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Determination of phenothiazine derivatives in human urine by using ionic liquid-based dynamic liquid-phase microextraction coupled with liquid chromatography

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ABSTRACT

A simple and rapid method for the determination of seven phenothiazines derivatives (chlorpromazine, promethazine, levomepromazine, prochlorperazine, trifluoperazine, fluphenazine and thioridazine) in human urine samples is presented. The analytes are extracted from the sample in 50 μL of the ionic liquid 1-butyl-3-methyl-imidazolium hexafluorophosphate working in an automatic flow system under dynamic conditions. The chemical affinity between the extractant and the analytes allows a good isolation of the drugs from the sample matrix achieving at the same time their preconcentration. The separation and detection of the extracted compounds is accomplished by liquid chromatography and UV detection. The proposed method is a valuable alternative for the analysis of these drugs in urine within the concentration range 0.07–10 μg mL^{-1}. Limits of detection were in the range from 21 ng mL^{-1} (thioridazine) to 60 ng mL^{-1} (levomepromazine). The repeatability of the proposed method expressed as RSD ($n = 5$) varied between 2.2% (levomepromazine) and 3.9% (chlorpromazine).

1. Introduction

Phenothiazine derivatives are a group of basic substances which have been widely used as antipsychotic, antiparkinsonian, and antihistaminic drugs [1]. Biochemically, they are antagonists of dopamine receptors whose action mechanism is based on the blockade of nervous impulses from the central nervous system. The common chemical structure of phenothiazines consists of a three-ring structure in which two benzene rings are joined by a sulfur and nitrogen atom at nonadjacent positions. Depending on the substituents attached at the 2 and 10 positions, three kinds of derivatives may be distinguished, namely: aliphatic compounds (e.g. promazine), piperazines (e.g. fluphenazine) and piperidines (e.g. thioridazine). These groups of compounds present different profiles of therapeutic properties.

The analytical determination of these compounds is desirable taking into account that their overdose may cause coma, miosis, and respiratory depression, among other disorders [2]. According to the literature, their determination has been accomplished in different biological samples such as such as blood [3], plasma [4], and urine [5], and in gastric contents, bile [6], brain [7] and hair [8] to a lesser extent. Due to the low concentration of the analytes in the biological samples and the large number of potential interferences presents in them, the isolation and preconcentration of the target analytes is a crucial step in the developed methodologies. Traditional liquid–liquid extraction (LLE) [9] and solid-phase extraction (SPE) using conventional sorbents such as C_{18} [10] or with more selective sorbents like MIPs [11], have been proposed for these purposes. Besides these well-established approaches, new extraction strategies including the so-called solventless techniques, such as solid-phase microextraction (SPME) [12] and liquid-phase microextraction (LPME) [13], are gaining importance.

LPME emerged in the mid-to-late 1990 when Liu and Dasgupta [14] and Jeannot and Cantwell [15] almost simultaneously, proposed for the first time the use of solvents in the low microliter range as extractants. The technique can be considered a simple, low cost and rapid procedure requiring lower sample and extractant volumes [16]. According to the hydrodynamic features, LPME procedures can be divided into static and dynamic ones. In the static mode, the extractant is suspended in a large volume of sample phase and the transference of the analytes to the extractant is passively carried out [17]. In the dynamic approach, proposed by He and Lee for the first time [18], the mass transfer of the analytes takes place between the solvent microfilm formed on the inner surface of the extraction unit (usually a microsyringe) and the sample (or its headspace) providing higher enrichment factors (EFs) [19,20].

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In this work, a simple, rapid and almost solventless method for the determination of seven phenothiazine derivatives (chlorpromazine, promethazine, levomepromazine, prochlorperazine, trifluoperazine, fluphenazine and thioridazine) in human urine samples is presented for the first time. The target analytes are isolated from the sample matrix by means of a dynamic liquid-phase microextraction (dLPME) procedure using the ionic liquid (IL) 1-butyl-3-methyl-imidazolium hexafluorophosphate as extractant. The dLPME, which is mechanized in a flow system, takes advance of the special properties of the IL (mainly its viscosity, density and chemical affinity to the analytes) to achieve an effective extraction and preconcentration of the analytes prior their determination by liquid chromatography.

