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Journal of Chromatography B, 798 (2003) 127–135
JOURNAL OF CHROMATOGRAPHY B
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Three-phase liquid-phase microextraction of weakly basic drugs from whole blood

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Received 14 February 2003; received in revised form 25 August 2003; accepted 9 September 2003

Abstract

The behaviour of weak basic analytes in liquid-phase microextraction (LPME) and the optimisation of parameters in whole blood are described. Benzodiazepines and non-benzodiazepine drugs were chosen as model substances. Liquid-phase microextraction based on disposable polypropylene hollow fibres was used in the three-phase extraction of five weak bases from whole blood. The sample work up with the liquid-phase microextraction technique can be impeded by low recovery due to incomplete trapping in the acceptor phase of weakly basic drugs and the complexity of the whole blood matrix. Different parameters related to this problem were experimentally studied. Additionally the stability of the analytes was examined because of low pH in the acceptor phase. The investigation resulted in optimised LPME conditions for the extraction of weak bases from whole blood. The parameters limiting the recovery were evaluated.

Keywords: Three-phase liquid microextraction; Weak bases

1. Introduction

Preparation of biological samples has traditionally been carried out by liquid-liquid extraction (LLE) or solid-phase extraction (SPE) prior to chromatography and electrophoresis. A miniaturised device for LLE was recently introduced [1–8]. This technique; liquid-phase microextraction (LPME) is based on porous hollow polypropylene fibre. When using two-phase LPME the analytes are extracted from an aqueous sample matrix into an organic acceptor phase, which is similar to two-phase LLE. The acceptor phase can then be analysed by gas chromatography (GC) or high performance liquid chromatography (HPLC) in the normal phase mode. Three-phase LPME involves extraction from an aqueous sample matrix, through an organic phase in the pores of the hollow fibre, immiscible with water and back into a new aqueous phase inside the lumen of the hollow fibre. This process is similar to LLE with back extraction. Analytical techniques such as reversed phase HPLC and aqueous capillary electrophoresis (CE) can be used for the analyses of the aqueous acceptor phase in three-phase LPME. Substantial sample clean up occur in both two- and three-phase LPME. Thus, proteins, salts, neutral compounds, acidic or basic compounds (acidic compounds when extracting basic drugs and vice versa) are prevented from entering the acceptor phase. The hollow fibres utilised are used only once, which eliminates problems related to carry-over effects. This single use adds high demands on the reproducibility of the manufacturing of the hollow fibre.

The ratio between the volume of the donor phase (sample volumes of 0.5–2 ml) and the acceptor phase (15–25 μl) governs enrichment of analytes in the acceptor phase. A high volume ratio promotes high enrichment. The partitioning of analytes is controlled by physicochemical properties of the analyte itself, in addition to the three phases in LPME; the donor, organic and acceptor phase. To promote extraction through the organic phase, the pH of the sample phase is adjusted to a value where the analytes are uncharged prior to extraction. Another critical parameter is the pH value of the acceptor phase. This pH should ideally be set to a value where the analytes are highly charged and thereby fully trapped in the acceptor phase.

For extraction of basic substances the maximal trapping of the extracted analytes in ionised form require that the pH of the acceptor phase is at least 3.3 pH units below

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doi:10.1016/j.jchromb.2003.09.021

the pK_a of basic analytes [9]. In other studies it was found that the fraction of analytes in the acceptor phase had to be lower than 0.0005 ($-\log 0.0005 = 3.3$) to have negligible effect on extraction efficiency [9–14]. However, a non-ideal situation occurs when LPME must be carried out on weak bases, which have pK_a values lower than 4. In these cases, the acidic capacity in the acceptor phase is a limiting factor.

The main objective of this paper was to investigate the non-ideal situations by studying model substances like benzodiazepines and non-benzodiazepine drugs with special emphasis on the optimisation of parameters in real biological matrix such as whole blood. This was done by studying various parameters which might affect the extraction recovery.

