

Quantitative analysis of phenobarbital in biological fluids: Analyte enrichment by an electrically-assisted microextraction technique

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An electrically-assisted microextraction method called electromembrane extraction, followed by a simple high performance liquid chromatography and ultraviolet detection was developed and validated for determining phenobarbital in biological samples. The major parameters influencing the electromembrane extraction procedure including solvent composition, voltage, pH of acceptor and donor solutions, salt effect, and time of extraction were evaluated and optimized. The drug was extracted from the donor aqueous sample solution (pH 9) to the acceptor aqueous solution (pH 13). The donor and acceptor phases were separated by a hollow fiber dipped in 1-octanol as a supported liquid membrane. A voltage of 40 V during 20 minutes was applied as the driving force. The enrichment factor was obtained >51 which enhanced the sensitivity of the instrument. Limit of detection and limit of quantitation were 7.5 and 25 ng/mL, respectively. The method was linear over the range of 25-1000 ng/mL for phenobarbital ($R^2 > 0.9998$) with repeatability (%RSD) between 0.4% and 6.8% ($n = 3$). The proposed method was successfully applied to human plasma and urine samples with relative recovery of 70-80% and %RSD < 6.8%.

Keywords: Biological samples. Electromembrane extraction. HPLC-UV. Microextraction. Phenobarbital.

INTRODUCTION

Nowadays, application of new microextraction methods for analytical purposes has become an interesting alternative to conventional sample preparation methods in pharmaceutical analysis. Miniaturized microextraction techniques have been recently used in areas of pharmaceutical analysis, analytical toxicology, clinical pharmacology, environmental chemistry, and food analysis. (Sarafraz-Yazdi, Amiri, 2010; Rutkowska *et al.*, 2014; Flanagan *et al.*, 2006; Maham *et al.*, 2014; Ocaña-González *et al.*, 2016).

Electrically-assisted microextraction or electromembrane extraction (EME) has recently been introduced as a new miniaturized sample preparation technique for pharmaceutical and biomedical analysis (Pedersen-Bjergaard, Rasmussen, 2006; Balchen *et al.*, 2007). In this method, the charged analyte is extracted from donor solution into the acceptor solution. The donor and acceptor phases are separated by a hollow fiber dipped in

an organic solvent. This separator is called supported liquid membrane (SLM). In donor and acceptor solutions, the charged analytes are obtained by pH adjustment. A hollow fiber (HF) segment is first immersed in an organic solvent and then the lumen of HF is filled by the acceptor solution. A platinum electrode is placed in the donor solution and another platinum electrode is inserted into the acceptor solution inside the lumen of the hollow fiber. Meanwhile, an electric field or voltage is utilized as the driving force. This technique provides high analyte extraction within a short period of time without any time-consuming and expensive procedures. Use of very low volumes of an organic solvent makes this method environmentally friendly (Gjelstad, Pedersen-Bjergaard, 2011; Marothu, Gorrepati, Vusa, 2013; Gjelstad, Pedersen-Bjergaard, 2013; Yamini, Seidi, Rezazadeh, 2014; Huang *et al.*, 2015).

EME-combined analytical methods have been recently used for determining and quantifying drugs, dyes, amino acids, peptides, heavy metals, and environmental pollutants from different sample matrices (Huang *et al.*, 2015; Fotouhi *et al.*, 2015; Yaripour *et al.*, 2018; Asadi, Nojavan, 2016; Zahedi *et al.*, 2016; Eibak *et al.*, 2012; Yaripour, Mohammadi, Nojavan, 2016; Nojavan *et al.*, 2016; Kubán *et al.*, 2011; Villar-Navarro *et al.*, 2016).

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Phenobarbital (Figure 1) is an anticonvulsant drug which is useful in the treatment of partial seizures and generalized tonic-clonic seizures especially when attacks are difficult to control. Phenobarbital enhances GABA-mediated inhibition and reduces glutamate-mediated excitation. The therapeutic actions of the drug are relevant to the concentrations of phenobarbital in biological fluids (Katzung, Masters, Trevor, 2012). Therefore, the quantification of phenobarbital in biological samples is important for pharmacokinetic, therapeutic, and toxicological studies. The drug concentration measurement in body fluids as an aid to the management of drug therapy always requires a sample preparation step prior to being introduced into instrumental analysis.

