



Electromembrane extraction as a new approach for determination of free concentration of phenytoin in plasma using capillary electrophoresis

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Abstract

Purpose

Electromembrane extraction is a new membrane-based extraction method in which charged compounds are extracted by an electric field. So far, this method has been used to extract and isolate a variety of acidic and basic drugs from various samples, including blood and plasma. However, in this procedure, it is not yet clear whether only unbound fraction of a drug is extracted or the total drug. The aim of this study is to reveal the nature of drug extraction in the presence of plasma proteins.

Methods

To determine the nature of the extraction, the electromembrane extraction was performed from plasma solutions of phenytoin with concentrations 0.03 and 1.0 µg/mL, then the result was compared with the values obtained from the electromembrane extraction of ultrafiltrate of the same solutions (free concentration) and protein-free ultrafiltrate of plasma with final concentration of 0.03 and 1.0 µg/mL (total concentration). For this purpose, EME followed by capillary electrophoresis coupled with diode array detection was optimized and validated.

Results

The results showed that the electromembrane extraction method was only able to extract the unbound fraction of phenytoin from plasma samples. The method was validated over a concentration range of 0.03–4 µg/mL. The inter and intra-assay precisions were less than 6.7%. The phenytoin protein binding was also determined to be in agreement with the literature data and confirms the validity of this method.

Conclusion

This sensitive and quick EME approach for determining the free concentration of a phenytoin, can be a good alternative to classic methods for therapeutic drug monitoring and pharmacokinetic studies.

Keywords Antiepileptic · Microextraction · Plasma protein binding · Unbound fraction

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Introduction

Phenytoin (PHT) is one of the most widely used antiepileptic drug which is used in all types of epilepsy except absent seizure [1]. But since the PHT therapeutic index is narrow, therapeutic drug monitoring (TDM) is needed to reduce its side effects and increase therapeutic effects. Since the therapeutic range for total PHT is 10 to 20 µg/mL and protein binding approximately 90%, so the free concentration is in the range of 1.0 to 2.0 µg/mL [2]. For TDM, the drug concentration is measured in whole blood, serum or plasma samples. But given that drugs in the blood are available in both the free (unbound) and bound (by protein) fractions and only the free drug is pharmacologically active, as a result, the therapeutic range

of a drug is better expressed in terms of the concentration of free drug. Especially for high protein binding drugs like PHT [3, 4]. However, it is difficult to measure the free drug concentrations in these samples. Therefore, the therapeutic ranges of drugs are usually expressed in terms of total concentration. Nevertheless, in the case of PHT, several papers have been published indicating that the free concentration of PHT has a better correlation with the therapeutic efficacy and toxicity than the total concentration. Especially for patients with uremia and liver disease and in the presence of some known conditions, that is, hypercholesterolemia, hypoalbuminemia and interaction with other high protein-bound drugs [5–19].

Two steps are required to determination of free drug concentration. Firstly the separation of the free and bound fractions. Variety of methods are available for determining the free drug concentration in plasma, including equilibrium dialysis (ED), ultrafiltration (UF), ultracentrifugation (UC), microdialysis and parallel artificial membrane permeation assay (PAMPA). ED is the most widely used method to determination of free drug concentration and study of drug–protein interactions as the reference method. However, it suffers from many drawbacks, including long equilibration times (typically 12–48 h) [20], volume shift [21], nonspecific adsorption of drugs on the dialysis membrane [22] and the Donnan effect [23]. UF is a faster method than dialysis but like dialysis, there are several disadvantages such as the Donnan effect, nonspecific binding and protein leakage [24]. UC does not have the disadvantages of Donnan effect and membrane adsorption due to the absence of the membrane. However, the equipment used for UC is expensive. There are also some other disadvantages such as sedimentation and back diffusion [25, 26]. All techniques are well reviewed in the literature [24, 27]. So far, plasma free PHT concentration has been quantified by ED, UF and gel permeation chromatography methods [28–31]. PAMPA is a kind of three phase liquid-liquid microextraction in which compounds are extracted based on passive diffusion into the acceptor phase. PAMPA was originally devised to predict of drugs passive permeability through biological membranes. But, for the first time the use of a PAMPA to measure binding constants was reported by Lázaro et al. [32]. In this method, a thin liquid membrane coated on a porous filter is used to separate sample solution (donor phase) and buffer solution (acceptor phase) compartments. This method has several important advantages over the above mentioned methods including faster than traditional ED, no change in the volume of oncotic pressure due to water impermeable membrane and self-corrected nonspecific adsorption [24]. However, this method also takes a long operation time (about 2 h) [32].