2. Experimental

2.1. Reagents and samples

All reagents were of analytical grade or better. Acetonitrile, acetic acid, triethylamine (Scharlab, Barcelona, Spain) and Milli-Q ultrapure water (Millipore Corp., Madrid, Spain), were employed as components of the chromatographic mobile phase. 1-Butyl-3-methylimidazolium hexafluorophosphate ($bmimPF_6$) from Solvent Innovation (Cologne, Germany) was used in the dLPME procedure.

Phenothiazines derivatives (chlorpromazine, promethazine, levomepromazine, prochlorperazine, trifluoperazine, fluphenazine and thioridazine) were purchased from Sigma–Aldrich (Madrid, Spain). Stock standard solutions of each analyte were prepared in methanol at a concentration of 5 g L^{-1} and stored at 4 °C. Working solutions of phenothiazines were prepared by dilution of the stocks in Milli-Q water.

Blank urine samples were collected from healthy individuals and stored in polytetrafluoroethylene (PTFE) flasks at −20 °C until analysis. Prior to the dLPME, each sample was adjusted to pH 8.0 with sodium hydroxide and filtered through a disposable nylon filter (0.45 μm of pore size, Millipore, Madrid, Spain).

2.2. Chromatographic conditions

Chromatographic analyses were performed on an HP1100 series liquid chromatograph (Agilent, Palo Alto, CA) equipped with a binary high pressure pump for mobile phase delivery, a high pressure injection valve (Rheodyne 7725, Cotati, CA) fitted with a 20 μL loop, and a single wavelength photometer (HP1100 series) for analytes determination. Data analysis was performed using HP ChemStation software.

Chromatographic separation was achieved on a tandem LiChrosorb C_8 (4.6 mm × 150 mm)–LiChrosorb C_{18} (4.6 mm × 150 mm) cartridge columns using acetonitrile/water/acetic acid/triethyl amine 40/40/20/2 (v/v/v/v) as mobile phase, previously degassed in an ultrasonic bath (Selecta, Barcelona, Spain) during 30 min (50 W, 60 Hz). Separation was done at room temperature using a constant flow rate of 0.5 mL min^{-1}, the analytes being monitored at 250 nm.

2.3. Dynamic liquid-phase microextraction manifold and procedure

The manifold employed for the dLPME procedure, which is depicted in Fig. 1, consists of a Cavro XP 3000 syringe pump (Sunnyvale, CA) equipped with a 1 mL syringe connected to a Pasteur pipette which acts as the extraction unit. PTFE tubing of 0.5 mm i.d. and standard connectors are also employed. The setup is computer controlled by Sagittarius 3.0 software package (obtained from the working group on Chemical Analysis and Vibrational Spectroscopy, Vienna University of Technology, Vienna, Austria) which manages the volume and flow rate of the different solutions employed.

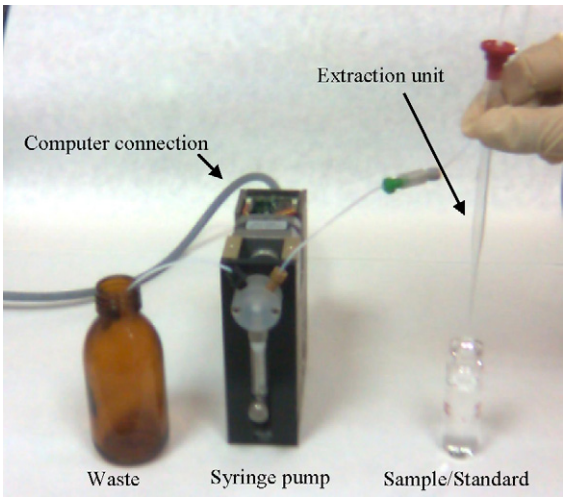

Fig. 1. Photograph of the dynamic liquid-phase microextraction manifold employed.

