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Development of a simple in-vial liquid-phase microextraction device for drug analysis compatible with capillary gas chromatography, capillary electrophoresis and high-performance liquid chromatography

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Abstract

A simple, inexpensive and disposable device for liquid-phase microextraction (LPME) is presented for use in combination with capillary gas chromatography (GC), capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). 1–4 ml samples of human urine or plasma were filled into conventional 4-ml vials, whereafter 15–25 μl of the extraction medium (acceptor solution) was filled into a short piece of a porous hollow fiber and placed into the sample vial. The drugs of interest were extracted from the sample solutions and into the small volumes of acceptor solution based on high partition coefficients and were preconcentrated by a factor of 30–125. For LPME in combination with GC, the porous hollow fiber was filled with 15 μl *n*-octanol as the acceptor solution. Following 30 min of extraction, the organic acceptor solution was injected directly into the GC system. For LPME in combination with CE and HPLC, *n*-octanol was immobilized within the pores of the hollow fiber, while the internal volume of the fiber was filled with either 25 μl of 0.1 *M* HCl (for extraction of basic compounds) or 25 μl 0.02 *M* NaOH (for acidic compounds). Following 45 min extraction, the aqueous acceptor solution was injected directly into the CE or HPLC system. Owing to the low cost, the extraction devices were disposed after a single extraction which eliminated the possibility of carry over effects. In addition, because no expensive instrumentation was required for LPME, 10–30 samples were extracted in parallel to provide a high number of samples per unit time capacity.

Keywords: Liquid-phase microextraction; Extraction methods; Hollow fibers; Benzodiazepines; Amphetamines; Nonsteroidal anti-inflammatory drugs

1. Introduction

Drug analysis in human urine and blood plasma is often complicated by low analyte concentrations (0.1–10 ng/ml level), by complex sample matrices, and by limited sample volumes available for the determinations (normally 0.5–1 ml for plasma). Because of this, sample preparation is crucial in drug analysis and includes both analyte preconcentration and sample clean-up. Sample preparation is traditionally carried out by liquid–liquid extraction (LLE) or by solid-phase extraction (SPE), while the final analysis in most cases is accomplished by either high-performance liquid chromatography (HPLC) or capillary gas chromatography (GC).

In LLE, typically 0.5–1 ml of a biological sample

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is extracted by a similar volume of an organic solvent. In a subsequent step, most of the solvent is evaporated or the sample is back-extracted into a new aqueous phase to preconcentrate the analyte. For practical reasons, the final volume containing the analytes normally has to exceed 50 μl and consequently, the analytes are typically preconcentrated by a factor of less than 10–20 prior to the final chromatographic analysis. Alternatively, the sample preparation may be carried out by SPE. With this technique, typically 0.5–1 ml of a biological sample is loaded onto the SPE column and subsequently the analytes are eluted by at least two bed volumes of eluent. For 100 mg SPE columns, this eluent volume corresponds to approximately 250 μl, and consequently, the analytes are preconcentrated by a factor of only 2 to 4 unless a final evaporation is carried out.

In several cases, preconcentrations of 2–20-times as discussed above are sufficient; drugs may be present at relatively high concentration levels or may be analyzed by HPLC with a high sensitivity because the complete extract may be injected into the separation system. For GC and CE in contrast, only a small fraction of the sample (e.g., 1 μl and 10 nl, respectively) is normally injected and in these cases, higher preconcentration factors may be required to detect and quantitate the analytes of interest. In addition, some drugs and their metabolites are present at very low concentration levels (below 1 ng/ml) requiring high analyte preconcentrations regardless of the separation technique in use.

Obviously, alternative sample preparation methods for drug analysis is of high interest which are capable of high analyte preconcentration. Basically, the problem may be solved by either increasing the volume of sample extracted (from e.g., 1 to 10 ml) or by reducing the volume of the final extract (from e.g., 50 to 5 μl). While the former concept is of little interest in drug analysis due to strongly limited volumes of sample material, the latter solution involving microextraction is of high interest. Currently, most microextraction is carried out by solid-phase microextraction (SPME), and SPME has been evaluated for the determination of drugs in both human urine and plasma [1–20]. The technique is commercially available, totally solvent-free, and is capable of extracting drugs automatically prior to GC analysis. Potentially, high preconcentration factors are possible for hydrophobic drugs, but the technique has not been widely implemented in the field of drug analysis.