The EME method was introduced for the first time in 2006 [33]. In EME, the charged compound is extracted from the donor aqueous phase to the acceptor aqueous phase under the influence of an electric field and through a supported liquid

membrane (SLM). EME like PAMPA is a three-phase liquid-liquid microextraction method, except where the electric field is applied. Consequently given that in the EME, compounds are extracted under electric field and by active diffusion, so EME is a fast and highly selective method for complex matrices such as whole blood and plasma compared to PAMPA [33]. On the other hand, EME due to the small volume of the acceptor phase yields large enrichment factors (EF) which increases the sensitivity of the method.

The second step for determination of free drug concentration is a separation and quantification of the desired drug in the presence of other substances. Spectroscopic methods, chromatographic and related techniques are commonly used for this purpose. The use of spectroscopic approaches is limited because of the low sensitivity and poor selectivity [22]. On the other hand; capillary electrophoretic techniques have advantages such as high efficiency and separation selectivity, low sample and reagent consumption, high speed of analysis and ease of automation that makes them a favorite method. However, capillary electrophoresis (CE) also has limitations, such as the risk of adsorption of protein on the capillary wall and also low sensitivity of commonly used UV detectors [24], but its combination with EME that has a high EF and efficient clean-up, eliminates these limitations.

According to aforementioned points, a method that does not have the disadvantages of membrane-based methods such as ED and UF is needed to separate free drug fraction. On the other hand, the method should be capable of concentrating the drug so that drugs that are in low concentration due to high protein binding or low dosage can be detected [31].

The aim of this study was to investigate the extraction mechanism in EME whether this method extracts only free drug fraction or whether in the presence of the electric field the bound fraction is removed from the protein and the whole drug is extracted. To this end, an experiment was designed to obtain the extraction mechanism. Therefore, a method for the determination of the free fraction of PHT in plasma was developed employing EME for separation and CE- diode array detection (CE-DAD) for quantitation of the unbound drug. As well as protein binding for PHT was obtained using this method.

Experimental

Chemicals and reagents

Sodium phenytoin was kindly supplied by Alhavi pharmaceutical company (Tehran, Iran). Analytical grade acetone, 1-octanol, ethanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from Shahid Ghazi Pharmaceutical Company (Tabriz, Iran).

Standard solutions and biological matrices

Two separate stock solutions of 1 mg/mL PHT in ethanol were used for preparation of calibration standards and quality control samples (QCs) and were stored at 4 °C protected from light. Drug-free human plasma was obtained from Iranian Blood Transfusion Organization (Tabriz, Iran), and was stored at –20 °C protected from light and thawed before use. Plasma dilution was used in the current study to minimize matrix effect. All working standard solutions of plasma and QCs were prepared daily by dilution of the stock solution in plasma:water (1:4, v:v).

Equipment for EME

The sample compartment was a 7 mL homemade glass, screw-capped vial with internal diameter of 10 mm and height of 7 cm. The porous hollow fiber used for housing the acceptor solution was a PP Q3/2 polypropylene hollow fiber (Membrana, Wuppertal, Germany) with an internal diameter of 0.6 mm, wall thickness of 200 µm, and a pore size of 0.2 µm. The DC power supply used was a PS858 model (S.KAR, Tabriz, Iran) with programmable voltage in the range of 0–300 V, and with a current output in the range 0–1000 mA. Platinum wires (diameter of 0.2 mm) with an average inter-electrode distance of 3 mm were used as electrodes in the sample and acceptor solutions and were connected to the power supply. A multimeter Model M890C⁺ (Zhangzhou Weihua Electronic Co., Ltd. China) was used for current monitoring during extraction. During the experiments, the sample solution was stirred using a 50×2 mm magnetic bar by a heater-magnetic stirrer model RCT basic from IKA Company (Germany).