The overall scheme of the dLPME procedure can be described by three sequential steps. In the first step, 100 μL of the mixture ionic liquid:acetonitrile (50:50, v/v) are picked up in the Pasteur pipette at a flow rate of 0.5 mL min^{-1}. Secondly, the pipette is immersed in the sample vial to draw in a fixed volume of sample (10 mL) at a flow rate of 0.5 mL min^{-1}. The sample is continuously introduced in the system and passes through the ionic liquid plug, which remained in the lower part of the pipette due to its high density (see Fig. 2), allowing a dynamic extraction of the analytes. Once the extraction

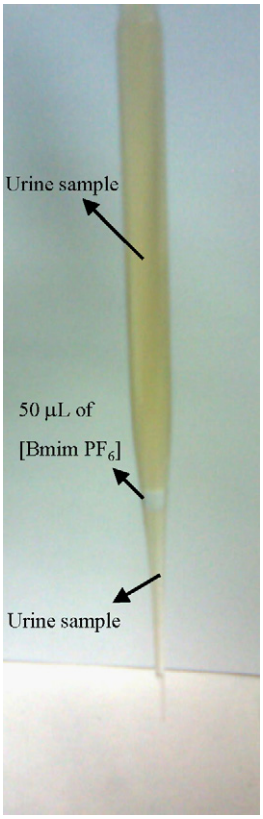

Fig. 2. Location of the IL section on the extraction unit during the sample introduction step.

Table 1
Chemical and spectroscopic properties of the ionic liquid 1-butyl-3-methyl-imidazolium hexafluorophosphate.

Property	Value
Molecular weight	284 g mol^{-1}
Density	1.37 g mL^{-1}
Viscosity	352.2 mPa s
Solubility in water[a]	0.13% (v/v)
Max UV absorbance[a]	210 nm
Absortivity at 210 nm[a]	4.08×10^5
Absortivity at 254 nm[a]	3×10^2

[a] Obtained from Carda-Broch et al. [23].

has been completed, the 50 μL of [bmimPF$_6$] are pumped out at a flow rate of 0.05 mL min^{-1} and recovered in an eppendorf vial containing 50 μL of acetonitrile. Finally, 20 μL of the mixtures was injected for the subsequent chromatographic analysis.

Taking into account that in the sample loading cycle, the flow rate of the syringe emptying step was fixed to 5 mL min^{-1}, the whole process takes place in less than 35 min which is compatible with the chromatographic analysis.

Considering that the Pasteur pipette is changed for each new extraction, no carry-over between samples is observed.

3. Results and discussion

Ionic liquids (IL), which are ionic media resulting from the combination of organic cations and various anions [21], have been proposed as alternative to the conventional organic solvents in LPME procedures [22]. Their high density and viscosity as well as their low water solubility make them an excellent alternative of choice when dynamic LPME is developed. On the one hand, the high density and viscosity values allow the easy location of a small section of the ionic liquid in the extraction unit avoiding its dragging by the sample stream. On the other hand, the low water solubility permits to pass high volumes of sample through the extractant, increasing the extraction efficiency, with negligible losses of extractant. In this sense, Table 1 summarizes the main chemical characteristics of [bmimPF$_6$] which are the ionic liquid selected for this application [23].

Moreover, imidazolium-based ionic liquids present a high chemical affinity to substances with one or more aromatic rings in their structure. Fig. 3 represents the generic chemical structures of the analytes and the extractant. The phenothiazines derivatives are extracted from the sample matrix through CH-π hydrogen bonds between the C$_2$H of the imidazolium ring and the aromatic parts of the analytes [24]. The extracted analytes are conveniently preconcentrated due to the high sample/extractant volumes ratio.

3.1. Optimization of dynamic LPME procedure

The different variables involved in the dynamic LPME procedure were studied in depth and optimized following a univariant approach. For this purpose, spiked urine samples containing all the

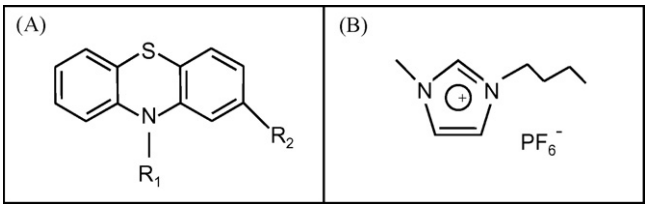

Fig. 3. (A) Generic chemical structure of the phenothiazine derivatives. (B) Chemical structure of 1-butyl-3-methyl-imidazolium hexafluorophosphate.

