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Three phase liquid phase microextraction of phenylacetic acid and phenylpropionic acid from biological fluids

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Abstract

Three phase liquid phase microextraction (three phase LPME) technique coupled with HPLC-UV has been applied as a sensitive and efficient sample preparation method to determine phenylacetic acid (PAA) as a biomarker of depressive disorders and phenylpropionic acid (PPA) in biological fluids. The compounds were extracted from 3.0 ml aqueous solution with the adjustment of pH at a fixed value in the range of 2.0–3.5 (donor solution) into an organic phase (1-hexanol) layered on the surface of the donor solution and finally back-extracted into 4.0 μl of the acceptor microdrop (pH 11.1) located at the end of the microsyringe needle. After a prescribed back-extraction time, the acceptor microdrop was withdrawn into the microsyringe and then directly injected into the HPLC system. In order to achieve maximum extraction efficiency, different parameters affecting the extraction conditions were optimized. At the optimum conditions (donor solution: 2.3 M Na_2SO_4, pH 2.0–3.5; organic membrane: 95 μl of 1-hexanol; acceptor solution: 4.0 μl of 0.1 M NH_3/NH_4^+ with pH 11.1; donor solution temperature: 45–50 °C; extraction time: 20 min and back-extraction time: 12 min), up to 110-fold enrichment factor was obtained. The calibration curve for these analytes was linear in the range of 1–5000 μg/l with $r^2 > 0.998$. The intraday and interday RSD% were below 6.5% and the limits of detection (LODs) for both analytes were 0.2 μg/l (based on S/N = 3). The proposed technique is a low cost, simple and sensitive method with highly clean-up effect. Finally, this technique was successfully utilized for the detection of target analytes in human urine, serum and plasma.

Keywords: Three phase liquid phase microextraction; Phenylacetic acid; Phenylpropionic acid; High performance liquid chromatography

1. Introduction

Phenyl ethylamine (PEA), an endogenous neuroamine, is similar in structure and behavioral pharmacology to the psychostimulant, amphetamine. It improves concentration, elevates mood and alleviates depression as rapidly as amphetamine but does not produce tolerance. An abnormal brain PEA metabolism has been proposed as an etiological factor in some forms of schizophrenia and major depression [1–3].

It has been proved that monoamine oxidase B selectively metabolizes PEA to phenylacetic acid (PAA) [1]. Since PEA turnover is very fast and PAA levels in biological fluids are far higher compared to PEA levels, it has been suggested that PAA excretion is a better measure than PEA for examining the modulatory role of PEA [1]. PAA is markedly reduced in the biological fluids of unipolar and bipolar depressed and schizophrenia subjects and increased in schizoaffective subjects following the administration of antidepressants [4–8]. Also, the intestinal bacterial action on phenylalanine causes the appearance of phenylacetate and phenylpropionate in urine. They are products of unidentified specific strains of bacteria, marking a state of bacterial overgrowth when they appear elevated in urine [9].

Free PAA plasma levels in humans range between 30 and 300 μg/l (0.22–2.2 μmol/l) [10]. Mean total plasma PAA concentrations obtained by Gusovsky et al. were 536.18 ± 54.99 μg/l for a healthy population ($N = 10$) and 327.64 ± 45.44 μg/l in the depressed patients. Also, Sabeli et al. studied a 24 h urinary PAA excretion in 48 healthy volunteers (141.1 ± 10.2 mg PAA/24 h) and in 144 patients with major depression (78.2 ± 41.0 mg PAA/24 h) [7,11].

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It is known that normal urine and normal phenylketonuric urine contain very little phenylpropionic acid. Whereas a single urine specimen from an atypical phenylketonuric patient contains a large amount of phenylpropionic acid (87 mg/l for a typical phenylketonuric 3 months patient), probably due to the action of gut bacteria on poorly absorbed phenylalanine and escaped oxidation because of a temporary overloading of the oxidation pathways [12].

