Fig. 3 shows typical chromatograms after SLM extraction of spiked blood plasma and a plasma blank, trapping on the ion-exchange column and reverse phase chromatography followed by biosensor detection. The peak shapes are not very attractive, but the separation is sufficient considering the use of a very selective detector and the constraints of flow-rate and mobile phase composition. It is suspected that approximately 7 μg/l phenol and 4 μg/l *p*-cresol are present in the blank plasma sample. Furthermore, an unknown compound (U) is found, which due to the selectivity of the biosensor should be a phenol.

Binding of analytes to proteins in blood plasma is a problem with many available sample handling techniques [23]. For the SLM approach, this influences the extraction efficiency, as shown in Table 1. Lindegård et al. [11] has shown that a major part of the binding of analytes to protein are broken by the concentration gradient over the SLM and that it is probably possible to evaluate protein binding constants with the SLM technique [11].

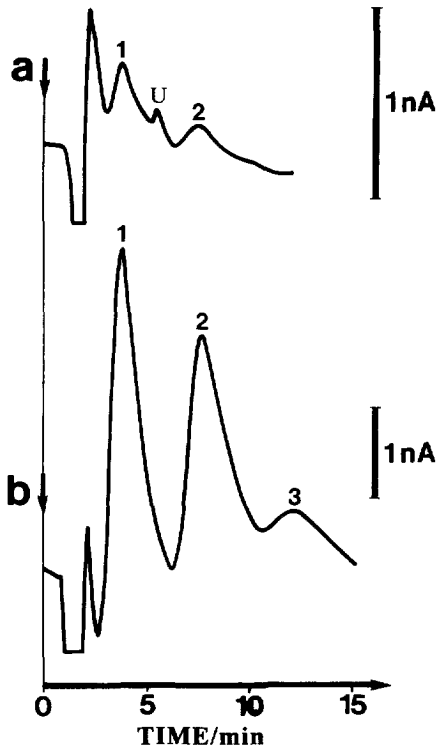

Fig. 3. Chromatogram after on-line SLM extraction of plasma sample at a donor flow-rate of 0.15 ml/min using the biosensor as selective detection unit. Peaks: phenol (1), *p*-Cresol (2) and 4-chlorophenol (3), unknown (U). (a) Plasma blank; (b) plasma, spiked to 100 μg/l with the phenol mixture.

Fig. 4 shows the stability of the response for *p*-cresol in the fully integrated system with different methanol concentrations. The other model compounds show similar patterns.

3.6. Linearity and limit of detection

The response of a similar biosensor was found to be linear with concentration. The extraction efficiency of the membrane extraction in the concentration range of interest, is also largely concentration independent. Furthermore, the protein binding may be considered constant in the concentration range of interest, since the concentration of proteins in plasma (for albumin 35–55 g/l [24]) is much larger than the concentration of analytes (<200 μg/l). However, the fact that the stability of this particular biosensor is poor compared to other LC detectors has to be considered for the linearity study. Table 2 shows the linear correlation, in the 50–150 μg/l range, for SLM extraction of phenols in blood plasma followed by trapping and separation as described above with the biosensor replaced by an UV detector. Centrifuged plasma was spiked with different concentrations of a mixture of the three model compounds and analysed in random order.

By extracting and analysing a similar sample containing the three phenols, each one, 100 μg/l, using the complete system with the biosensor for detection, the limits of detection (LOD) were estimated. LOD (Table 2) was calculated as three times the baseline noise. The LOD values vary over more than one order of a magnitude mainly due to the different response of the biosensor to various phenols.