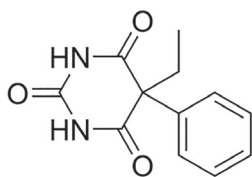


FIGURE 1 - Chemical structure of phenobarbital (Chemical formula: $C_{12}H_{12}N_2O_3$, Molecular weight: 232.2 g/mol, pKa: 7.4 and Log *P*: 1.5).

Several analytical methods such as HPLC (Ferreira *et al.*, 2014; Romanyshyn *et al.*, 1994; Asadi *et al.*, 2015), LC-MS (Karinen *et al.*, 2015; Hori *et al.*, 2006; Hoshina *et al.*, 2009; Qu *et al.*, 2016), and GC (Roveri, Paranhos, Yonamine, 2016; Menck de Almeida *et al.*, 2012; Queiroz *et al.*, 2002) were reported in literature for determining phenobarbital in different biological samples. Some of the utilized instruments such as LC-MS and LC-MS/MS are expensive and unavailable for analysis of biological samples in routine laboratories. Some of the reported methods require time-consuming and complicated sample preparation processes. Considering the advantages of electromembrane extraction technique, in the present research for the first time, a rapid and simple EME-HPLC-UV method was developed and validated for quantification of phenobarbital in human plasma and urine samples. The major parameters associated with the efficiency of EME were further evaluated and optimized.

MATERIAL AND METHODS

Chemicals and reagents

Phenobarbital BP (99.9%) was supplied by Nantong General Pharmaceutical Factory (Nantong, China). Acetonitrile, methanol, hydrochloric acid, sodium hydroxide, glacial acetic acid, ammonium

acetate, cetyl trimethylammonium bromide (CTAB), 2-ethylhexanol, 1-heptanol, and 1-octanol were purchased from Merck (Darmstadt, Germany). Accurel PP 300/1200 polypropylene hollow fiber with the inner diameter of 1200 μm , wall thickness of 300 μm , and pore size of 0.2 μm that was purchased from Membrana (Wuppertal, Germany). HPLC grade water to prepare all solutions was obtained using a Fistream cyclon system (Leicestershire, UK).

Stock and standard solutions and plasma sample

A stock solution containing 1 mg/mL of phenobarbital was prepared in methanol using ultrasonication and stored at 4 °C while kept off the light. Sample standard solutions were prepared via dilution of the stock solution using distilled water. The stock solution was also used to spike human plasma and urine in defined concentrations. Drug-free human plasma was obtained from the Iranian Blood Transfusion Organization (Tehran, Iran) and drug-free human urine was obtained from a 25-year-old male volunteer. The spiked biological samples were freshly prepared before the extraction.

HPLC conditions

The HPLC-UV analysis was carried out using a modular HPLC system consisted of a Knauer HPLC Pump K-1001, a UV Detector K-2600, a Knauer injection system and a Knauer solvent degasser (Berlin, Germany). Data acquisition was performed using ChromGate® Chromatography Data System Version 3.1.7 (Berlin, Germany). An HS-5 C8 Perkin Elmer column (5 μm particles, 125 mm \times 4.6 mm i.d.) (Waltham, USA) was used for chromatographic separation at 24 ± 2 °C. The mobile phase was a mixture of acetonitrile and 50 mM ammonium acetate buffer (pH = 6.8) which was used in a 35:65 ratio (v/v). The buffer was filtered through 0.45- μm membrane filter prior to use. The flow rate was 0.7 mL/min and UV detection was performed at 240 nm. The volume of injection was 10 μL .

Electromembrane extraction procedure

The DC power supply EPS-Universal model from Paya Pajohesh Pars Company (Tehran, Iran) was used to aid the EME procedure. A 3-mL homemade glass tube with a screw cap was used as an EME compartment. Platinum wires (diameter 0.2 mm) were utilized as the electrodes. Aliquots of 3 mL of the sample solutions were filled in the sample extraction vial. A 25-mm piece of

polypropylene hollow fiber was dipped into the organic solvent and sonicated for 1 min to impregnate the hollow fiber. The excess organic solvent was removed from the inner and outer of the hollow fiber through water washing by a micro-syringe. The lower end of the hollow fiber was closed by thermal and mechanical pressure and the lumen of the HF was filled with 20 μ L of the acceptor solution via a micro-syringe. The hollow fiber, including acceptor solution, was inserted into the sample solution. Based on the charge of the molecules, negative electrode (cathode) was placed in the sample solution while positive electrode (anode) was placed directly in the acceptor solution. Subsequently, the power supply was switched on and then the required voltage was applied across the electrodes for a pre-defined time. During all extractions, the sample solution was stirred using a stirrer at 1100 rpm. At the end of the extraction procedure, the acceptor solution was collected by a micro-syringe and analyzed by HPLC-UV.