EME procedure

Hollow fibers were cut into 40 mm pieces, washed with acetone in an ultrasonic bath and dried; the upper end of fiber was connected to a spinal needle tip as a guiding tube. Then, to impregnate the organic solvent in the pores of hollow fiber, the prepared fiber was placed in 1-octanol for 10 s. Excess of solvent in the lumen and surface of hollow fiber was then removed by blowing with a medical syringe and medical wipe, respectively. 10 µL of acceptor phase (pH 13.0 aqueous solution) was introduced into the lumen of the hollow fiber via the guiding tube, by a microsyringe and then the lower end of hollow fiber was sealed with a small piece of nonconductive plastic bar. 3.5 mL sample solution with pH 11.0 was filled into a glass vial (sample compartment). The hollow fiber and the negative electrode were inserted into the sample solution, and through a guiding tube, the positive electrode was located into the acceptor solution. Subsequently, the electrodes were connected to the power supply, and a voltage of

15 V was applied between two electrodes and the extraction was carried out for 15 min. During the extraction, the sample solution was agitated at 750 rpm. Finally, the hollow fiber was taken out and the acceptor solution was collected using a microsyringe and placed into a CE microvial to be injected into the CE system through the autosampler unit.

Capillary electrophoresis conditions

The CE analyses were performed on an Agilent capillary electrophoresis system equipped with a DAD (190–600 nm). Data acquisition was performed using ChemStation (Agilent Technologies). The separations were performed on a 48.5 cm bare fused silica capillary with 40 cm effective length and an internal diameter of 50 µm (Agilent Technology, Waldbronn, Germany). Prior to the first use, the capillary was washed with 1 M NaOH for 30 min, deionized water for 30 min and background electrolyte (BGE) solution for 30 min. At the beginning of each day, the capillary was washed with 0.1 M NaOH for 10 min, deionized water for 10 min and BGE solution for 10 min. Between runs, the capillary was flushed with 0.1 M NaOH for 2 min, deionized water for 2 min and BGE solution for 4 min. The capillary was flushed for 10 min with DI water and the capillary tips were kept inside DI water vials at the end of a working day. The optimal conditions of electrophoretic separation were as follow; the BGE solution was a 150 mM borate buffer adjusted to pH 9.3, sample injections were performed hydrodynamically at a pressure of 0.5 psi for 10 s. A constant voltage of +25 kV was applied during the analyses and the capillary temperature was set at 25 °C. Before use, all BGE and rinsing solutions were filtered through a 0.20 µm pore size PTFE syringe filter (Chromafil, Germany). Detection of the PHT was set at 200 nm.

Sample preparation for total and unbound PHT with UF

In order to obtain unbound PHT fraction, a 5 mL PHT-spiked plasma sample at two concentrations (0.03 and 1.0 µg/mL) with pH 11.0 was added to the sample reservoir of the UF with an Amicon Centrifree® Filter System (molecular weight cut off 30 kDa) to separate the unbound drug from the protein-bound drug in plasma. Subsequently, sample-filled devices were counterbalanced in a centrifuge rotor containing adapters. The samples were centrifuged at 5000 g for 10 min. After repeating this step for five times, approximately 20 mL of ultrafiltrate of free PHT was collected in the collection tube. The same route was taken to obtain protein-free ultrafiltrate with the difference that there was no PHT in the sample solution. The driving force for ultrafiltration was provided by centrifugation (Universal 320 centrifuge, Hettich, Germany). To investigate the nonspecific adsorption of ultrafiltration membranes, the aqueous solution of PHT with

concentration of 1 µg/mL (pH = 11.0) was injected directly into the CE before and after filtration and their peak areas was compared.