The chemical structures and physicochemical properties of the model substances are listed in Table 1. Zolpidem is an imidazopyridin derivative with properties as a weak base. The weak basic properties of 1,4-benzodiazepines are due to the nitrogen at position four, which can undergo protonation. Nitrazepam, *N*-desmethyldiazepam and diazepam are keto derivatives of 1,4-benzodiazepines. The unsubstituted nitrogen at position one in nitrazepam and *N*-desmethyldiazepam may have an acidic character at very high pH values. Alprazolam is a triazolo derivative of 1,4-benzodiazepines. In addition to the nitrogen at position four in the seven-membered ring, alprazolam contains other weakly basic centres on the 1,2,4-triazole. The presence of different substituent groups on the 1,4-benzodiazepines can change the physicochemical properties of these analytes when pK_a and pH changes. This may cause differences between analytes during extraction and chromatographic analysis.

Benzodiazepines are widely prescribed as sedative, hypnotic, anxiolytic and anticonvulsive drugs. These compounds have frequently been shown to produce dependence and can be potentially abused. Other non-benzodiazepine hypnosedative drugs, like zopiclone and zolpidem have been introduced in hope of reduction in side effects [15,16]. A large number of analytical procedures have been developed for the measurement of these drugs [17,18] in biological matrices.

2. Experimental

2.1. Chemicals

Zolpidem was obtained from Synthelabo (Brøndby, Denmark), alprazolam from Pharmacia Upjohn (Somerset County, NJ, USA), nitrazepam, *N*-desmethyldiazepam and diazepam from Alltech (Deerfield, IL, USA). All other chemicals used were of analytical grade.

2.2. Standard solutions and biological samples

Stock solutions of zolpidem, nitrazepam, alprazolam, *N*-desmethyldiazepam and diazepam were prepared in methanol (2 μmol/ ml and 50 nmol/ml). Standard whole blood samples (500 μl) were spiked with analytes (50 nmol/ml in methanol) to a concentration of 1 nmol/ml. All solutions were stored at 5 °C protected from light.

2.3. Liquid-phase microextraction device

The disposable device for LPME is illustrated in Fig. 1. LPME was carried out in conventional 1.5 ml sample vials with screw tops and silicone septums (Chromacol Ltd., Trumbull, CT, USA). A moulded polypropylene precision tip specially designed for Finnpipettes™ (Labsystems, Helsingfors, Finland) was inserted through the silicone septum. The tip functioned to support the hollow fibre, to introduce the acceptor phase into the hollow fibre prior to

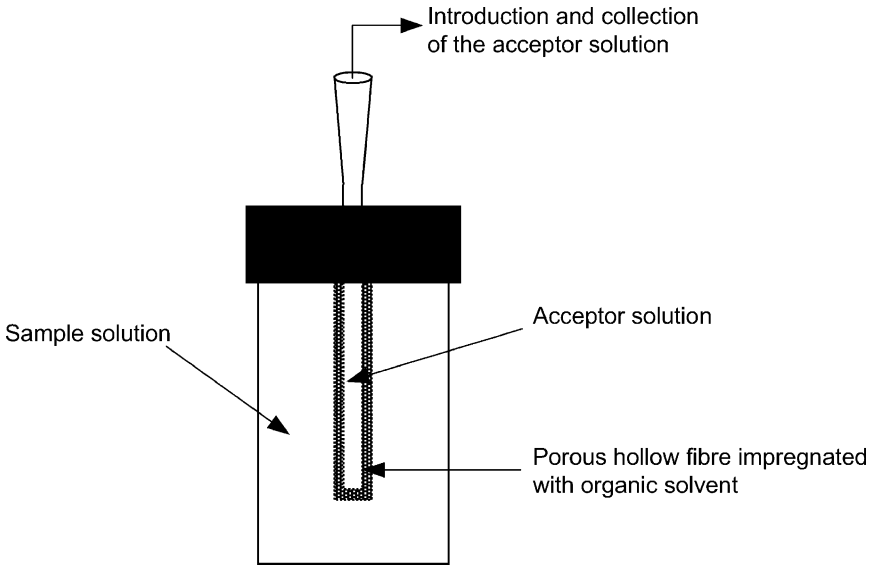
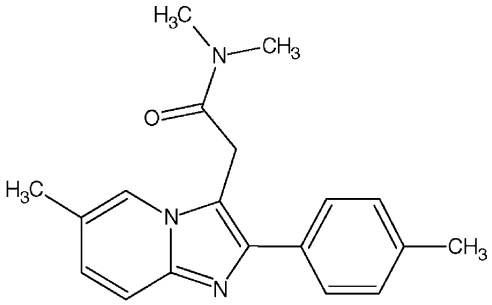
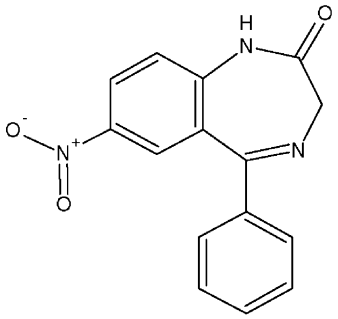
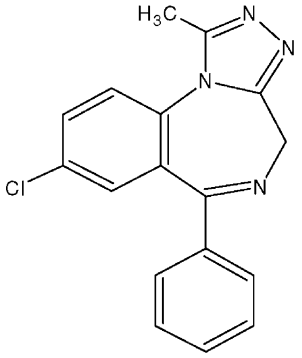
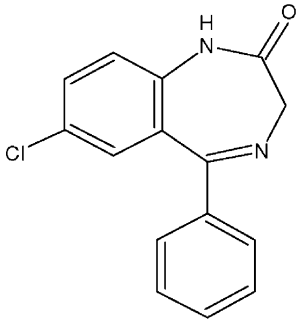
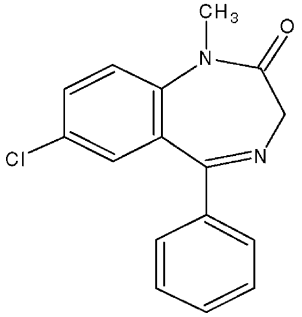