Calculation of enrichment factor, extraction recovery and relative recovery

The enrichment factor (*EF*) is defined as the ratio of the final analyte concentration in the acceptor solution (C_a) to the initial concentration of analyte in the donor solution (C_d) which is calculated using equation 1:

$$EF = \frac{C_a}{C_d} \quad (1)$$

The extraction recovery (*ER*) is defined as the percentage of the number of moles of analyte originally present in the donor sample (n_d), which was extracted to the acceptor solution (n_a). *ER* is calculated according to equation 2:

$$ER = \frac{n_a}{n_d} \times 100 = \frac{C_a \times V_a}{C_d \times V_d} \times 100 = EF \left(\frac{V_a}{V_d} \right) \times 100 \quad (2)$$

The relative recovery (*RR*) is calculated for biological samples using equation 3:

$$RR = \frac{C_f - C_r}{C_s} \times 100 \quad (3)$$

where, C_f is the concentration of analyte found after addition of known amount of standard into the real sample, C_r is the concentration of analyte in real sample, and C_s is the concentration of known amount of standard, spiked into the real sample.

RESULTS AND DISCUSSION

In order to ensure the maximum extraction efficiency, the parameters influencing on the EME procedure including membrane composition, pH of donor and acceptor solutions, applied voltage, and extraction time were evaluated, and whereby suitable conditions for maximum extraction were obtained.

Supported liquid membrane (SLM) composition

The composition of the membrane may influence the extraction recovery by affecting the diffusion coefficient of the analyte of interest. The organic solvent used in EME should exhibit adequate electrical conductivity, low vapor pressure, and water immiscibility (Yamini, Seidi, Rezazadeh, 2014; Huang *et al.*, 2015). There are many solvents used in EME procedure reported in the literature. Phenobarbital is a weak acid and according to previous reports, acidic compounds are compatible with aliphatic alcohols in EME (Yamini, Seidi, Rezazadeh, 2014; Huang *et al.*, 2015). Solvents such as 1-octanol, 1-heptanol, and 2-ethylhexanol were evaluated as potential components of the SLM. The effect of addition of a cationic surfactant like cetyl trimethylammonium bromide (CTAB) as a carrier in SLM composition was also evaluated, with the results illustrated in Figure 2. The results indicated that 1-octanol is the best solvent offering the most efficient extraction. Addition of 1% (w/v) CTAB in 1-octanol led to elevated electrical current in the system up to 3 mA, and caused electrolysis and instability of EME.

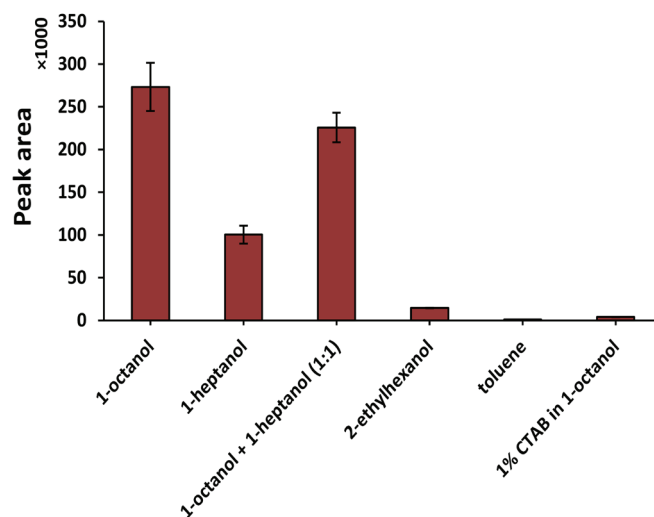


FIGURE 2 - Effect of SLM composition on EME efficiency (concentration of phenobarbital in donor: 1 μ g/mL, pH of donor: 8, pH of acceptor: 13, time of extraction: 10 min and voltage: 40 V).