Determination of EME extractability from plasma samples for unbound and bound fractions

Some papers have suggested that applying an electric field in EME would reduce the protein binding of drug [34]. In other words, in the presence of an electric field the protein-bound fraction is detached from the protein and extracted by EME. But a large number of literature indicate that recovery for drugs with high protein binding are lower than for drugs with low protein binding [35–37]. This means that only the free fraction of the drug in plasma is extracted, not the total drug or it may be an intermediary between the two. To understand this, we designed an experiment that once EME was performed from the free PHT solutions (free drug) obtained in section (sample preparation for total and unbound PHT with UF). Once again, EME was performed from the total drug (PHT-spiked protein-free ultrafiltrate) solutions with a final concentrations of 0.03 and 1.0 µg/mL. In addition, these results were compared with the results obtained from EME of PHT from plasma samples at concentrations of 0.03 and 1.0 µg/mL. If the result obtained for the plasma sample is the same as that obtained for the free drug concentration, it shows that the EME only extracts unbound fraction. If the obtained value is greater, then it can be deduced that in the presence of the electric field the PHT is detached from the protein. Moreover, if it is equal to the value obtained for the total concentration of the PHT, that is, the electric field can detach all the protein bound drugs.

Calculation of plasma protein binding

By knowing the free and total concentrations of a drug in plasma, the plasma protein binding (PPB) can be calculated using:

$$PPB\% = \frac{C_{total} - C_{free}}{C_{total}} \times 100 \quad (1)$$

In section (determination of unbound fraction of PHT), it is shown that EME can extract unbound fraction of the drug, and only the free drug concentration can be extracted and measured by this method. Therefore, for the analysis of the unbound concentrations of PHT, EME was realized from plasma samples and for the analysis of total concentrations of PHT, protein-free ultrafiltrate of plasma sample was spiked with PHT at concentrations of 0.03 and 3.0 µg/mL and then EME was performed. As in dilute plasma solutions, the concentration of free drug is increased. So, in order to calculate PPB for undiluted plasma from the data obtained for diluted

plasma, Musteata et al. proposed a simple model for determination of drug plasma protein binding by solid phase microextraction (SPME). Due to the fact that the extraction bases in both SPME with liquid coatings and EME methods is similar and based on the distribution of the drug between the sample solution and the sorbent phase. Therefore, the same concepts will be used to calculate the protein binding here, which are as follows: considering whole plasma as a matrix of single binding proteins (P) with the concentration of C_p and the binding constant of K between drug (D) and matrix. Since the free and bound drug in the presence of P are in equilibrium, then the equilibrium equation for binding constant (K) can be written as follows [38]:

$$K = \frac{[PD]}{[D] \times [P]} = \frac{C_{total} - C_{free}}{C_{free} \times (C_p - C_{total} + C_{free})} \quad (2)$$

when the drug concentration (C_{total}) is very low compared to the protein concentration (C_p), ($C_{total} - C_{free} = C_{bound}$) is negligible with respect to (C_p) and Eq. 2 becomes

$$K = \frac{C_{total} - C_{free}}{C_{free} \times C_p} \quad (3)$$

This assumption applies in cases where the concentration of the drug is much lower than the concentration of proteins, in other words, in experiments where plasma dilution factor is low, such as in the present work. Some studies suggest that binding sites are not limited even at higher plasma dilutions and higher drug concentrations [38, 39]. From the rearrangement of Eq. 1 we have:

$$1 - \frac{PPB\%}{100} = \frac{C_{free}}{C_{total}} \quad (4)$$

by dividing the numerator and the denominator of the Eq. 3 by C_{free} then by inverting the sides of the equation:

$$\frac{C_{free}}{C_{total}} = \frac{1}{1 + (C_p \times K)} \quad (5)$$

Finally, the value of PPB% can be easily deduced from combining of Eqs. 4 and 5:

$$\begin{aligned} 1 - \frac{PPB\%}{100} &= \frac{C_{free}}{C_{total}} = \frac{1}{1 + (C_p \times K)} \Rightarrow PPB\% \\ &= \frac{100}{1 + \{1/(C_p \times K)\}} \end{aligned} \quad (6)$$