Fig. 1. Principle of LPME.

Table 1
Structures and physiochemical properties

Drug	Structure	pK_a	log P	Protein binding (%)
Zolpidem		6.91	2.61	92
Nitrazepam		3.19, 11.35	2.84	85
Alprazolam		2.39	2.50	70
N-desmethyldiazepam		3.40, 11.72	3.15	97
Diazepam		3.40	2.96	98

extraction and to collect the acceptor phase after the extraction is accomplished. A manually cut 18 mm piece of a polypropylene hollow fibre (Varian, Harbor City, CA, USA) was placed at the end of the tip. The inner diameter of the fibre was 1.2 mm, the pore size 0.2 μm and the thickness of the wall was 200 μm. The fibre was closed with a pair of pincers. Cyanoacrylic super glue (Loctite, München, Germany) fastened the fibre to the tip and sealed the free end of the fibre. Each piece of hollow fibre and tip were used for once and discarded after extraction.

2.4. Liquid-phase microextraction procedure

Prior to extraction, the sample vial was filled with 0.5 ml whole blood and diluted with 1.0 ml 0.1 M phosphate buffer (pH 7.5) to a total volume of 1.5 ml. For each extraction a new 18 mm length of the polypropylene hollow fibre with a sealed end was placed at the end of the tip and for 5 s dipped in an organic solvent to immobilise the solvent in the pores of the hollow fibre. Excess of solvent was removed by 15 s of ultra sonification in water. After immobilisation, 17 μl of acceptor solution was injected into the hollow fibre using a micro-litre syringe (Hamilton, Bonaduz, Switzerland). The assembly was then placed into the sample solution. During extraction the unit was vibrated utilising a Vibramax® 100 (Heidolph, Kelheim, Germany) at 1500 rpm. After extraction 10 μl of the acceptor solution was withdrawn from the fibre by the micro-litre syringe and collected into a 0.1 ml micro-insert (Merck Eurolab, Leuven, Belgium) The acceptor solution was then diluted with 100 μl 0.05 M ammonium acetate buffer, pH 5.5. An aliquot of 50 μl of this mixture was introduced to the HPLC system.

2.5. High performance liquid chromatography analysis

The automated injection was performed by an ASTED®-Dilutor 401 unit (Gilson, Viliers-le-Bel, France). The chromatographic separation was carried out on an XTerra™ MS C_8 analytical column (2.1 mm × 150 mm, 3.5 μm particles) from Waters (Milford, MA, USA). The mobile phase, acetonitrile–0.05 M ammonium acetate buffer, pH 5.5 (28:72, v/v) was delivered by a Shimadzu LC-9A HPLC pump (Shimadzu, Kyoto, Japan) at a flow rate of 0.25 ml/min. The buffer solution was filtered before mixing with acetonitrile. The analytes were detected using UV detection (SPD-6AV, Shimadzu) operated at 240 nm. Data acquisition was performed using a Class-VP Chromatography Data System (Shimadzu).