The most common procedure for PAA measurement is liquid–liquid extraction into an organic solvent and final analysis by GC or HPLC [11,13,14]. This method has some disadvantages such as high consumption of organic solvent and also loss of PAA due to volatilization as the organic solvent is dried. Other methods have been reported for PAA measurement including anion-exchange resin separation followed by UV detection [15], ion pair chromatography [16], GC/MS [17], column-switching HPLC after PAA derivatization [18] and using Nile blue (NB) as a precolumn derivatization reagent followed by HPLC-VDLIF (visible diode laser induced fluorescence) [2]. However, these methods generally involve long extraction procedures or require expensive equipment. Thus, a simple, reliable and reproducible method for the determination of PAA concentration in human blood and urine is needed for screening monitoring of both healthy and depressed subjects.

Recently a miniaturized format of LLE, called liquid-phase microextraction (LPME), has been developed to reduce solvent usage in sample preparation [19]. Like solid phase extraction (SPE), LPME is not an exhaustive extraction procedure and only a small fraction of the analytes is extracted for analysis. LPME can be classified as two phase and three phase microextraction [20–25]. Three phase LPME is performed as hollow fiber or droplet based mode. In hollow fiber based three phase LPME, the analytes are first extracted from an aqueous sample matrix into the thin layer of the organic phase inside the wall pores of a hollow fiber and then back-extracted into the acceptor phase located inside the hollow fiber. In three phase LPME, based on the hanging droplets, the receiving phase is a microdrop of aqueous phase suspended from the tip of a microsyringe and located inside the organic phase [25–28].

Three phase LPME is a simple, fast and inexpensive technique. In this method, high preconcentration may be achieved because the analytes are transferred by passive diffusion from a relatively large sample volume (1–5 ml) into a microdrop (2–50 μl). It uses minimal amounts of organic solvent that enables the extraction and concentration steps to be carried out simultaneously [29,30]. An important advantage of three phase LPME is excellent clean-up that enables the extraction of analytes from complex matrixes such as biological fluids.

Since the excretion of PAA alters in serious illnesses such as schizophernia, phenylketonuria and major depressive disorders and due to the importance of PPA in some forms of phenylketonuria, availability of a simple method to measure PAA contents seems to be clinically significant. In the present study, three phase LPME was applied combined with HPLC for the extraction and preconcentration of phenylacetic acid (PAA, $pK_a = 4.31$, $\log P_{o/w} = 1.41$) and phenylpropionic acid (PPA, $pK_a = 4.66$, $\log P_{o/w} = 1.84$) from biological liquids. Due to relatively high preconcentration factor that can be achieved by the proposed method, it is possible to dilute the samples and simplify their matrixes prior to extraction.

In order to have high extraction efficiency different factors, affecting the extraction, were studied and optimized by the sequential single factor analysis method. Finally, the proposed method was used to extract PAA and PPA from human urine, serum and plasma to demonstrate its feasibility.

2. Experimental

2.1. Chemicals and reagents

Phenylacetic acid (PAA), 3-phenylpropionic acid (PPA) and HPLC grade acetonitrile were obtained from Merck (Darmstadt, Germany), 1-naphthalene acetic acid (NAA) was purchased from Sigma (St. Louis, MO., USA) and HPLC grade methanol was bought from Caledon (Georgetown, Ont., Canada). All the other reagents were of analytical grade and obtained from Merck. The water used was purified on a Younglin ultrapure water purification system (Aqua MAX^{TM} – Ultra, Korea).

Proper amounts of PAA and PPA were separately dissolved in deionized water to obtain stock solutions of each analyte with a concentration of 250 mg/l. Working standard solutions were freshly prepared by diluting the mixed standard solution of the analytes with the deionized water to the required concentration. Also a stock solution of 1-naphthalene acetic acid (NAA) with the concentration of 1000 mg/l was prepared in 40% (v/v) methanol: H_2O, and secondary stock solution of NAA with the concentration of 100 mg/l was prepared by diluting this solution with the deionized water. All the stock solutions were stored at 4 °C and kept stable at least for 4 weeks. Concentration of the analytes in the preliminary optimization experiments was 1 mg/l and after the achievement of suitable enrichment factor, it was reduced to 0.2 mg/l.