It is obvious that by diluting the plasma d times, C_p becomes C_p/d and Eq. 6 can be written as:

$$PPB_d\% = \frac{100}{1 + \{d/(C_p \times K)\}} \quad (7)$$

The value of $PPB_d\%$ can be determined experimentally for diluted plasma, and since the product $C_p \times K$ is the same in Eqs. 6 and 7; so, $C_p \times K$ can be obtained from Eq. 7 and then introduced into Eq. 6 for calculating $PPB\%$ for undiluted plasma:

From Eq. 7

$$C_p \times K = \frac{d \times PPB_d\%}{100 - PPB_d\%} \quad (8)$$

And by putting the equivalent of $C_p \times K$ in Eq. 6:

$$PPB\% = \frac{100 \times d \times PPB_d\%}{100 + \{PPB_d\% \times (d-1)\}} \quad (9)$$

As a result, the protein binding for undiluted plasma is easily obtained from Eq. 9 by the use of protein binding in diluted plasma sample.

Method validation

To quantify the unbound PHT fraction in plasma was necessary to validate bioanalytical method. The method was validated according to the ICH guidelines with respect to linearity, accuracy and precision, selectivity, specificity, recovery, dilution integrity, carry-over and stability [40].

Human samples

The method was applied to the analysis of PHT in plasma samples taken from patients receiving the drug. The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (code of ethics committee: IR.TBZMED.REC.1398.406) and competent authorities and performed in accordance with the Declaration of Helsinki. A signed written informed consent form was obtained from patients or their parents.

Results and discussion

Optimization of EME conditions

In a previous work, the variables affecting the EME of PHT from aqueous solution were investigated and optimum conditions were obtained [41]. In the present work, since the extraction was performed from the plasma, several experiments were tested to find new optimal conditions in this matrix. For this purpose, the one-factor-at-a-time (OFAT) strategy was used to investigate the effect of changing each of the variables on the response around the former optimal conditions. The results showed that the optimum conditions in the plasma sample did not differ from that of the aqueous sample (data not shown), so the same conditions were used to

continue. The optimal conditions are as follows: extraction time of 15 min, stirring speed of 750 rpm, pH of donor 11.0, pH of acceptor 13.0 and extraction voltage of 15 V.

Validation

Calibration curve and range

A calibration curve was plotted with eight calibration points ($n = 3$) over a concentration range of 0.03–4 $\mu\text{g/mL}$ for PHT. An acceptable linear range (0.03–4 $\mu\text{g/mL}$) and linearity of 0.9998 were obtained which covers the therapeutic range of free PHT concentration. The representative regression equation of PHT was $Y = 403.8X - 1.39$. A signal-to-noise ratio (S/N) of three was used for estimating limit of detection (LOD) and was 0.005 $\mu\text{g/mL}$. Lower limit of quantification (LLOQ) was the concentration providing accuracy and precision within $\pm 20\%$, and at least an S/N of 10. The upper limit of quantification (ULOQ) is the highest concentration of the calibration curve which its accuracy and precision should be within $\pm 20\%$. LLOQ and ULOQ were 0.03 and 4 $\mu\text{g/mL}$, respectively.

Selectivity and specificity

The selectivity of the method was determined using drug-free plasma from six different sources. For this purpose, the samples were extracted as described in section (EME procedure) and then analysed. The selectivity of the method was evaluated by comparing electropherograms of blank drug-free plasma samples and plasma spiked with PHT at LLOQ level. No interfering peaks of the endogenous components were found at the migration time of PHT in the electropherograms of blank samples. The specificity assay was established using four potentially co-administered drugs, which are used in neurosurgical patients, including ranitidine, dexamethasone, vancomycin and clindamycin. For this purpose, four drugs were spiked in drug-free plasma at concentration of 0.1 $\mu\text{g/mL}$ and then EME was performed. No interfering peak was observed for PHT.

Accuracy and precision

Repeatability (intra-day precision, $n = 5$), reproducibility (inter-day precision, $n = 15$) and accuracy of the method were evaluated at three concentration levels of QC samples (LLOQ, LQC and ULOQ). Acceptable accuracy (bias within $\pm 15\%$ or $\pm 20\%$ at the LLOQ) and precision ($RSD \leq 15\%$ or $\leq 20\%$ at the LLOQ) for the determination of PHT in plasma were demonstrated. The results are reported in Table 1.