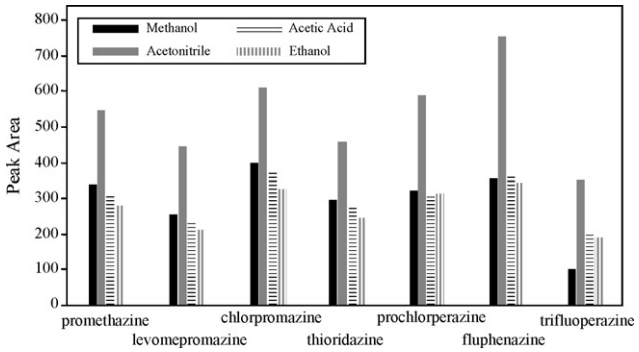

Fig. 4. Influence of the solvent used for ionic liquid dissolution on the extraction efficiency of phenothiazines derivatives.

analytes at a concentration of 1 μg mL^{-1} were used, the chromatographic peak area of each analyte being considered as analytical signal for optimization purposes.

3.1.1. Extractant composition and volume

The ionic liquid [bmimPF$_6$] presents a high viscosity value (see Table 1) which difficult its introduction in the flow system in a reproducible way. In order to overcome this problem, it must be diluted in an appropriate organic solvent prior to the dLPME procedure. This solvent should act favouring the introduction and release of the IL in the extraction unit. According to these considerations, the solvent must be miscible with water (sample solvent) and with the IL. Different solvents were evaluated, namely: ethanol, acetonitrile, methanol and acetic acid. The results, which are represented in Fig. 4 shows almost the same tendency (acetonitrile > methanol > acetic acid > ethanol) for all the analytes. Therefore, acetonitrile was selected as the IL solvent for further studies. Different acetonitrile/IL ratios were evaluated; 1:1 ratio being the optimum value in terms of reproducibility of the volume pumped.

In LPME procedures, the amount of extracted analytes increases with the volume of extractant employed. In this sense, four different volumes of IL have been evaluated, namely: 25, 50, 100 and 200 μL (diluted 1:1, v/v in acetonitrile). According to the results, 50 μL of IL lead to the best extraction results for all the analytes. This behaviour can be explained taking into account two facts. On the one hand, during the extraction the IL is located in the bulbous part of the Pasteur pipette. On the other hand, in the dynamic procedure the mass transfer of the analytes takes place between the solvent microfilm formed on the inner surface of the extraction unit and the sample. The ratio between the microfilm length and the solvent volume seems to play a key role in the extraction process. In fact, volumes higher than 50 μL produce an evident increment of the microfilm length but also an excessive increment of the extractant volume. The use of volumes lower than 50 μL involves a great irreproducibility in the extraction process since, in some cases; the location of small volumes in the extraction unit is difficult. This fact involves losses of micro-drops of the extractant during the dLPME which directly reduces the extraction values. For this reason, 50 μL was selected as the optimum IL volume, which was mixed with an equal volume of acetonitrile, as it was previously optimized.

3.1.2. Sample conditions and volume

The sample pH is a crucial parameter since it affects to the form in which the analytes are presented in the sample. This variable was studied in the range from 2 to 10 by adding the appropriate microvolumes of hydrochloric acid or sodium hydroxide solutions

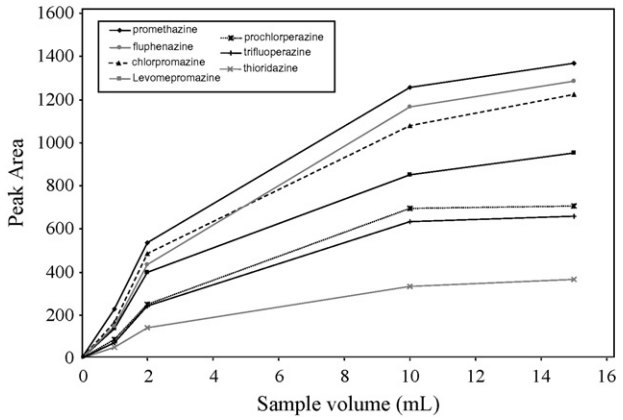

Fig. 5. Effect of sample volume on the extraction of phenothiazine derivatives.

to the urine samples. The obtained results show that the peak areas for all the phenothiazine derivatives increased when increasing the pH in the intervals 2–7, remaining almost constant in the range 7–10. pH 8 was selected as the optimum value for further studies.