pH of donor and acceptor solutions

In an EME procedure, the analyte should be in ionic form in the donor solution to ensure maximum extraction efficiency. The pH of donor and acceptor solutions can impact the ionic balance in the system. It was observed that the total ionic concentration in the donor solution to that in the acceptor solution (χ , ion balance) has a direct impact on the flux across the membrane (Gjelstad *et al.*, 2007). In order to evaluate the effect of pH, in the first step, the effect of pH (7-12) in the donor solution was evaluated whilst retaining the pH of the acceptor solution at 13. As presented in Figure 3A, the best pH for the donor solution was 9.0. In the second step, the effect of different pH values (10-13) in the acceptor solution was also evaluated whilst the donor solution pH was kept at 9.0. Based on the experiments, the maximum extraction efficiency was observed at a pH of 13 for the acceptor solution (Figure 3B). The pKa value of phenobarbital is 7.4 and it is a weak acid. Therefore, most of the molecules in the donor solution are likely to be ionized at pH 9.0 and above. The pHs higher than 9.0 resulted in greater ionization of the molecules, but the extraction of analytes diminished.

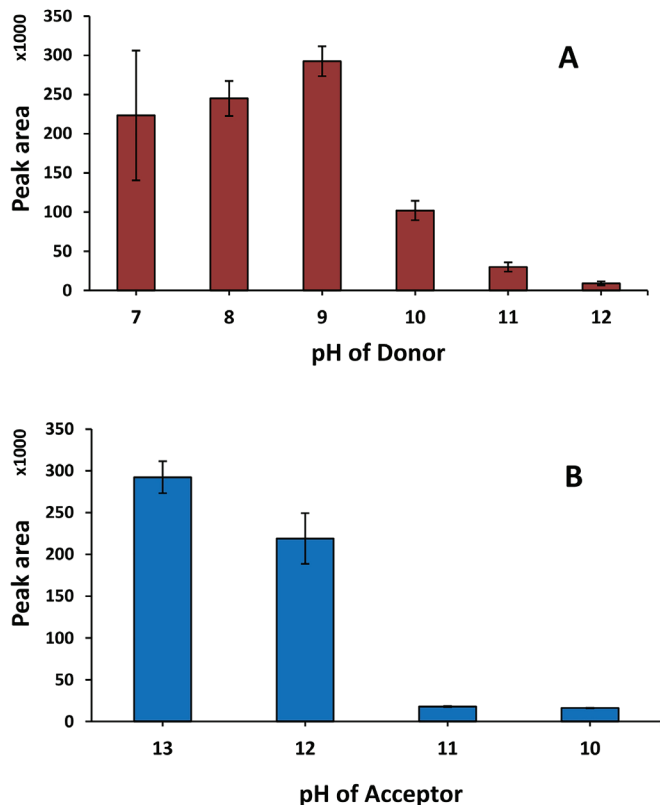


FIGURE 3 - Effect of (A) pH of donor solution, and (B) pH of acceptor solution on EME efficiency (extraction conditions; concentration in donor: 1 $\mu\text{g/mL}$, SLM: 1-octanol, pH, time of extraction: 10 min and voltage: 40 V).

Elevation of the donor pH seems to have caused enhanced ionic balance (χ) in the system. Therefore, the decrease in extraction was observed at higher pHs. Consequently, pH of 9.0 and 13 were selected for the donor and acceptor solutions, respectively. It seems that under this condition, effective ionization of molecules and minimal effective ion balance were established in EME system.

Applied voltage

In the EME method, voltage is the main driving force to transport the analytes across the liquid membrane. Indeed, the applied voltage affects the flux of analytes across the SLM (Yamini, Seidi, Rezazadeh, 2014). The extraction of phenobarbital was evaluated using different applied voltages (0 to 100 V), with the results depicted in Figure 4. The extractability of molecules grew as the voltage was elevated up to 40 V, but no further enhancement was observed using higher voltages. The growth of voltages led to amplification of the electric current in the system and resulted in electrolysis, bubble formation, and instability of EME. So, no further extraction efficiency was observed at voltages higher than 40 V. Accordingly, 40 V was selected as the optimum voltage and driving force for the subsequent experiments.

Extraction time

The effect of extraction time was also assessed to ensure the maximum extraction efficiency. As displayed in Figure 5, an increase in the extraction efficiency was observed up to the first 20 min, after which the extraction reached a steady state. Membrane saturation during EME procedure and loss of organic solvent in the SLM as a consequence of heat generation in the system were also the possible reasons of the diminished recovery (Yamini, Seidi, Rezazadeh, 2014; Huang *et al.*, 2015). Therefore, 20 min was selected for the extraction time.