2.2. HPLC system

Separation and determination of the analytes were performed on the Waters HPLC system (Millipore, Co, Milford, MA, USA), consisted of a Rheodyne 7725 injector (Cotati, CA, USA) equipped with a 5 μl sample loop, a Waters 600E system controller and a Waters 486 tunable absorbance detector. Chromatographic data were recorded and analysed using a Waters 746 data module integrator. Separations were accomplished using a C_{18} Teknokroma analytical column (150 mm × 4.6 mm i.d., Barcelona, Spain) packed with 5 μm particles. All the chromatographic separations were performed in isocratic mode at room temperature. The mobile phase was a mixture of acetonitrile, methanol, ortho phosphoric acid and water (15:30:0.1:55, v/v). It was prepared daily, filtered, degassed before use and delivered at the flow rate of 1 ml/min and detection wavelength of 210 nm.

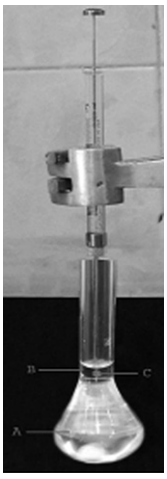

Fig. 1. A schematic diagram of the extraction device: (A) donor solution (B) organic membrane (C) acceptor phase.

2.3. Three phase LPME procedure

The basic experimental setup is shown in Fig. 1. The sample vial was manufactured by modifying a 5 ml volumetric flask using an 8 mm I.D. glass tube in the neck of the volumetric flask in order to reduce the consumption of organic solvent. The sample solution (donor phase), containing PAA and PPA in known trace concentrations, 100 μg/l NAA as internal standard and 2.3 M Na_2SO_4, was adjusted to be acidic with the pH range of 2.0–3.5 by adding HCl. Also the acceptor phase, containing 0.1 M NH_3, was adjusted to be basic by adding NaOH (pH 11.1) in order to ionize the analytes. Briefly, three phase LPME consisted of the following steps: (1) a water bath was placed on a Hidolph magnetic stirring plate (MR 3001 K, Kelheim, Germany) and the water temperature was maintained at 45–50 °C; (2) a 3 ml aliquot of the sample solution was placed in the sample vial with a 6 mm × 3 mm magnetic stirrer bar; (3) 95 μl of the organic membrane solvent (1-hexanol) was withdrawn into a 250 μl Hamilton syringe (Bondaduz, Switzerland) and carefully added on the top of the sample solution. The organic solvent was immiscible with the water, thus it served as an efficient barrier between the donor phase and the acceptor phase; (4) a piece of aluminum foil was used to cover the sample vial in order to prevent evaporation of the organic membrane; (5) the stirrer was then switched on and the stirring speed was set at 1200 rpm. The sample was stirred for 20 min to facilitate mass transfer of the analytes from the donor to the acceptor phase (extraction time); (6) after extraction time out, 5 μl aliquot of the acceptor solution was withdrawn into a 25 μl flat-cut Hamilton HPLC syringe. This syringe was employed to suspend the microdrop of the acceptor solution in the organic phase and also for the injection of the extractant into the HPLC system; (7) the syringe needle was inserted into the organic phase and the syringe plunger was then slowly pushed out to form a 4 μl aqueous microdrop in the organic membrane phase; (8) when the desired back-extraction time was elapsed, the stirrer was switched off and the plunger was slowly withdrawn to take the aqueous microdrop back into the needle; (9) finally, the entire analyte-enriched extractant was injected into the HPLC system.

3. Results and discussion

3.1. Basic principles

The important aims of three phase LPME are to separate and clean-up the analytes from the complex matrixes and to provide efficient preconcentration prior to HPLC or CE determination. It involves the extraction of ionizable compounds from the aqueous sample (donor phase) at suitable pH into the organic solvent (organic membrane) immiscible with water and layered over the donor phase, followed by back-extraction into the receiving aqueous phase (acceptor phase) by adjusting the pH on the desired value.