Recently, we developed a microextraction device based on a liquid microphase for CE [21]. The idea of using a liquid microphase for extraction was to increase the sample capacity as compared with SPME and consequently to enhance analyte preconcentration from the biological samples. The concept was based on a 4-ml vial containing a small piece of a porous polypropylene hollow fiber with the liquid microphase and was developed into a simple, inexpensive and disposable device. In the present work, this liquid-phase microextraction (LPME) concept is further developed into a device compatible with GC, HPLC and capillary electrophoresis (CE).

2. Experimental

2.1. Liquid-phase microextraction

The principle of the disposable LPME device is illustrated in Fig. 1. The sample solution was filled into a 4-ml vial with a screw top/silicon septum (Supelco, Bellefonte, PA, USA). Two conventional 0.8 mm O.D. medical syringe needles (guiding needles) were inserted through the silicon septum in the screw top and the two ends were connected to each other by a 4 or 8 cm piece of Q3/2 Accurel

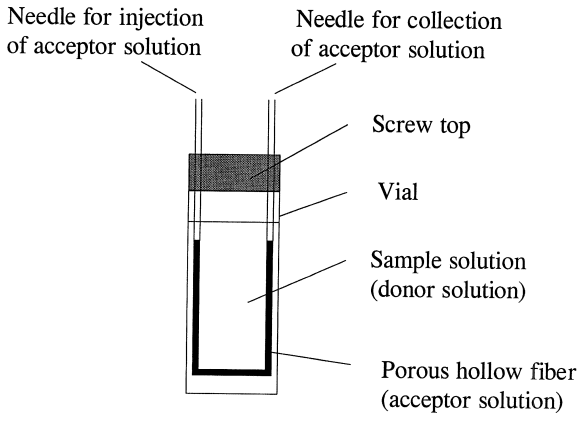

Fig. 1. Schematic illustration of a LPME extraction unit.

KM polypropylene hollow fiber (Akzo Nobel, Wuppertal, Germany). The latter served to contain the μl volume of extracting solution (acceptor solution). 4-cm pieces were used in combination with capillary GC, while the length of the hollow fiber was 8 cm in cases of CE or HPLC. The inner diameter of the hollow fiber was 600 μm, the thickness of the wall was 200 μm, and the pore size was 0.2 μm.

For extraction in combination with GC, the hollow fiber was filled with 15 μl of *n*-octanol. This was easily accomplished by a μl syringe through one of the guiding needles. Subsequently, the hollow fiber was placed in the sample solution present in the 4-ml vial. During extraction, each extraction unit was effectively vibrated (1000 rpm) for a 30 min period utilizing a Vibramax 100 vibrator (Heidolph, Kelheim, Germany). For extraction in combination with CE or HPLC, the hollow fiber mounted on the guiding needles was first dipped for 5 s into a small beaker with *n*-octanol to immobilize the solvent in the pores. Subsequently, excess *n*-octanol was removed by 15 s of ultra sonication in a water bath, and 25 μl of the aqueous acceptor solution was filled into the hollow fiber by a μl syringe. Following this, the fiber was placed in the sample and extraction was performed for 45 min supported by vibration (1000 rpm) as for the GC experiments. After extraction, the acceptor solutions were either withdrawn by a GC or HPLC syringe for manual injection in GC or HPLC, or the acceptor solutions were collected in microvials by application of a small head pressure on one of the guiding steel needles for automated analysis by GC or CE.

2.2. Capillary gas chromatography

Capillary GC was performed with a Varian Star 3400 CX gas chromatograph equipped with a Varian 8200 CX GC autosampler and a nitrogen–phosphorus detection (NPD) system (Varian, Walnut Creek, USA). Separations were performed in a 30 m×0.25 mm, 0.25 μm SPB-1 polydimethylsiloxane column (Supelco). 99.998% helium (AGA, Oslo, Norway) was used as carrier gas at a flow-rate of 1.0 ml/min. The NPD system was operated at 250°C. Injections were performed in the splitless mode at 300°C. The oven temperature was programmed from 180°C (1 min) at 20°C/min to 300°C.