2.6. Liquid chromatography–mass spectrometry analysis

The liquid chromatography–mass spectrometer (LC–MS) system consisted of an SpectraSystem® AS3000 auto sampler, a SpectraSystem® P4000 HPLC pump and a Finnigan LCQduo™ ion trap instrument (all Finnigan, San Jose, CA, USA). Electro spray (ESI) was used as ionisation mode and operated in the positive mode. The analytical column, mobile phase and flow rate were the same as mentioned under Section 2.5. All samples were injected by an auto sampler and the analyses were carried out with the analytical column at room temperature. Between each analysis the sample syringe was flushed with 1 ml of water:methanol (50:50) containing 1% acetic acid.

The following ESI source conditions were applied: Sheath gas (nitrogen) flow rate 40 arbitrary units (approximately 0.6 l/min), auxiliary gas (nitrogen) flow rate 5 arbitrary units (approximately 1.5 l/min), spray voltage 5 kV, capillary temperature 250 °C, capillary voltage 15 V, and tube lens offset 0 V. The octapole 1 and 2 offset, lens voltage and octapole RF amplitude values were set to −475, −8, −20, and 400 V p–p, respectively after automatic tuning.

2.7. Calculation of extraction recoveries

The extraction recovery (R) for LPME was expressed as the percentage of total analyte amount $n_{s,initial}$ (number of mole of analyte originally present in the sample), which was transferred to the acceptor phase at the end of the extraction ($n_{a,final}$)(number of mole finally collected in the acceptor phase):

$$R = \left(\frac{n_{a,\,final}}{n_{s,\,initial}}\right) 100\% = \left(\frac{V_a}{V_s}\right)\left(\frac{C_{a,\,final}}{C_{s,\,initial}}\right) 100\% \qquad (1)$$

where V_a and V_s are volumes of the acceptor phase and the sample matrix (donor phase), respectively. $C_{a,final}$ is the final concentration of analyte in the acceptor phase, and $C_{s,initial}$ the initial analyte concentration within the sample. $C_{a,final}$ was determined by HPLC; peak areas from analysis of the LPME acceptor phases were compared with peak areas from standard solutions prepared in similar solution as the acceptor phases.

3. Results and discussion

The three-phase LPME system with simultaneous extraction and back extraction resulted in efficient sample clean up. Fig. 2 shows the chromatogram of zolpidem (ZOL), nitrazepam (NIT), alprazolam (ALP), *N*-desmethyldiazepam (ND) and diazepam (DIA) present at 1 nmol/ml level in whole blood, and a blank of whole blood extracted by LPME (procedure as in 2.4) and subsequently analysed by HPLC within the elution window of the drugs. The absence of extra peaks in the chromatogram of blank whole blood illustrate the cleaness of the LPME extract.

3.1. Effect of extraction time on analytes behaviour in the LPME system

As described above, the LPME process involved two reversible extractions. Analyte partitioning was controlled by the physicochemical properties of the analyte, the sample