The beneficial effect of the ionic strength in LLE procedures has been widely reported in the scientific literature. For this reason, its effect was evaluated in the range 0–60 g L^{-1} sodium chloride using as electrolyte. The peak area of the extracted analytes dramatically increased when increasing the ionic strength up to 1 g L^{-1} and decrease slightly in the range 1–60 g L^{-1}. This unexpected decrease has been previously reported when ILs are used as extractant [25]. According to these results, 1 g L^{-1} was selected as the optimum value to study the effect of the sample volume in the extraction of the analytes. This variable was evaluated by extracting different volumes of the same spiked urine sample (1 μg mL^{-1}), maintaining constant the volume of [bmimPF_6] at a value of 50 μL. Fig. 5 shows that the peak area of the analytes increased by increasing the sample volume in the interval 1–10 mL remaining almost constant in the range 10–15 mL. This parabolic behaviour can be explained in view of two facts. On the one hand, higher sample volumes lead to an increasing of the preconcentration factors achieved. On the other hand, higher sample volumes reduce the recovered IL volume after the dLPME procedure due to the partial dissolution of the extractant in the sample. This fact is not problematic from the analytical point of view because, once the sample volume has been fixed, the volume of IL recovered in the eppendorf remained constant. Finally,

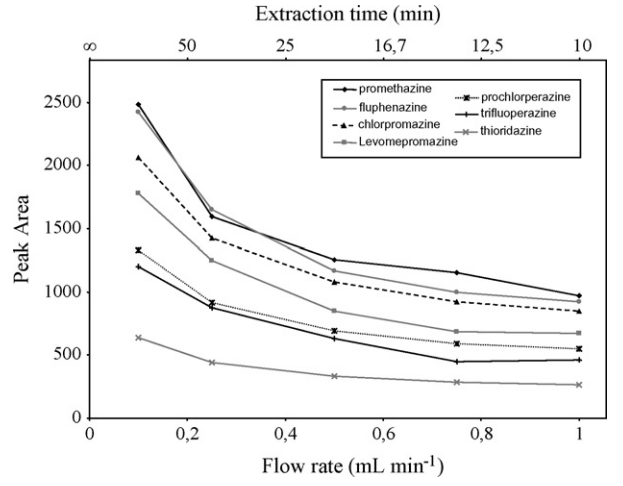

Fig. 6. Effect of sample flow rate on the extraction of phenothiazine derivatives. The data are obtained for a sample volume of 10 mL.

Table 2
Enrichment factors obtained for the target analytes in the dLPME process.

Analyte	Equation[a]	R	EF ± SD[b]
Promethazine	$S' = 13.19 \times S_0 - 0.4$	0.999	13.19 ± 0.02
Levomepromazine	$S' = 14.88 \times S_0 - 0.4$	0.999	14.88 ± 0.02
Chlorpromazine	$S' = 11.92 \times S_0 - 0.3$	0.999	11.92 ± 0.01
Thioridazine	$S' = 20.62 \times S_0 - 0.1$	0.999	20.62 ± 0.03
Prochlorperazine	$S' = 10.57 \times S_0 - 0.1$	0.999	10.57 ± 0.02
Fluphenazine	$S' = 15.64 \times S_0 - 0.2$	0.999	15.64 ± 0.02
Trifluoperazine	$S' = 12.88 \times S_0 - 0.3$	0.999	12.88 ± 0.01

[a] S', concentration after LPME; S_0, initial concentration in the sample.
[b] EF, enrichment factor; SD, standard deviation.

a volume of 10 mL of sample was selected as a compromise between sensitivity and extraction time.

3.1.3. Hydrodynamic variables

The sample flow rate directly affects the extraction of the analytes from the sample as it is closely related to the residence time of them in the extraction unit. This variable was studied within the interval 0.1–1.0 mL min^{-1} by passing 10 mL of a spiked urine sample (1 μg mL^{-1}) through the extraction unit. The results, depicted in Fig. 6, pointed out a clearly decrease of the peak areas for all the analytes with increasing flow rates through of the extraction times, as it is noted in the upper part of the figure.