In this study, prior to the extraction owing to the acidic property of the analytes, the donor phase solution was adjusted to acidic condition (at a fixed pH in the range of 2.0–3.5), so that the analytes were deionized and their solubility in the sample solution was reduced. Under stirring, the neutral analytes were extracted into the organic membrane, layered over the donor phase. After 20 min, a microdrop of the acceptor phase (pH 11.1) was suspended in the organic phase, at the same time back-extraction of the analytes into the microdrop occurred. Due to basic nature of the acceptor solution, the analytes were ionized at the interface of the organic and acceptor phases and transferred into the acceptor solution [31]. Since the volume of the acceptor solution was very small comparing to the donor solution; the target compounds were preconcentrated in the aqueous acceptor phase.

3.2. Method development

In the proposed procedure, to achieve maximum extraction efficiency, various parameters affecting the extraction efficiency were optimized using sequential single factor analysis approach. The preliminary experiments showed that it was better to use an internal standard in the donor phase. The results showed very good repeatabilities with RSDs lower than 6.5%.

Optimization of the parameters was performed based on the absolute signal (peak area) of each analyte. Then NAA was used as internal standard in the donor phase and quantification occurred at the optimum conditions in the presence of the internal standard.

3.2.1. Selection of organic solvent

The choice of proper organic solvent in the three phase LPME is of great importance. Proper organic solvent has to satisfy the following requirements: (1) solubility of the analytes in the organic solvent should be higher than in the donor phase but lower than in the acceptor phase so that the analytes could be transferred from the donor into the acceptor phase with high extraction efficiency [32,33]; (2) it should be of low volatility to prevent solvent loss and be immiscible with water to avoid dissolution during the extraction and serve as a barrier between the donor and acceptor phases; (3) solvents with low viscosity are preferred due to larger diffusion coefficient of the analytes; and finally (4) the solvent should have no toxicity.

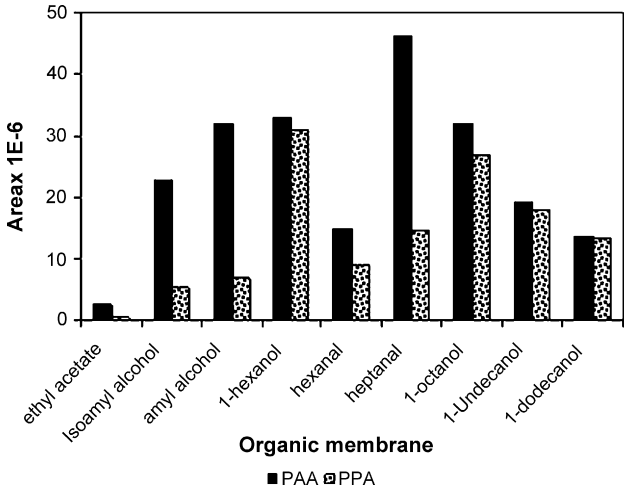

Fig. 2. Effect of the organic solvent on the extraction of analytes: Extraction conditions: 1 mg/l of the analytes; donor phase: 3 ml of the solution containing 1.0 M Na_2SO_4 (pH < 1); donor phase temperature: 50 °C; organic phase: 150 μl of 1-hexanol; acceptor phase: 3 μl of the aqueous solution (pH > 11.5); extraction time: 15 min; back-extraction time: 8 min.

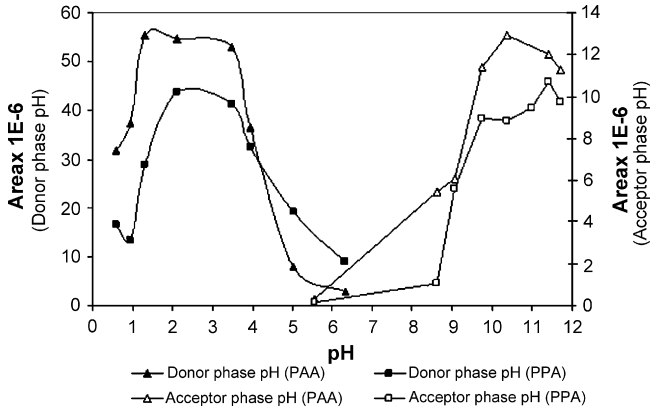

Fig. 3. Effect of the donor and acceptor phases' pH on the extraction of analytes: Extraction conditions: 1 mg/l of the analytes; donor phase: 3 ml solution containing 1.0 M Na_2SO_4; donor phase temperature: 50 °C; organic phase: 150 μl of 1-hexanol; acceptor phase: 3 μl of the aqueous solution; extraction time: 15 min; back-extraction time: 8 min.