2.3. Capillary electrophoresis

CE was performed with a MDQ instrument (Beckman, Fullerton, CA, USA) equipped with a UV detector. Separations were performed in a fused-silica capillary of 40 cm (effective length 30 cm)×75 μm I.D. (Beckman). 25 m*M* sodium phosphate adjusted to pH 2.75 with phosphoric acid was utilized as the running buffer for the basic drug, while the acidic drug was separated with a separation buffer consisting of 30 m*M* acetate adjusted to pH 4.75 with acetic acid. Samples were introduced by hydrodynamic injection at 0.5 p.s.i. for 5 s (1 p.s.i.= 6894.76 Pa). Detection was accomplished at 200 nm for the basic drug and at 214 nm for the acidic drug utilizing a 800×100 μm slit.

2.4. High-performance liquid chromatography

HPLC was accomplished with a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7010 injector (Rheodyne, Berkeley, CA, USA) equipped with a 20-μl loop, and a Shimadzu RF-551 fluorescence detector. Separations were accomplished with a HyPurity Elite C_{18} column (Hypersil, Cheshire, UK) and a mobile phase of acetonitrile–5 m*M* ammonium phosphate (30:70, v/v). The flow-rate of the mobile phase was 1 ml/min. Excitation was accomplished at 249 nm and emission was measured at 302 nm.

2.5. Reagents and standards

Diazepam, prazepam and naproxen were obtained from Sigma (St. Louis, MO, USA). Methamphetamine was purchased from Norsk Medisinaldepot (Oslo, Norway). Citalopram and *N*-desmethylcitalopram were obtained from H. Lundbeck (Copenhagen, Denmark). Sodium phosphate, sodium hydroxide, sodium acetate and hydrochloric acid (all analytical grades) were obtained from Merck (Darmstadt, Germany). *n*-Octanol (99%) was from Sigma.

2.6. Calculation of preconcentration factors and extraction efficiencies

The preconcentration factor (PF) was defined as the ratio between the final analyte concentration

$C_{a,final}$ in the sample extract (acceptor phase) and the initial concentration of analyte $C_{s,initial}$ within the sample:

$$PF = C_{a,final}/C_{s,initial} \quad (1)$$

The preconcentration factor was determined by peak area measurements and by calibration with standard solutions containing the drugs at a 100-fold higher concentration level than in the samples. The extraction efficiency (EE) was defined as the percentage of the total analyte amount $n_{s,initial}$ (originally present in the sample) which was transferred to the extract (acceptor phase) at the end of the extraction ($n_{a,final}$):

$$EE = (n_{a,final}/n_{s,initial}) \cdot 100\% = (V_a/V_s)PF \cdot 100\% \quad (2)$$

where V_a and V_s are the volumes of acceptor solution (extracting phase) and sample solution (donor solution), respectively. The extraction efficiency was determined from PF, V_a and V_s as shown in Eq. (2).

3. Results and discussion

3.1. Basic principle

The basic concept of the disposable LPME device is illustrated in Fig. 1. The sample solution (donor solution) was filled into a conventional 4-ml vial. The vial contained a small piece of a porous polypropylene hollow fiber with 15–25 μl of the extraction medium (acceptor solution). The experimental conditions were adjusted to promote a high analyte solubility within the acceptor solution while the solubility was suppressed within the donor solution by an appropriate pH adjustment; owing to the porous nature of the hollow fiber, the analytes penetrated the hollow fiber from the donor solution and were preconcentrated within the acceptor solution. The hollow fiber was connected to two steel needles placed in the screw cap of the vial; the needles were utilized for introduction and collection of the acceptor solution. The acceptor solution was easily filled into the hollow fiber with a μl syringe through one of the steel needles. The final collection of the acceptor solution was accomplished by pressurizing one of the steel needles or by removal with a μl syringe. During the extraction, each LPME device was placed in a vibrator to effectively promote analyte preconcentration within the acceptor solution; vibration served to continuously replenish both the donor and acceptor solutions close to the wall of the porous hollow fiber.

The price of each extraction unit was low and comparable with the price an SPE column. Thus, each LPME device was utilized only for a single extraction. The disposable nature of LPME totally eliminated both the possibility of sample carry-over effects and the need for regeneration of the porous hollow fiber. The latter aspect served to save time and maintained a high reproducibility because the chemistry of each extraction device was not affected by previous use. Owing to the low price and the simplicity of the technology, it was possible to operate a large number of extraction units in parallel; frequently we extracted 10–30 samples simultaneously.