Recovery

The absolute recovery of PHT in plasma was calculated by comparing the peak areas ratio of spiked samples before and after the extraction at two QC levels (LLOQ and ULOQ, $n = 5$). For this purpose, the acceptor solution of EME at concentrations of 0.03 and 4 $\mu\text{g/mL}$ were injected directly into the CE. Then, the plasma solutions with the same concentrations were injected after EME. The mean recovery and its corresponding EF were 8% and 28, respectively.

Carry-over

The carryover was assessed by analysing of three EME from blank plasma samples after the analysing of the three EME from highest calibration sample (ULOQ). No peak was observed at the migration time of PHT. Therefore, no carryover was detected for the PHT.

Dilution integrity

As regards, in current work diluted plasma solution (plasma:water, 1:4, v:v) was used in all experiments. So, the integrity of the dilution should be monitored. For this purpose, first, a solution of whole plasma was prepared at concentration of 20 $\mu\text{g/mL}$ of PHT, then 1 mL of this solution was diluted to final volume 5 mL by water and pH was adjusted to 11.0. Subsequently, EME was performed from this diluted solution (five replicate). The results obtained were precise (2.3%) and accurate (1.6% bias).

The stability of PHT in different conditions

1 Stability of stock solution

The stability of stock solution of PHT was assessed by the direct injection of a freshly diluted solution ($t = 0$) and diluted solution from stock solution after 30 days storage

at 4 °C with a concentration of 1 $\mu\text{g/mL}$ and then the detector response was compared for them ($n = 5$). A paired t-test was used to compare the mean of the measurements at the confidence level of 95%. The results showed that there was no significant difference between the averages and the stock solution was stable for at least one month.

2 Bench top and freeze thaw stability

The stability of PHT in plasma was investigated after QC samples had been exposed to two different conditions, including storage at room temperature (30 °C) for 5 h and after four freeze-thaw cycles. For freeze-thaw, the QC samples were frozen at -20 °C for a minimum of 12 h and then thawed at room temperature for 2 h. By analysing two different QC samples (LQC and ULOQ; five replicates per level) under these condition, the stability of PHT was demonstrated and the precision (RSD %) and accuracy (relative error) are allowed within $\pm 15\%$. The results are shown in Table 2.

Application to clinical samples

The current method for the extraction of unbound fraction of PHT, as well as the CE-DAD methodology for the quantification of unbound concentration of PHT have been successfully applied to the samples from the neurosurgery patients. Blood samples from patients were collected into sodium citrate tubes after 1 h of PHT administration in the morning. Immediately after collection, the samples were centrifuged at 6000 rpm for 15 min. The obtained plasma was then subjected to EME. Concentrations of the PHT were obtained in patients in the range of 0.2–1.1 $\mu\text{g/mL}$. Figure 1 shows the electropherograms obtained for patients.

Table 1 Precision and accuracy for EME of PHT from plasma samples

	Nominal concentration ($\mu\text{g/mL}$)	Measured concentration (mean \pm SD)	RSD%	RE% ^a
Intra-day ($n = 5$) ^b	0.03	0.033 \pm 0.0012	3.63	+10.00
	0.10	0.088 \pm 0.0059	6.70	–12.00
	4.00	4.00 \pm 0.064	1.60	0.00
Inter-day ($n = 3$) ^c	0.03	0.032 \pm 0.0016	5.00	+6.67
	0.10	0.089 \pm 0.0057	6.40	–11.00
	4.00	4.01 \pm 0.089	2.21	+0.25

$$^a RE = \left[\frac{\text{measured value} - \text{true value}}{\text{true value}} \right] \times 100$$