Analytical method validation and biological sample analysis

To evaluate the performance of the proposed EME procedure for quantifying phenobarbital, the enrichment factor, extraction recovery, linearity, repeatability, limit of detection (LOD), limit of quantification (LOQ), and accuracy were determined using the optimized extraction conditions. The results are summarized in Table I. The enrichment factor (EF) and extraction recovery (ER) were 51 and 34, respectively. The LOD and LOQ were estimated according to signal-to-noise ratios of 3 and

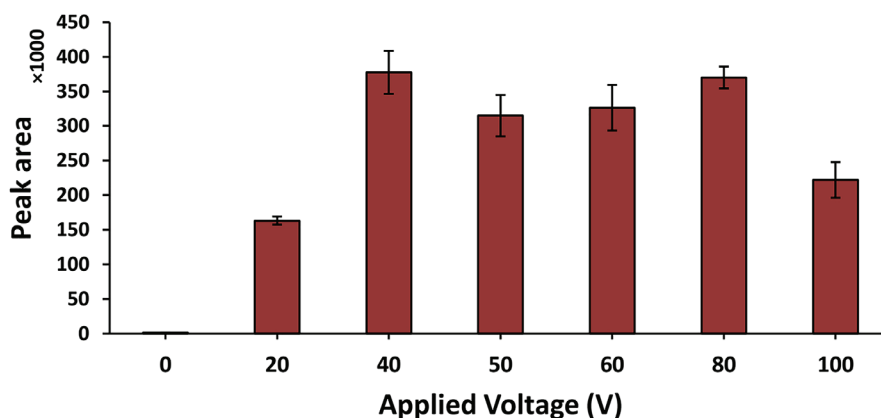


FIGURE 4 - Effect of voltage on EME efficiency (extraction conditions; concentration in donor: 1 $\mu\text{g/mL}$, SLM: 1-octanol, pH of donor: 9.0, pH of acceptor: 13 and time of extraction: 10 min).

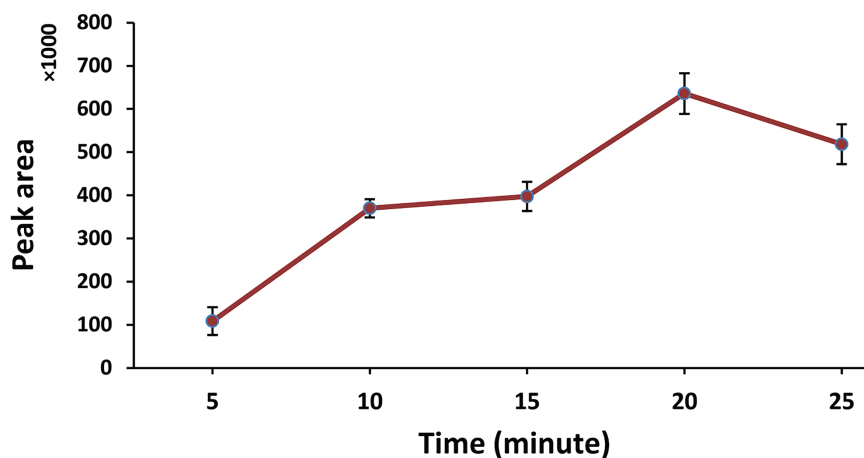


FIGURE 5 - Effect of extraction time on EME efficiency (extraction conditions; concentration in donor: 1 $\mu\text{g/mL}$, SLM: 1-octanol, pH of donor: 9.0, pH of acceptor: 13 and voltage: 40 V).

10, respectively. Five standard solutions were prepared for the calibration. The linearity was obtained within the range of 25-1000 ng/mL and the resultant R^2 value was 0.9998. The parameters of system suitability test for chromatographic separation were calculated and tabulated in Table II. The repeatability and relative error were determined at five different concentration levels, with these data summarized in Table III. The relative standard deviations (RSD%) ranged between 0.4% and 6.8%. The precision determined at each concentration level should not exceed 15% based on US FDA guidelines for bioanalytical method validation. A minimum of three concentrations within the range of expected concentrations is recommended for determination of accuracy. The mean should be $\pm 15\%$ of the actual value (US FDA Guidance for Industry, 2018). As can be seen in Table III, The precision and accuracy data have been acceptable for this EME-HPLC-UV method considering previously reported EME experimental data and reference acceptance criteria. The proposed EME method was applied for determining

phenobarbital in human plasma and urine samples to establish the applicability of the extraction method. In order to minimize the matrix effect, plasma and urine samples were diluted by distilled water in 1:19 ratio with pH adjusted to 9.0 before the EME process. In order to calculate the relative recovery, biological samples were spiked with defined concentrations of phenobarbital (100 ng/mL). Then, the relative recoveries were obtained as $70.9 \pm 4.8\%$ for plasma samples and $80.1 \pm 3.7\%$ for urine samples. The precision of the method for plasma and urine samples analyses was established as RSDs% following triplicate analyses ranging between 4.6 and 6.8%. Typical chromatograms following the EME of plasma and urine samples are demonstrated in Figure 6.