3.2. Selection of hollow fiber

Because the extraction units should be compatible with both aqueous solutions and a broad range of organic solvents, polypropylene was selected as the material for the porous hollow fiber. Commercially available hollow fibers with an internal diameter of 600 μm were appropriate for the μl volumes of acceptor solution used in the present work. The wall of the hollow fibers were relatively thick (200 μm) which simplified the preparation of the extraction units; the mechanical stability of the hollow fibers was excellent and the fibers were easily connected to the two syringe needles of the simple extraction unit. A pore size of 0.2 μm was selected to ensure efficient penetration of small molecules (analytes).

Commercially available vials (4 ml) with screw cap were utilized for the extraction units. Two medical needles were penetrated through each screw cap and were connected to the ends of the porous hollow fiber; one served to guide a μl syringe for introduction while the other was utilized for collection of the acceptor solution after extraction. For the GC and HPLC experiments, the acceptor solution was collected by a μl syringe followed by direct injection into the GC/HPLC systems. For CE, the

acceptor solution was collected in micro-vials by application of a small head-pressure on one of the steel needles, whereafter the micro-vials were placed in the sample tray for automated CE analysis. With the LPME extraction units, no expensive instrumentation was required; in addition to the extraction units, LPME was accomplished with a μl syringe (for GC or HPLC), an inexpensive 2.5-ml medical syringe (to flush the acceptor solution into micro-vials), and a vibrator with a multi-sample tray.

3.3. LPME for capillary GC

For the compatibility studies of LPME with capillary GC, the two benzodiazepines diazepam and prazepam were selected as model compounds. For successful LPME–GC, the acceptor solution in the LPME device should be an organic solvent capable of direct injection. The solvent should be selected to provide a high solubility for the analytes of interest (good extraction solvent) and for practical reasons, it is an additional advantage to utilize a solvent of relatively low volatility. Based on these considerations, *n*-octanol was utilized as acceptor solution in the present work for the extraction of diazepam and prazepam [10]. A 15-μl volume of *n*-octanol was introduced into the porous hollow fiber by a μl syringe and this solvent served both to fill the pores of the polypropylene hollow fiber and to fill the internal volume of the hollow fiber. Because the hollow fiber was made of polypropylene, the *n*-octanol present in the pores of the fiber was effectively immobilized and served as an active barrier to the aqueous donor solution. Thus, no leakage of *n*-octanol to the aqueous donor solution was observed during extraction. The majority of the injected *n*-octanol was filled inside the internal volume of the hollow fiber and served as the principal acceptor solution. Diazepam and prazepam were extracted from the aqueous sample solutions (1.5 ml) and into the *n*-octanol acceptor solution (15 μl) forced by a high partition coefficient.

Extraction of diazepam and prazepam at the 1.5 μg/ml level from human plasma is illustrated in Fig. 2. In order to deionize the two analytes within the sample and consequently to ensure high analyte preconcentration, the pH of the plasma was adjusted to 5.5 by sodium acetate prior to the extraction.

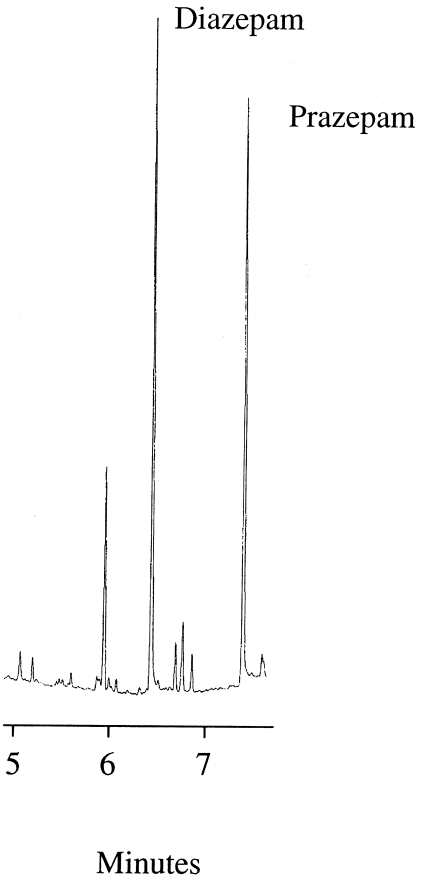

Fig. 2. LPME–GC of human plasma spiked with 1.5 μg/ml of each of diazepam and prazepam.