^b n = Sample number

^c n = Day number

Table 2 Results of stability for the analysis of unbound PHT in human plasma

Storage condition	Nominal concentration (µg/mL)	Calculated concentration (µg/mL)	Relative error (RE %)	Precision (RSD %)
–20 °C /4 freeze-thaw cycles	0.10	0.09	–10.00	6.30
	4.00	4.28	+7.00	3.00
5 h at room temperature	0.10	0.092	–8.00	5.70
	4.00	4.17	+4.25	3.20

Determination of unbound fraction of PHT

The results of experiments in section (determination of EME extractability from plasma samples for unbound and bound fractions) showed that the extraction values for PHT from PHT-spiked plasma and ultrafiltrate of PHT-spiked plasma (free drug) were the same. The equality of these two values indicates that EME can only extract the free fraction of the drug and has no effect on the bound fraction. Thus, EME is a method for determination of the free concentration of drug in the plasma sample. In this respect, it has advantages over conventional methods. As in this method, organic solvent is used as SLM which is immiscible with water, so the water cannot pass through it and thus there will be no change in the donor and acceptor volume due to the flow of water under oncotic pressure. As a result, given that all calculations performed through the calibration curve under optimal conditions, non-specific binding to the SLM are self-corrected and will not affect the results. On the other hand, applying the electric field in the extraction process increases the extraction kinetics and thereby decreases the extraction time and also increases the selectivity of the method. According to the mentioned characteristics, EME can be a suitable technique to extract free concentration of the drug from other biological samples such as whole blood and urine. Due to the low volume of the acceptor phase used in this method, a high EF is obtained which enables the coupling of this method to instruments with low sensitivity detectors. This feature is especially important for drugs with high protein binding that their free concentration is very low.

Protein binding determination

From Eq. 6, it can be seen that at very low concentrations of drug relative to protein, the value of PPB is independent of drug concentration and only depends on the protein concentration. Subsequently, PPB changes significantly when protein concentration changes. Also, most binding studies indicate the expected decrease in PPB when plasma is diluted [42–45]. As a result of dilution, the plasma protein concentration decreases, leading to increased free drug concentration and reduced protein binding. Plasma dilution was used in the current study to reduce the matrix effect. Based on what is described in section

(determination of EME extractability from plasma samples for unbound and bound fractions), the free PHT concentrations were obtained by EME from plasma samples at concentrations 0.03 and 1.0 µg/mL and the total concentration of PHT was obtained by EME from PHT-spiked protein-free ultrafiltrate of plasma solutions at concentrations 0.03 and 1.0 µg/mL. Consequently, with the free and total concentration of the PHT, PPB% can be obtained using Eq.1 which in this work represents the extraction in diluted solution, then Eq. 9 can be used to convert the obtained value into protein binding in

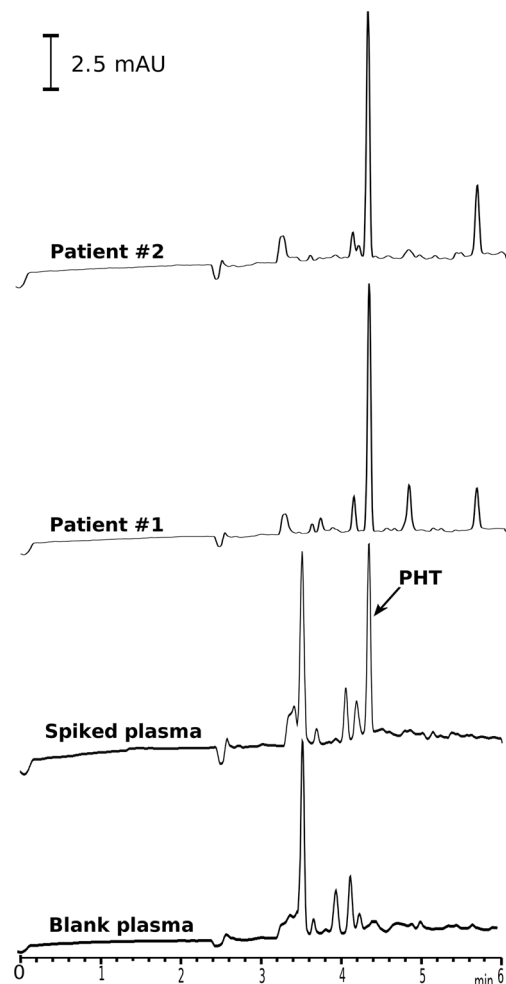


Fig. 1 Electropherograms of blank plasma sample, spiked plasma sample with PHT and real plasma samples of patients after EME under optimal conditions