Short extraction and analysis time, consumption of small volumes of organic solvent ($<15 \mu\text{L}$), no need to time-consuming complicated sample preparation, as well as inexpensive and simple analytical technique all make this method highly practical for analyzing phenobarbital in biological fluids.

TABLE I - Figures of merit of EME-HPLC-UV analysis for phenobarbital

Calibration equation	Linearity (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Enrichment factor ^a	Extraction recovery ^a	RSD ^a % (n = 3)
$Y = 682.6X - 5703$	25-1000	0.9998	7.5	25	51	34	0.4

^aEnrichment factor, extraction recovery, and RSD% were calculated for determining phenobarbital at concentration of 500 ng/mL.

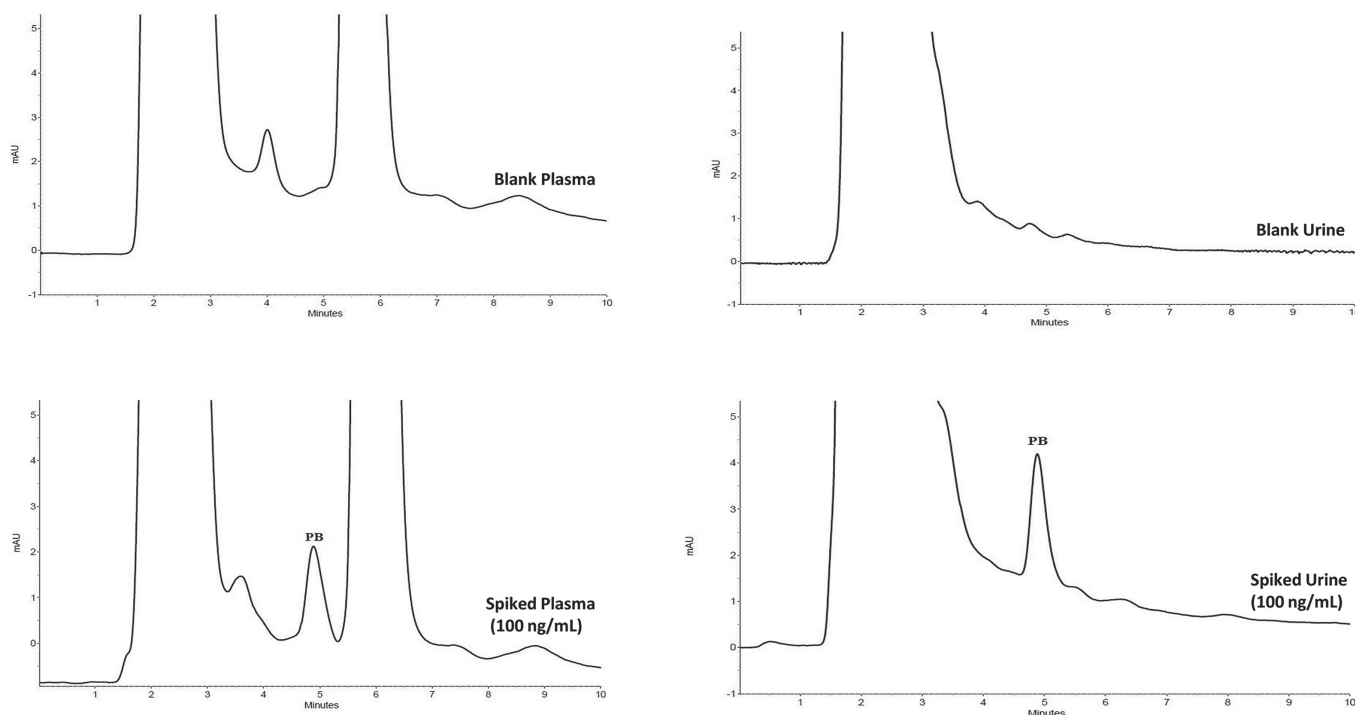
TABLE II - HPLC System suitability test

Parameter	Calculated data	Acceptance criteria
Resolution (with the nearest peak)	2.5	>2
Capacity factor	2.2	>2
Number of theoretical plate	2050	>2000
Asymmetry	1.3	<2
Repeatability of retention time	1	<2