In the selection of the optimum value for this variable, two facts have to be taken into account, namely: extraction efficiency and expeditiousness. In this sense, low flow rates provide higher extraction values at the expense of the extraction time. According to these conflicting factors, the optimum value was set at 0.5 mL min^{-1} as a compromise.

3.2. Analytical performance

Some analytical characteristics of the proposed method such as EF, linear range, correlation coefficient, limits of detection, precision, recovery and chromatographic retention times were investigated for each analyte.

The EF permits the evaluation of the global extraction efficiency of the method. It was calculated according to the next formula:

$$\mathrm{EF} = \frac{C'}{C_0}$$

where C' is the concentration of the analyte in the extracts obtained after dLPME and C_0 is the initial concentration in the sample. This equation can be also expressed in terms of signal considering the negligible contributions of the intercepts compared to the slopes values of the calibration graphs for all the analyte:

$$\mathrm{EF} = \frac{S'}{S_0}$$

Table 3
Figures of merit of the proposed method.

Analyte	LD[a]	LQ[b]	RSD[c]	RT ± SD[d]
Promethazine	47.7	159.2	3.3	17.3 ± 0.1
Levomepromazine	60.0	200.0	2.2	20.4 ± 0.1
Chlorpromazine	30.6	102.0	3.9	25.6 ± 0.1
Thioridazine	21.4	71.3	2.6	29.4 ± 0.1
Prochlorperazine	57.1	190.2	2.5	31.7 ± 0.1
Fluphenazine	32.4	108.1	2.7	33.3 ± 0.2
Trifluoperazine	33.6	112.2	2.7	36.7 ± 0.2

[a] Limit of detection in ng mL^{-1}.
[b] Limit of quantification in ng mL^{-1}.
[c] Relative standard deviation.
[d] RT, chromatographic retention time; SD, standard deviation.

Table 4
Recovery study of the proposed method for the determination of seven phenothiazine derivatives in four independent urine samples at four different levels of concentration.

Samples	Urine sample 1		Urine sample 2		Urine sample 3		Urine sample 4		Average values
Concentration added	2 μg mL^{-1}		1 μg mL^{-1}		0.4 μg mL^{-1}		0.2 μg mL^{-1}		
Analytes	Found[a]	R^b (%)	Found[a]	R^b (%)	Found[a]	R^b (%)	Found[a]	R^b (%)	R^b (%) ± SD[c]
Promethazine	1.99 ± 0.05	99.5	1.05 ± 0.03	105.0	0.37 ± 0.01	92.5	0.19 ± 0.01	95.0	98 ± 5
Levomepromazine	1.83 ± 0.04	91.5	0.92 ± 0.02	92.0	0.39 ± 0.01	97.5	0.18 ± 0.01	90.0	93 ± 3
Chlorpromazine	1.94 ± 0.06	97.0	0.94 ± 0.04	94.0	0.38 ± 0.01	95.0	0.17 ± 0.01	85.0	93 ± 5
Thioridazine	1.31 ± 0.03	65.5	0.75 ± 0.02	75.0	0.31 ± 0.01	77.5	0.14 ± 0.01	70.0	72 ± 5
Prochlorperazine	1.50 ± 0.03	75.0	0.73 ± 0.03	73.0	0.32 ± 0.01	80.0	0.14 ± 0.01	70.0	74 ± 4
Fluphenazine	1.44 ± 0.02	72.0	0.80 ± 0.01	80.0	0.32 ± 0.01	80.0	0.15 ± 0.01	75.0	77 ± 4
Trifluoperazine	1.34 ± 0.03	67.0	0.75 ± 0.02	75.0	0.30 ± 0.01	75.0	0.14 ± 0.01	70.0	72 ± 4

[a] Concentrations in μg mL^{-1} obtained for three replicates.
[b] Recovery percentage.
[c] Standard deviation.

where S_0 is obtained by direct injection of standards containing the analytes at a specific concentration (C_0) while S' is calculated by injecting the extracts obtained when these standards are processed by the dLPME procedure. S' is calculated for different initial concentrations (S_0), the results being adjusted to a linear equation in which the slope is the EF:

$$S' = \mathrm{EF} \times S_0 + a$$

Table 2 summarized the results obtained for all the phenothiazines derivatives assayed. The linear fittings obtained were acceptable ($R > 0.999$) which indicates that the EF remains constant through the whole concentration range evaluated. Moreover, the intercept values of the models were negligible compared with the slopes (in the range 1–0.1%). EFs varied between 10.57 (for prochlorperazine) and 20.62 (for thioridazine).