Table 3 Comparison of the EME-CE-DAD method with other methods for determination of the PHT in plasma samples

Method	Linear range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	RSD%	Ref.
DLPME-SI-SFOD-HPLC-UV ^a	2.0–400	0.65	2.20	4.10	[50]
DMSPE-HPLC-UV ^b	3.0–500	0.80	2.80	4.50	[51]
MEPS-HPLC-DAD ^c	0.3–30	0.05	0.30	< 6.20	[52]
MIPSPE-HPLC-UV ^d	2.5–40	—	2.50	< 12.80	[53]
SFODM-HPLC-UV ^e	1.0–200	0.30	1.00	4.60	[54]
SPME-HPLC-UV ^f	2.0–40	2.00	5.00	< 15	[55]
SPME-GC-TSD ^g	0.05–25	—	0.05	< 6.77	[56]
EME-CE-DAD ^h	0.03–4.0	0.005	0.03	< 6.70	This work

^a Dispersive liquid phase microextraction-sequential injection-solidified floating organic drop-high performance liquid chromatography-ultraviolet

^b Dispersive micro-solid phase extraction-high performance liquid chromatography-ultraviolet

^c Microextraction by packed sorbent-high performance liquid chromatography-diode array detection

^d Molecularly imprinted solid-phase extraction-high performance liquid chromatography-ultraviolet

^e Solidified floating organic drop microextraction-high performance liquid chromatography-ultraviolet

^f Solid-phase microextraction-high performance liquid chromatography-ultraviolet

^g Solid-phase microextraction-gas chromatography-thermionic specific detector

^h Electromembrane extraction-capillary electrophoresis-diode array detection

undiluted plasma. As mentioned, first the free and total concentrations of PHT were determined by EME in diluted plasma samples at concentrations 0.03 and 1.0 $\mu\text{g/mL}$, then by using Eq. 1 the protein binding for PHT was obtained 65.40 and 68.75, respectively. Subsequently, by placing these values instead of $PPB_d\%$ and 5 (dilution factor) instead of d in Eq. 9 protein binding was obtained 90.43 and 91.66, respectively for undiluted plasma. According to the results of protein binding data, it is observed that by diluting, the concentration of plasma proteins decreases and as a result, $PPB\%$ decreases. Therefore, corrected equations should be used in studies with protein binding in diluted plasma solutions. $PPB\%$ values obtained with the current EME method (90.43 and 91.66) correlate well with average literature values [46–49]. Also, based on the results, no non-specific adsorption of the PHT to UF membrane was observed (data not shown).

Comparison of the proposed method with others

Comparison of important analytical parameters of the developed EME-CE-DAD method with those reported in the literature for measuring PHT in plasma samples (Table 3) shows that the sensitivity of this method is higher than other methods and also exhibits wide dynamic linear range and good repeatability (RSD%). Hence, the proposed method can be suitable for TDM.

Conclusions

EME method for the measurement of free PHT fraction in human plasma was successfully developed and validated.

Due to the clinical importance of therapeutic drug monitoring in patients as well as pharmacokinetic studies of drugs in both drug discovery and in clinical phases of drug development, there is an increasing need to measurement of drug free concentration. This study details a new approach for determining the free fraction of drug based on the partitioning of a drug between a hollow fiber and plasma matrix. As regards in EME, a water-immiscible membrane is used, as a result, some of the most important limitations of currently available methods such as volume shift and protein leakage, for determination free concentration of drugs have been eliminated. On the other hand, due to the use of electric field in this method, the extraction time is much shorter than other methods. Also using EME, the protein binding for PHT was obtained which indicates the accurate estimation of this method.

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Compliance with ethical standards

Conflict of interest The authors claim no conflict of interest.

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