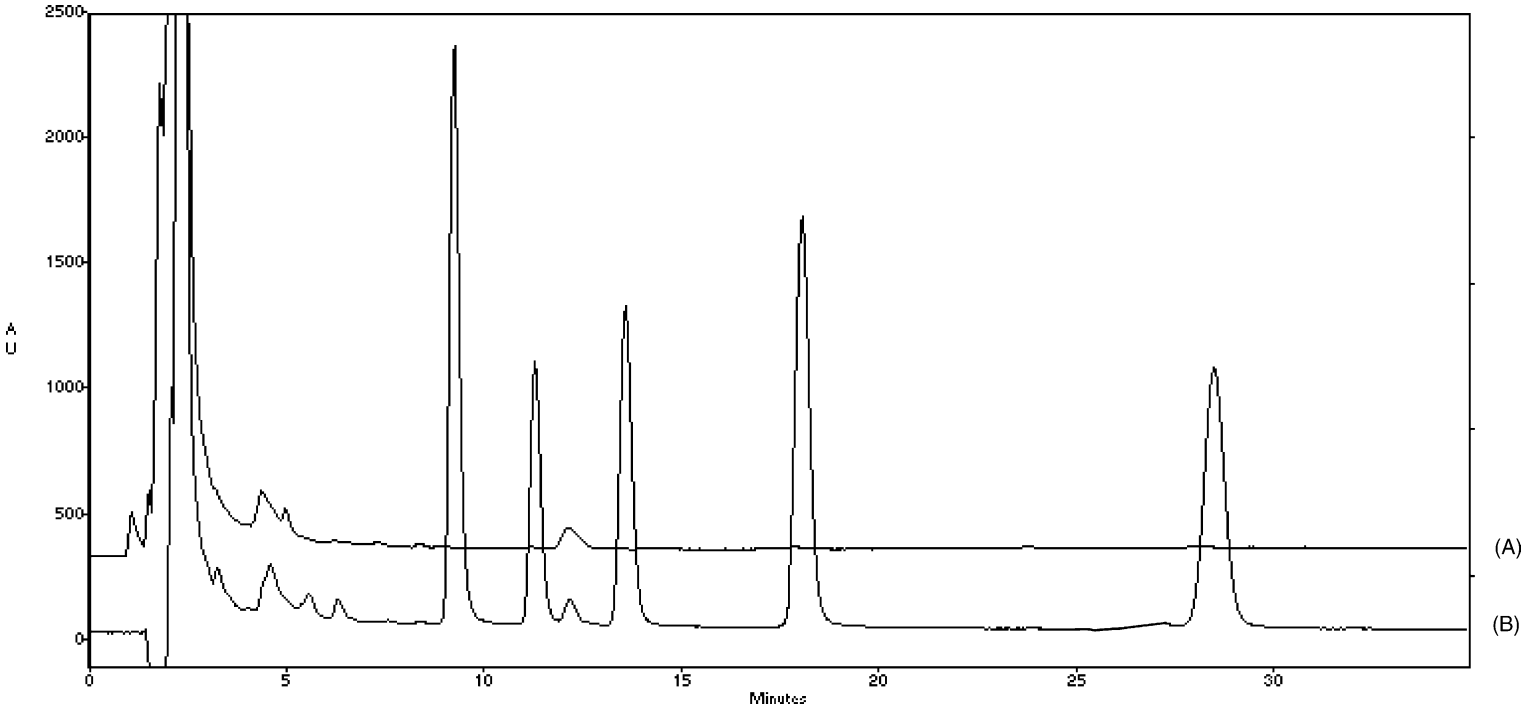


Fig. 2. Chromatograms of (A) drug-free whole blood sample and (B) a whole blood sample spiked with 1 nmol/ml zolpidem (peak 1), nitrazepam (peak 2), alprazolam (peak 3), *N*-desmethyldiazepam (peak 4) and diazepam (peak 5).

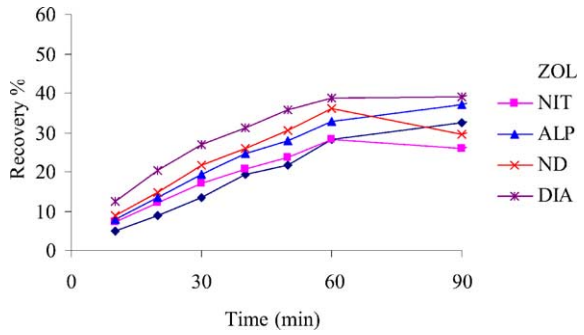


Fig. 3. Extraction recoveries of zolpidem (◆), nitrazepam (■), alprazolam (▲), *N*-desmethyldiazepam (×) and diazepam (✳) versus extraction time in whole blood.

matrix, the organic phase and the acceptor phase. When sufficient extraction time has elapsed for equilibrium to establish, further increase in extraction time showed minimal effect on the amount of analyte extracted. Recovery of ZOL, NIT, ALP, ND and DIA versus extraction time in whole blood with 0.4 M HCl as acceptor phase and nonanol as organic phase is shown in Fig. 3. These values are calculated from Eq. (1). The amount of analytes extracted by LPME increased slowly with increasing exposure time from 10 to 60 min, then the extraction recovery reached a plateau, as equilibrium had been established. The difference in physicochemical properties between the analytes had minor impact on the extraction time profile, as the profiles were quite similar. The high viscosity of whole blood is the main reason of an extraction time as long as 60 min together with a high degree of protein binding of the drug. This cause a reduced and slow diffusion of analytes to the acceptor phase. Previous LPME publications [3,8] show the relation between matrix viscosity and time of extraction on the extraction recovery. Due to parallel extraction of up to 30 samples, high extraction throughput was ensured despite the long extraction time.