LPME was carried out for 30 min; extraction times lower than 30 min resulted in reduced analyte preconcentration while no significant improvements were observed when extraction times exceeded 30 min. Both compounds were effectively extracted from the plasma sample by LPME; diazepam was preconcentrated by a factor of 69 (69% extraction efficiency), while the corresponding value for prazepam was 103 (103% extraction efficiency). Due to the effective analyte preconcentration, both drugs were detected down to the 2 ng/ml level in plasma ($S/N=2$) based on 1-μl injections on the GC–NPD system. The combined selectivity of LPME and NPD resulted in relatively clean chromatograms; only a limited number of peaks emerged from the matrix of the plasma sample.

A similar experiment was accomplished by SPME

with the two drug substances present at the 1.5 μg/ml level in human plasma. With a 100 μm polydimethylsiloxane SPME fiber and a 30 min extraction time, the preconcentration factors of diazepam and prazepam were in the range 10 to 20.

3.4. LPME for CE

To further develop and investigate the LPME extraction units, attention was secondly focused on the compatibility with CE. In this case, methamphetamine was selected as a basic model compound and was extracted from human plasma, while naproxen was selected as an acidic model compound and was extracted from human urine. In order to be directly compatible with CE, the LPME acceptor phase should be an aqueous solution because organic solvents immiscible with water are not injectable in conventional aqueous CE. In order to extract analytes from an aqueous sample into an aqueous acceptor solution, it was necessary to utilize a three-phase system with an organic phase separating the two aqueous phases. Thus, the pores of the hollow fibers were first immobilized with *n*-octanol by dipping the fiber for 5 s into the solvent. In this step, *n*-octanol penetrated the pores from the outside of the hollow fiber while no solvent was filled into the internal volume of the hollow fiber. The *n*-octanol phase served as an effective barrier between the two aqueous phases and prevented mixing of the donor and acceptor solutions during extraction. After hollow fiber impregnation with *n*-octanol, the internal volume of the hollow fibers were filled with an aqueous acceptor solution from a μl syringe, and the extraction was started by placing the hollow fiber into the sample solution. For the extraction of the basic drug methamphetamine from human plasma, 25 μl of 0.1 *M* HCl was utilized as the acceptor solution. Prior to the extraction, NaOH was added to the 2.5-ml plasma samples providing a pH value of approximately 13, and methamphetamine was extracted from the sample into the *n*-octanol phase inside the pores of the hollow fiber and further into the acceptor solution forced by increasing partition coefficients. For the acidic drug naproxen present in human urine, 25 μl of 0.02 *M* NaOH was utilized as the acceptor solution. In this case, the 4.0-ml samples were acidified by HCl (pH 1) prior to the extraction, to effectively promote analyte partition into the alkaline acceptor phase.

Extraction of the basic compound methamphetamine (100 ng/ml) from human plasma is illustrated in Fig. 3. In this experiment, extractions were performed for 45 min; lower extraction times resulted in reduced analyte enrichment while no significant improvements were observed following longer extraction times. With the current LPME set-up, approximately 75% of the methamphetamine present in the plasma sample was extracted into the acceptor phase. Since the analyte was extracted from a volume of 2.5 ml into 25 μl, a preconcentration of 75 was obtained during LPME. With UV detection at 200 nm, methamphetamine was detected down to the 3 ng/ml level in plasma ($S/N=2$). In addition to the high analyte preconcentration, LPME also accomplished a substantial sample clean-up; in spite of the low detection wavelength, only a few other peaks were present in the electropherogram. Macromolecules, proteins, acidic compounds and most neutral compounds were prevented from penetrating the pores of the hollow fiber and remained in the sample solution. A similar experiment was accomplished for naproxen (acidic drug) present at the 100 ng/ml level in human urine (Fig. 4). Also in this case, the

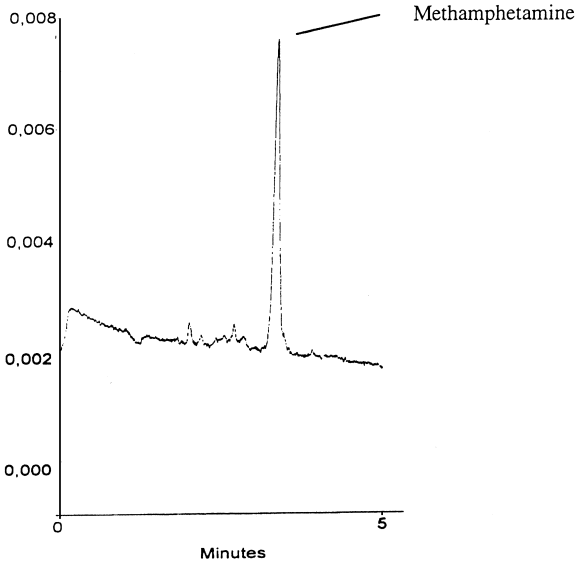

Fig. 3. LPME–CE of human plasma spiked with 100 ng/ml methamphetamine.