Preliminary trials showed that the use of non polar solvents such as benzene, toluene, heptane and dodecane considerably decreased the extraction efficiency. It was seen that by increasing the solvent polarity, the extraction efficiency was improved and in the presence of alcohols, the best extraction efficiencies were obtained. The reason was that the analytes had relatively high polarity and could be extracted into the solvents with sufficient polarity. Therefore, the solvents with higher polarity were examined as organic phase. The solvents such as ethyl acetate, amyl alcohol, isoamyl alcohol, 1-hexanol, 1-octanol, 1-undecanol, 1-dodecanol and also two aldehydes of hexanal and heptanal were examined. Fig. 2 shows that 1-hexanol and 1-octanol provided the best extraction efficiencies for the target analytes. 1-Hexanol was finally selected as the extraction solvent due to better chromatographic behavior comparing to 1-octanol.

3.2.2. Composition of the donor and acceptor solutions

The donor and the acceptor phases' pH plays an important role in three phase LPME. For ionizabale analytes, protonation is the most common reaction utilized to enhance $K_{a/d}$ and to facilitate analyte extraction from donor to acceptor phase. The pH difference between the donor and acceptor phases can promote the transfer of the analytes from the donor to the acceptor phase. For practical applications, pH should differ from the pK_a values of the analytes by at least 2 units. Since our analytes were weak acidic compounds (pK_a of PAA: 4.31 and pK_a of PPA: 4.66), the donor solution should be sufficiently acidic to maintain the neutrality of the analytes and consequently reduce their solubility within the donor phase. Also, the acceptor phase should be alkaline in order to promote dissolution of the acidic analytes. The experiments were conducted to optimize the pH of both donor and acceptor solutions. First, the effect of the donor phase's pH on extraction efficiency was investigated. Therefore, its pH was adjusted using HCl in the range of 0.5–6.5. On the other hand, pH of the acceptor phase was adjusted at 11.0 using NaOH. According to Fig. 3, it is clear that the extraction efficiency of both analytes was consistent at the donor phase's pH in the range of 2.0–3.5. At higher pH, the extraction efficiency decreased because protonation reaction was not complete and a large portion of the analytes existed in ionic form. Thus, in further experiments, pH of the donor phase was adjusted at a fixed value within the range of 2.0–3.5. Like the pH of donor phase, the acceptor phase's pH can affect extraction efficiency as well. Acceptor pH should be adjusted to pH values suitable for ionizing the analytes. To investigate the effect of acceptor pH on extraction efficiency, 0.1 M of NH_3 was used as acceptor phase and pH adjustment was carried out using NaOH and HNO_3 in the range of 5.5–12.0. As shown in Fig. 3, the best extraction efficiencies were observed in the pH range of 10.4–11.3. According to the pK_a values of the analytes, it was predictable that at pH higher than 7.0, the –COOH group of the analytes would be deprotonated to form ionized analytes that can be easily extracted into the acceptor phase. Therefore, pH 11.1 was chosen as optimum pH for the further extractions. In many cases, especially for polar compounds, the addition of salt can often improve extraction efficiency. In this study, in order to investigate the effect of ionic strength on extraction efficiency, a series of solutions with various concentrations of Na_2SO_4 in the range of 0.0–2.5 mol/l were prepared and the analytes were extracted. The results showed that addition of salt could promote the transport of the analytes into the acceptor phase (salting out effect). This can be explained by the participitation of more water molecules in the hydration spheres around the salt ions. These hydration spheres reduce the amount of the water molecules available to dissolve analyte molecules [34]. Based on these facts, 2.3 mol/l of Na_2SO_4 was added to the solutions in further experiments.