3.2. Effect of pH on analytes behaviour in the LPME system

3.2.1. Donor phase

The whole blood sample (500 μl) was diluted to 1500 μl to reduce the viscosity of the matrix, and to maintain a pH at which the analytes were unprotonated. To evaluate the pH of the donor phase 0.01 M NaOH and 0.1 M phosphate buffer were used ($n = 4$, R.S.D. 4–15%). The pH in the donor phase was then 12 and 7.5, respectively. A considerable lower recovery for NIT and ND was found when diluting the sample with NaOH (1.5 and 5.2%, respectively) compared diluting with phosphate buffer (28.1 and 36.1%, respectively). These analytes have a second pK_a around 11 and at pH 12 in the sample their nitrogen at position one may have a weak acidic character (amid). High pH gives NIT and ND an ionic character in the donor phase and therefore decreases the partitioning of analytes into the organic phase. ZOL was the only analyte that showed an increase in recovery using NaOH as diluter (35.7%), while DIA and ALP more than halved their recovery (17.2 and 13.8%, respectively). This can be explained by a certain degree of precipitation of proteins in the contact point between NaOH and the whole blood sample before mixing occurred. The phosphate buffer at pH 7.5 in the donor phase was shown to be optimal for the analytes except ZOL, which reduced its recovery from 35.7 to 28.1%. However, the recovery of ZOL at pH 7.5 was high enough to carry out additional experiments.

3.2.2. Acceptor phase

The pH of the acceptor phase was optimised, testing seven different molarities (0.01–1.0) of HCl. As illustrated in Fig. 4, an increase can be seen in recovery of all analytes as the pH decreases in the acceptor phase, except for ZOL. The recovery of ZOL is nearly unaffected in the pH range from 2 to 0. This can be explained by its pK_a of 6.9: at the pH values tested ZOL was totally ionised. DIA and ND showed similar behaviour with an increase in recovery from pH 2 to 0.4 of the acceptor phase and then reaching a plateau from pH 0.4 to 0. Again the differences in pK_a values of the substances might explain this similarity. Both DIA and ND have a pK_a value of 3.40. Although NIT has a lower recovery it has the same increase in recovery as DIA and ND as the acceptor phase pH was decreased from 2 to 1. The explanation is probably the lower pK_a value of 3.19 compared to 3.40 (ND and DIA). The behaviour of alprazolam with respect to the recovery is some different from

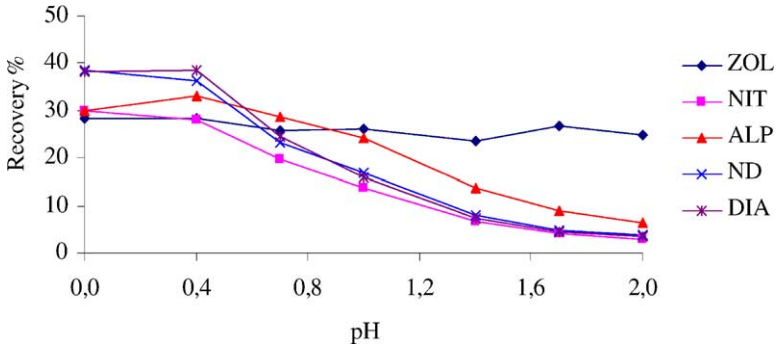


Fig. 4. Extraction recoveries in whole blood of zolpidem (◆), nitrazepam (■), alprazolam (▲), *N*-desmethyldiazepam (×) and diazepam (✳) versus pH in the acceptor phase.