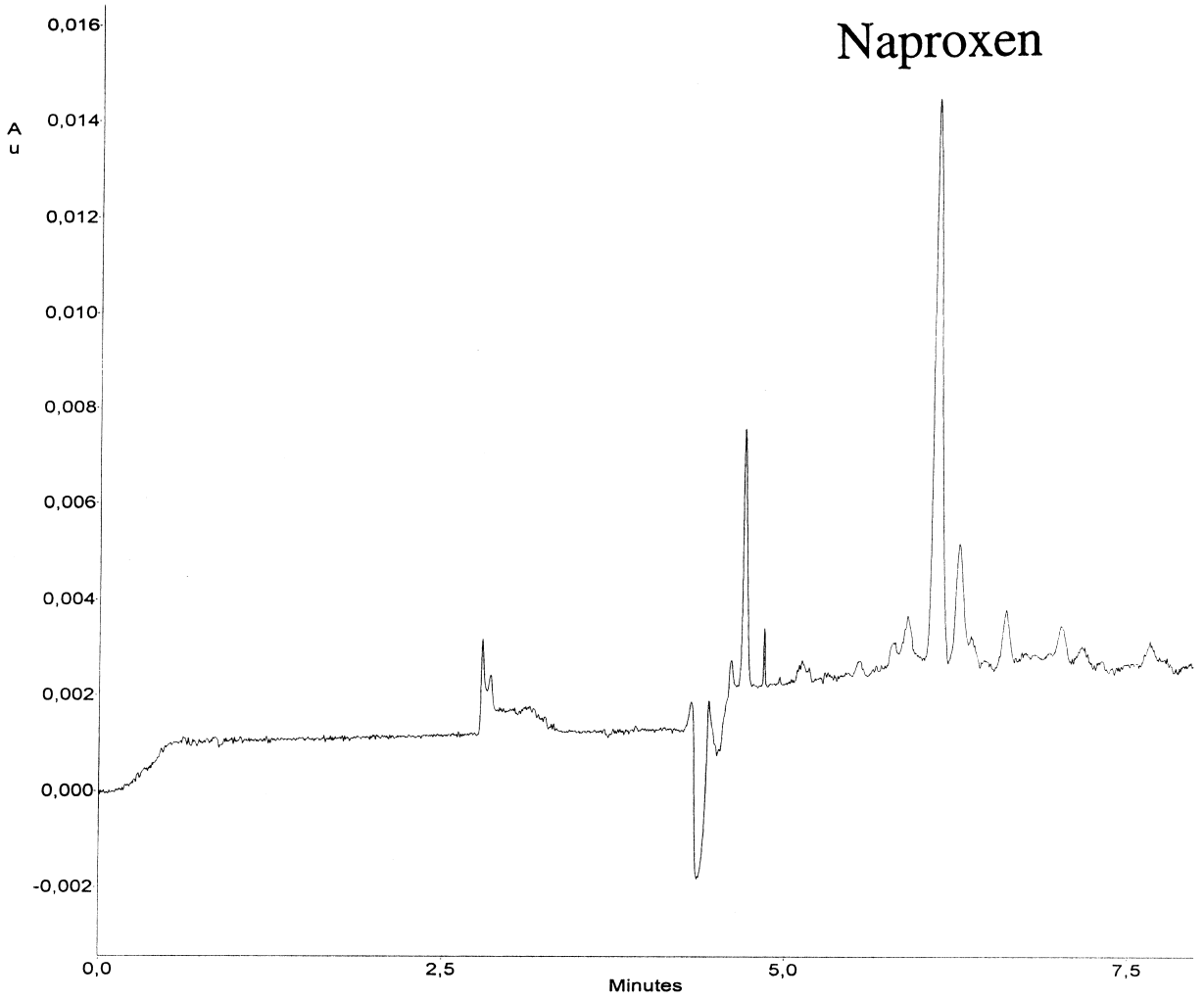

Fig. 4. LPME–CE of human urine spiked with 100 ng/ml naproxen.

analyte was extracted effectively following 45 min of LPME resulting in a 125 times preconcentration (78% extraction efficiency). This enabled naproxen to be detected down to the 1 ng/ml level by CE with UV detection at 214 nm ($S/N=2$). In the case of acidic drugs present in urine, some other peaks emerged in the electropherogram originating from the sample matrix. Although sample clean-up also occurred in this case, the presence of some acidic compounds in human urine somewhat reduced the selectivity generated by LPME. In such cases, selection of detection wavelength may be an important additional factor to control the selectivity.