TABLE III - Accuracy and precision data of the proposed EME-HPLC-UV method (n = 3)

Nominal concentration ^a	Measured concentration ^a (mean ± SD)	RSD ^b (%)	RE ^c (%)
25	27.4 ± 1.0	3.8	+9.4
50	53.4 ± 1.2	2.3	+6.8
100	99.0 ± 6.7	6.8	-1.0
500	490.6 ± 1.8	0.4	-1.9
1000	1004.5 ± 26.5	2.6	+0.4

^ang/mL, ^bRSD: Relative standard deviation, ^cRE: Relative error.

**FIGURE 6** - Typical chromatograms following the EME of plasma and urine samples (peak PB is phenobarbital).

Comparison of the proposed method to other analytical methods

Some analytical characteristics of developed method and other previously published methods for determining phenobarbital in various sample matrices

have been compared in Table IV. The obtained LOD in the presented method is comparable with those of the other mentioned methods. Repeatability of the developed method is satisfactory and its RSDs% are better than the others. In addition, time of extraction and sample preparation procedure in developed method is less than

TABLE IV - Comparison of the proposed method to other methods for determining phenobarbital

Method	Sample matrix	LR ^a (ng/mL)	LOD ^b (ng/mL)	RSD %	Time of sample preparation (min)	Sample treatment	Reference
SFO-DME-HPLC-UV ^c	Plasma, Urine	2-300	1	4.4	>75	Yes	Asadi <i>et al.</i> , 2015
MEPS-HPLC-DAD ^d	Plasma	200-40000	15	6.8-11.7	ND ^e	Yes	Ferreira <i>et al.</i> , 2014
RAM-MIP-CS-LC-MS/MS ^f	River water	5-500 ^g	5 ^g	1.9-4.6	ND	Yes	Hoshina <i>et al.</i> , 2009
PP-UPLC-MS/MS ^h	Blood	1200-46000	1160	3.9-20.4	>20	Yes	Karinen <i>et al.</i> , 2014
HF-LPME-GC-MS ⁱ	Blood	1000-10000	500	5.7-16.3	ND	Yes	Menck de Almeida <i>et al.</i> , 2012
EME-HPLC-UV ^j	Plasma, Urine	25-1000	7.5	0.4-6.8	20	No	This work

^a Linear range; ^b Limit of detection; ^c Solidified floating organic drop microextraction-high performance liquid chromatography-ultraviolet detection; ^d microextraction by packed sorbent-high-performance liquid chromatography-diode-array detection; ^e Not defined; ^f restricted access media-molecularly imprinted polymer- column switching-liquid chromatography-tandem mass spectrometry; ^g ng/L; ^h Protein precipitation-ultra performance liquid chromatography-tandem mass spectrometry; ⁱ Hollow-fiber liquid-phase microextraction-gas chromatography-mass spectrometry; ^j Electromembrane extraction- high performance liquid chromatography-ultraviolet detection.

the others. Some of the utilized instruments in those methods such as LC-MS and LC-MS/MS are expensive and unavailable for analysis of biological samples in routine laboratories. Some of the reported methods require time-consuming and complicated sample preparation processes and additional sample treatment steps such as filtration, sonication, precipitation, solvent evaporation, and reconstitution. The requirement of large volume of organic solvents, long time of sample treatment, and use of expensive sorbents (e.g. MIP, RAM, & C₁₈) are the major disadvantages of the mentioned methods. Overall our proposed method is easy-to-use, fast, sensitive, and environmentally friendly.

CONCLUSION

An efficient and simple EME-HPLC-UV method has been reported for the quantitative analysis of phenobarbital in human plasma and urine samples. Some parameters affecting the microextraction efficiency including SLM composition, pH of donor and acceptor solution, applied voltage, and time of extraction were evaluated and optimized. EME enriched the concentration of phenobarbital by more than 50-fold in samples and made the drug detectable at very low concentrations. The proposed EME-HPLC-UV method was a simple,

rapid, and environmentally friendly analytical method for determining phenobarbital in biological samples.

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