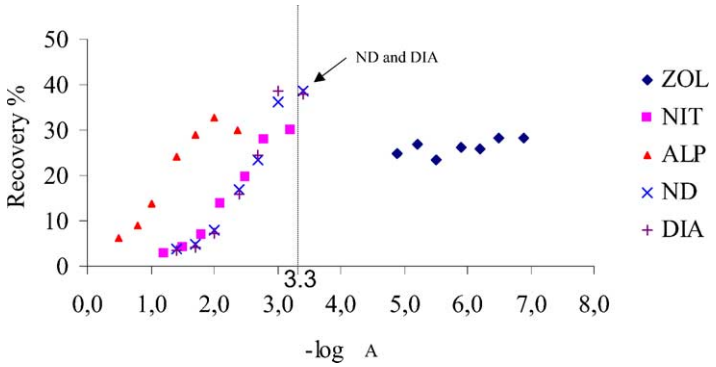


Fig. 5. Extraction recoveries in whole blood of zolpidem (◆), nitrazepam (■), alprazolam (▲), *N*-desmethyldiazepam (×) and diazepam (+) versus α_A in the acceptor phase.

the other analytes, but also for ALP a maximum recovery is observed.

Chimuka et al. [9] showed that a complete trapping of the extracted analytes in a stagnant acceptor phase in ionised form required that the acceptor pH should be at least 3.3 pH units below the pK_a of basic analytes. A measurement for protonation of the analytes in the acceptor phase is given by α_A:

$$\alpha_A = \frac{K_a}{H^+ + K_a} \quad (2)$$

From Eq. (2) it is clear that this value depends on the analyte pK_a and the pH of the acceptor phase. A situation where pH is 3.3 units lower than pK_a the negative logarithm of α_A is higher than 3.3. As can be seen from Fig. 5 there is only DIA, ND and ZOL, which meet these conditions at the acceptor pH of 0.4. ZOL is unaffected by the increase in α_A. The explanation is probably that the increase in acceptor pH from 2 to 0 gives a pH $<$ pK_a $-$ 3.3.

3.3. Effect of organic phase on analytes behaviour in the LPME system

Selection of an organic phase is one of the critical steps in LPME. The organic phase serves to separate the aqueous acceptor phase inside the hollow fiber from the aqueous donor phase in the sample vial. The organic phase must therefore be immiscible with both the acceptor and the donor phase. Second, the solubility of the analytes should be higher in the organic phase than the donor phase to promote extraction of analyte. A third criterion is that the solubility of the analyte should be lower in the organic phase compared to the acceptor phase, in order to achieve a high degree of recovery of analytes in the acceptor phase. The last criterion is that the organic solvent is easily immobilised in the pores of the hollow fiber and that it is non-volatile. Four different organic phases were investigated: octanol, nonanol, decanol and dodecanol. Octanol was not evaluated as organic phase in the extraction of whole blood as this solvent did not make a robust LPME system. When the sample was fresh whole blood (not frozen and thawed) the octanol did not fully serve the criteria for an organic phase as mentioned above, especially the criteria for immiscibility with both the acceptor and the donor phase.

Table 2
Extraction recoveries (%) ($n = 6$) (R.S.D.%) in whole blood of zolpidem (ZOL), nitrazepam (NIT), alprazolam (ALP), *N*-desmethyldiazepam (ND) and diazepam (DIA) using different alcohols as the organic phase (nonanol, decanol, dodecanol)

	ZOL	NIT	ALP	ND	DIA
Nonanol	28 (9)	28 (16)	33 (7)	36 (15)	38 (6)
Decanol	13 (13)	20 (12)	23 (10)	18 (16)	27 (11)
Dodecanol	9 (7)	23 (13)	12 (5)	21 (10)	26 (7)

Table 2 shows the different extraction recoveries obtained for the analytes in whole blood using different alcohols as the organic phase. A pronounced decrease was found in extraction recovery of all the analytes from whole blood using decanol (C_{10}) as organic phase. In increasing the C-length of the alcohol to C_{12} it was expected a further decrease in recovery. Extraction of whole blood samples gave decreased recovery of ZOL, ALP and DIA using dodecanol as the organic phase, but increased recovery for NIT and ND. The hydrophobicity of the analytes (log *P* values) is in the same order ranging from log *P* of 2.50 (ALP) to 3.15 (ND). For NIT and ND, the two analytes with an increase in recovery using dodecanol as the organic phase in whole blood, have one common characteristic, their secondary amine function at nitrogen at position one. This may influence their partitioning into the organic phase. Another possible suggestion may be contributions to the recovery from two competing distributions of analytes in the organic phase. One of the distributions promotes partitioning of analytes into the acceptor, while the other promotes the partitioning of analytes into the donor phase.