3.5. LPME for HPLC

To finish this brief evaluation of LPME, the technique was tested in combination with HPLC. In this case, citalopram and the corresponding metabolite *N*-desmethylcitalopram were selected as model compounds. In contrast to GC and CE, sample volumes in the range 10–25 μl are easily injectable in HPLC and consequently, with this technique, the whole micro extract may be analyzed potentially providing very low detection limits. In the case of HPLC, organic solvents with a higher elution capacity than the mobile phase (typically mixtures of

acetonitrile and water) are disadvantageous. Therefore, the three-phase concept of LPME utilized for CE and which provided aqueous micro extracts was tested in combination with HPLC. For the basic drug citalopram, LPME was accomplished with a 25 μl acceptor solution of 0.01 *M* phosphate (pH 3) and with the hollow fiber impregnated with *n*-octanol. In order to obtain high analyte preconcentrations, the samples containing the drug were adjusted to pH 13 with NaOH.

Preconcentration of 10 ng/ml of citalopram and *N*-desmethylcitalopram from 1 ml of human plasma is illustrated in Fig. 5. For both citalopram and the metabolite, a 30-times preconcentration was obtained following 45 min of LPME corresponding to a 75% extraction efficiency. In the case of HPLC, the total 25 μl volume of acceptor solution was injected into the HPLC system, and citalopram was detected down to the 700 pg/ml level by fluorescence detection ($S/N=2$). As reported in combination with CE above, LPME of basic compounds based on the three-phase system provided a chromatogram with only a few peaks from the matrix supporting a effective sample clean-up.

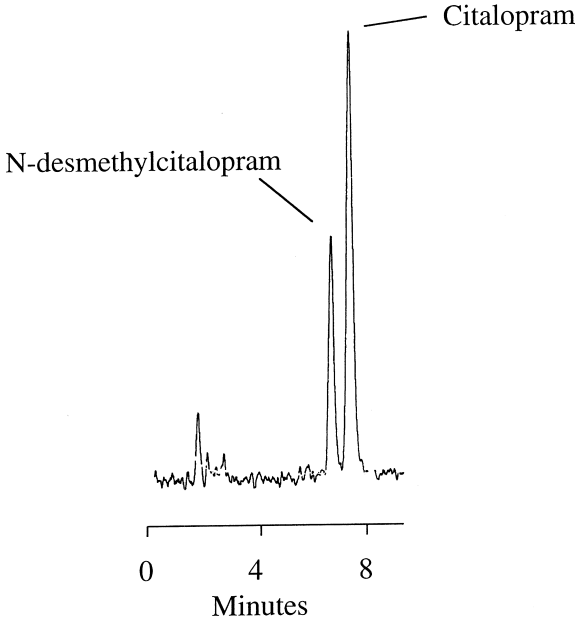

Fig. 5. LPME–HPLC of human plasma spiked with 10 ng/ml of each of citalopram and *N*-desmethylcitalopram.

4. Conclusions

In the present work, a simple, inexpensive and disposable device for LPME has been developed. The LPME concept was found to be compatible with GC utilizing a simple two-phase system (aqueous phase–organic phase). In addition to this, LPME was compatible also with CE and HPLC utilizing a relatively simple three-phase system (aqueous phase–organic phase–aqueous phase). LPME provided high preconcentration of several drugs present in both urine and plasma samples, and was capable of extensive sample clean-up. Because the extraction units were disposable, no carry over effects occurred. Because the price of each extraction unit was very low (comparable with the price of a SPE column), and because no expensive instrumentation was required to accomplish LPME, several (10–30) samples were extracted in parallel providing a high number of samples per unit time capacity. Work is in progress to further develop and evaluate LPME in combination with GC, CE and HPLC, and to develop a commercialized version of the device.

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