3.2.3. Influence of donor phase temperature

Solution temperature affects extraction kinetics. At higher temperatures, diffusion coefficients of analytes increase and the time required to reach equilibrium decrease. Also, in the case of carboxylic acids that can form hydrogen bonds with water molecules, enhancing temperature can disrupt these bonds and

facilitate extraction of polar analytes from donor to organic phase. In the present study, effect of the sample temperature was studied by changing the donor phase temperature from 27 to 60 °C. It was seen that the amount of the extracted analytes increased with the rising of temperature up to 45–50 °C. Upper temperatures can reduce extraction efficiency by affecting the acceptor microdrop, because, at higher temperatures, acceptor solution volume reduces due to higher solubility in 1-hxanol. Hence, the range of 45–50 °C was chosen as the optimum donor phase temperature in the present study.

3.2.4. Influence of organic phase volume

In three phase LPME, the effect of organic solvent volume on the extraction recovery can be shown as [35]:

$$R = \frac{100\, n_{eq,a}}{C_i V_d} = \frac{100 K_{a/d} V_a}{K_{a/d} V_a + K_{org/d} V_{org} + V_d}$$

where, $n_{eq,a}$ is the amount of the analyte present in the acceptor phase at equilibrium; C_i is the initial concentration of the analyte in the donor phase; V_d, V_{org} and V_a are the volumes of the sample (donor phase), organic and acceptor phases, respectively. Also, $K_{a/d}$, and $K_{org/d}$ are the partition coefficients between the acceptor phase and donor phase as well as between the organic phase and donor phase. According to the equation, since the organic phase can act as a receiving medium to increase the recovery rate, the organic phase volume should be reduced.

The effect of organic solvent volume on the extraction efficiency of the target analytes was investigated at the range of 80–150 μl. As shown in Fig. 4, the best results were obtained by using 90 μl volume of 1-hexanol as organic phase. By reducing the organic solvent volume from 150 to 90 μl, higher extraction efficiencies were achieved. At smaller volumes of the organic solvent, the organic layer was thin and therefore, introduction of the acceptor microdrop into the membrane phase was difficult. Also, smaller volumes of the organic solvent tended to cause instability in the aqueous drop during the stirring. Hence, to achieve sufficient extraction with good repeatability, 95 μl of 1-hexanol was employed as membrane solvent in further experiments.

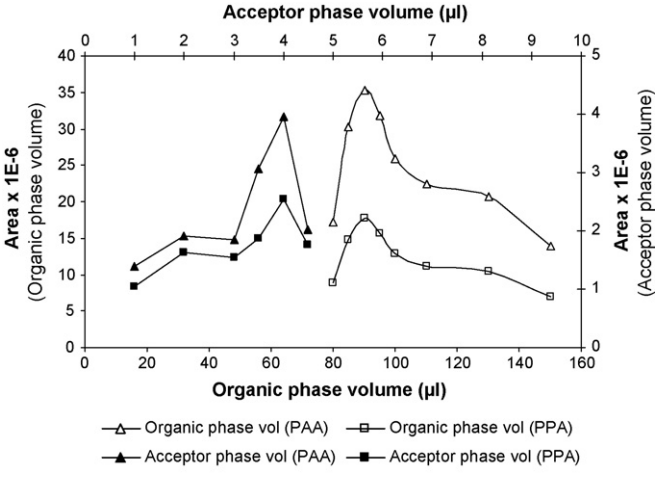

Fig. 4. Effect of the organic and acceptor phases' volumes on the extraction of analytes: Extraction conditions: 1 mg/l of the analytes; donor phase: 3 ml of the solution containing 2.3 M Na_2SO_4 (pH in the range of 2.0–3.5); donor phase temperature: 45–50 °C; organic phase: 1-hexanol; acceptor phase: 0.1 M of NH_3, pH 11.1; extraction time: 15 min; back-extraction time: 8 min; (due to overlapping signals of organic phase volume for two analytes, the signal related to organic phase volume for PPA was multiplied by 0.5 only for better resolution of the signals).