Table 1
The difference in extraction efficiencies (with 95% confidence intervals) between extractions of the phenol mixture (100 μg/l) in reagent grade water and in human plasma. Donor flow-rate 0.15 ml/min

Matrix	Phenol	*p*-Cresol	4-Chlorophenol
Reagent water	0.123±0.004	0.169±0.005	0.226±0.007
Plasma	0.104±0.003	0.076±0.002	0.065±0.001

4. Conclusion

From the chromatograms and tables presented it is evident that the technique can be applied to blood plasma samples at phenol concentrations in the low μg/l level. Preliminary data show that the method might be extended to phenolic compounds other than the three compounds studied here, although different response factors for different phenols may be a problem.

In this study, no further sample pretreatment, except centrifugation, has been used since the goal is a fast and simple method for the determination of phenolic compounds in biological fluids.

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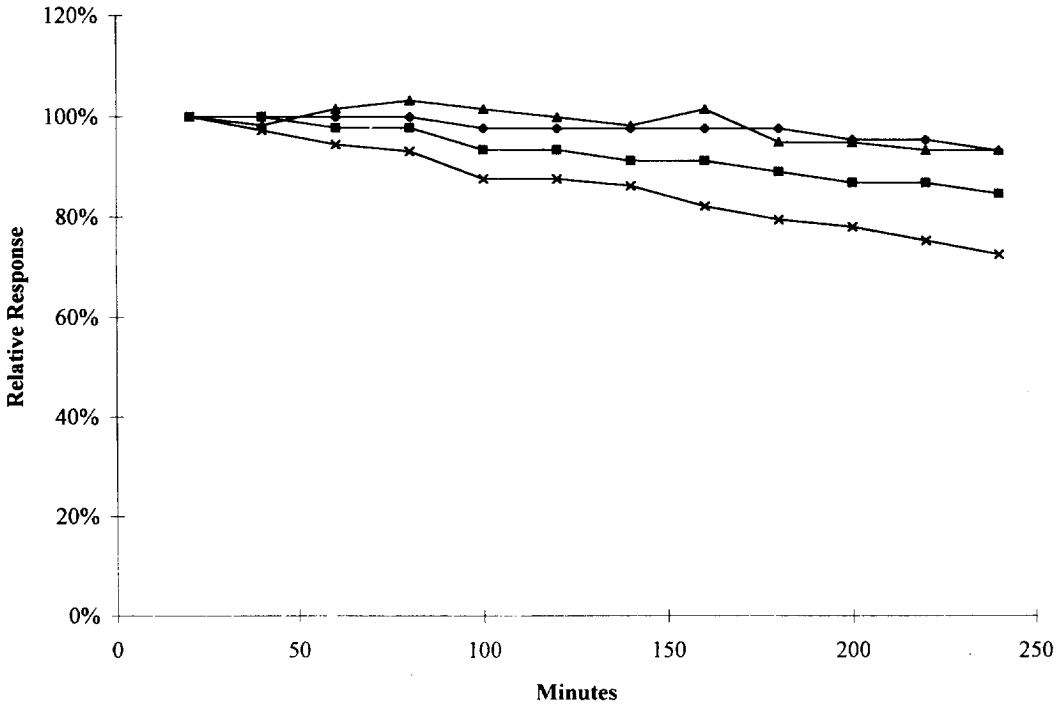

Fig. 4. Stability of the biosensor response after chromatography using different eluent compositions, containing 0% (♦), 5% (■), 10% (▲) and 15% (×) methanol. The relative response as a function of time is plotted for *p*-cresol, which shows a typical pattern for the three model compounds.

Table 2
Linearity and limit of detection (LOD) of phenols in blood plasma

Compound name	r^{2a}	Intercept[b]	LOD (μg/l)
Phenol	0.9988	1±3	2.6
p-Cresol	0.9998	−5±5	4.0
4-Chlorophenol	0.9997	1±1	42.8

Peak heights, obtained from SLM–LC–UV are plotted vs. spiked concentrations in the range 50–150 μg/l (n=3). The LOD values are three times the standard deviation obtained with SLM–LC–biosensor measurements.

[a] The square of the Pearson's product moment correlation coefficient

[b] 95% confidence interval.

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