Table 3 compiles the figures of merit provided by the proposed method for the phenothiazines derivatives selected. The linear calibration graphs ($R > 0.999$ in all cases) were constructed by analysing standards containing the analytes at 7 concentration levels in the range 0.07–10 μg mL^{-1}. Prior to the dLPME, each standard was adjusted to the optimized pH and ionic strength values. The standards were extracted in the flow configuration and the resulted extracts were analyzed by liquid chromatography.

Limits of detection were calculated according to the S/N = 3 ratio using in this case spiked blank urine samples. The obtained values were in the range from 21 ng mL^{-1} (thioridazine) to 60 ng mL^{-1} (levomepromazine). The precision of the method, expressed as relative standard deviation, was calculated from five replicates of a urine sample containing the analytes at concentration of 0.5 μg mL^{-1}. The values varied between 2.2% (levomepromazine) and 3.9% (chlorpromazine).

The precision of the chromatographic retention times, expressed as relative standard deviation, varied between 0.3% and 0.6%.

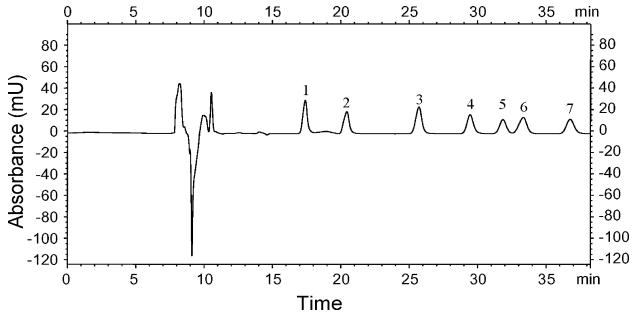

Fig. 7. Typical chromatogram obtained for a urine sample spiked with the seven phenothiazine derivatives: (1) promethazine; (2) levomepromazine; (3) chlorpromazine; (4) thioridazine; (5) prochlorperazine; (6) fluphenazine; (7) trifluoperazine.

3.3. Recovery study

In order to validate the proposed method, four independent free-analyte urine samples were spiked at four different concentration levels (0.2, 0.4, 1 and 2 μg mL^{-1}) and analyzed. The obtained peaks areas for each analyte were interpolated in the calibration graphs constructed using standards, the recovery value being calculated by the known equation: $\%R = [(\text{analyte found})/(\text{analyte added})] \times 100$. The obtained results, which are summarized in Table 4, are ranged in the interval 72–98%.

In Fig. 7 a chromatogram obtained for spiked urine sample (0.4 μg mL^{-1}) is presented. No interferences from endogenous matrix components were observed, which testifies to the high selectivity of the method developed. The absence of significant peaks coming from the matrix can be ascribed to the pH of the sample, the peak intensity being higher at acidic pH. On the other hand, cleaner extracts are obtained when the dLPME procedure is performed at alkaline condition.

4. Conclusions

According to the results presented, the developed method is a useful tool for the determination of seven phenothiazine derivatives in urine samples. In the same way, the ionic liquid 1-butyl-3-methyl imidazolium hexafluorophosphate has been proved to be an excellent choice for analytes isolation and preconcentration allowing an evident improvement in the selectivity and sensitivity of the HPLC method. In fact, the inherent properties of the ionic liquid such as viscosity, density and lower water solubility have been successfully exploited in the proposed dLPME procedure allowing the location of a small volume of the extractant in the extraction unit. Moreover, the high chemical affinity to the target analytes (especially to the aromatic rings present in it) permits the achievement of favourable analytical features for the selected application. In summary, compared with other analytical methods, the proposed one permits the obtaining of cleaner extracts (better selectivity) providing at the same time acceptable EFs for the targets analytes.

The method has been completely characterized studying in depth the influence of all the variables on the analytical signal. Furthermore, the methodology was successfully validated through a recovery study using four independent urine samples containing the analytes at four different concentration levels.

Acknowledgement

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