3.4. Addition of methanol to the donor phase and the effect on analytes behaviour in the LPME system

Drugs may bind to plasma proteins to varying degrees depending on their individual physiochemical properties.

Other biopolymers than proteins, e.g. red blood cells, may also cause reduced recovery from whole blood because of interactions with the analytes. Acidic and neutral drugs bind primarily to albumin while basic drugs in general bind to α-acid glycoprotein [19]. The interactions between drug and protein are either ionic, hydrophobic or polar interactions. Hydrophobic interactions may be suppressed by addition of organic solvent to the protein and drug-containing matrix. Methanol is an organic solvent with the ability to disrupt both hydrophobic and polar interactions [20,21]. Previous experience with methanol as suppressor of protein interactions in the LPME system have shown that this organic solvent is successful for the increase in extraction recovery for a lot of drugs, but not for all [3,6]. Dilution of the whole blood sample may increase the recovery of analytes by disrupting weak drug–protein bonds [21]. The analytes in this study have a relatively high degree of protein binding, varying from 70% (ALP) to 98% (DIA). To study the effect of methanol on the recovery of the test compounds varying concentrations of methanol (0–25%) were added to both spiked whole blood diluted with phosphate buffer and spiked phosphate buffer (protein free matrix). The extraction recoveries of the analytes in this study, were almost unaffected by the addition of methanol to whole blood that was diluted with 1 ml phosphate buffer. For phosphate buffer alone, similar results were obtained. Further addition of methanol made the LPME system unstable resulting in leakage of the hollow fibre. The reason for this leakage is not clear. With the analytes studied in this LPME system a dilution of the sample with phosphate buffer seem to give satisfactory displacement in the drug–protein equilibrium.

3.5. Stability of the analytes in an acidic acceptor phase

Many benzodiazepines will undergo hydrolysis in acidic aqueous solution, forming long- or short-lived intermediates (ring opening), which degrade further to end products (benzophenone). Intermediate forms have their molecular ion $m/z + 18$ higher than its parent, while the end products have molecular ion which is $m/z - 39$ lower than its parent [22–26]. Since the acceptor phase in this study was acidic to provide acceptable extraction recovery and enrichment in the three-phase LPME system, the stability of the analytes in 0.4 M HCl (pH 0.4) was examined by HPLC–UV and LC–MS. The LC–MS identification of ZOL, NIT, ALP, ND and DIA show that after 24 h, ZOL still is stable, while for NIT, ALP, ND and DIA the intermediate degradation products can be seen. Compounds with *m*/*z* values corresponding to *m*/*z* values of end products of NIT, ALP, ND and DIA are also seen. However, it is unclear if this is caused by in-source hydrolysis (low pH and high temperature, 250 °C) or by degradation of analytes in the acceptor solution. It is likely, however, that in-source hydrolysis is the cause for this presence, since parent and its corresponding end product (benzophenone) co-elute: Parent and its end-product have a too large log *P* difference to assume co-elution on reversed phase chromatography (benzophenone log *P* higher than the parent benzodiazepine). Chromatograms from HPLC–UV showed that there was no significant increase of degradation products within the timeframe (60 min) of extraction.

4. Concluding remarks

The present work demonstrates the behaviour of weak bases (benzodiazepines and a non-benzodiazepine as model substances) in LPME with varying physicochemical properties where not all experimental parameters can be optimised. The extraction time, pH and use of different organic phases were parameters investigated for their effect on analytes behaviour in the LPME system. Addition of methanol as a disruptor of the binding between protein and drug in whole blood had minimal effect on the recovery. Dilution of the whole blood sample with phosphate buffer gave satisfying results. Stability studies of the drugs in an acidic acceptor phase showed the formation of intermediates, but this was not significant within the timeframe of the extraction. This work shows that LPME can be used as an extraction technique for weak bases. However, it should be noted that especially for these types of compounds extensive optimisation was needed.

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