3.2.5. Effect of acceptor volume

In three phase LPME, higher enrichment factors can be resulted by decreasing the volume ratio of acceptor to donor phase. In the present work, the phase ratio of the acceptor to donor phase was changed by changing the volume of the acceptor phase in the range of 1.0–4.5 μl, whereas the volume of the donor phase was kept constant at 3.0 ml. Fig. 4 indicates that increasing the acceptor volume up to 4.0 μl improves the extraction efficiency. However, larger drops are difficult to manipulate and are less reliable. This observation was related to the higher surface area of the larger microdrop that had more interface area with the organic membrane and caused more analyte molecules to transfer into the acceptor microdrop. Accordingly, utilizing larger volumes of microdrop improves extraction efficiency whereas reduces enrichment factor. Since, sample volumes in the range of 5–25 μl are easily injected into the HPLC column, the whole acceptor phase may be analyzed, potentially providing higher sensitivity [36,37]. Hence, 3.0 ml of the donor phase, 95 μl of the membrane phase and 4.0 μl of the acceptor phase were used in further studies.

3.2.6. Effect of stirring rate

Stirring of donor phase accelerates the kinetics of extraction by decreasing the thickness of the Nernst diffusion film around the interface between the phases. This phenomenon enhances diffusion of analytes from donor to acceptor phase [38]. Agitation also reduces the time required to reach thermodynamic equilibrium and induces convection in membrane phase. Some studies have shown that extraction efficiency improves by increasing the stirring speed up to 1250 rpm, which is the highest speed that could be achieved by magnetic stirrer. Using larger magnets for sample agitation increases extraction but they are not suitable due to the production of vortex flow in the membrane phase that can reduce stability of the acceptor microdrop. Thus, stirring speed of 1250 rpm was applied in the subsequent experiments.

3.2.7. Effect of extraction and back-extraction time

In three phase LPME, there is a series of two extraction equilibria: the first equilibrium is between the donor and organic phases established at the extraction time, and the subsequent equilibrium is between the organic and acceptor phases obtained after the back-extraction time. The main objective in microextraction techniques is to achieve sufficiently high extraction efficiency within a relatively short period of time. During the extraction time, solute molecules pass through the interface between the donor phase and organic phase and preconcentrate in the organic membrane. In the present study, the influence of extraction time on the extraction efficiency of the analytes was

Table 1
Figures of merit of the proposed method

Analyte	Regression equation[a]	r^2	LOD (μg/l)	DLR (μg/l)	Enrichment factor (at 10 μg/l)	Recovery (%)[b]
PAA	$Y = 0.0031\,C\ (\mu g/l) - 0.0019$	0.9988	0.2	2.0–2500	110	11.0
PPA	$Y = 0.0029\,C\ (\mu g/l) - 0.0026$	0.9997	0.2	1.0–5000	104	10.4

[a] Each point in calibration curve was repeated at least three times and RSD% values for PAA and PPA were in the range of 0.5–4.5% and 1.4–10.1%, respectively.
[b] Recovery values were calculated as the ratio of the amount of analyte in the acceptor phase to its initial amount in the donor phase.

studied in the range of 0–25 min. The experiments were accomplished with 3 ml of the donor phase (containing 0.2 mg/l of PAA and PPA, 2.3 M Na_2SO_4 and Ph 3.0), 95 μl of 1-hexanol (as organic membrane) and 4.0 μl of the acceptor microdrop (0.1 M NH_3, pH 11.1). The stirring speed was set at 1250 rpm, bath temperature was 50 °C and back-extraction time was 8 min. The amount of the extracted analytes was found to increase with the increase of the extraction time in the range of 0–20 min and reached a maximum when the two phases were stirred for 20 min at 1250 rpm, but after 20 min, the extraction efficiency showed a soft decline. Thus, the extraction time of 20 min was considered as optimum extraction time. After extraction time out, the acceptor microdrop was suspended from the tip of the microsyringe into the organic membrane and at the back-extraction time, the analytes were back-extracted from the organic phase into the acceptor phase as ionic form.

The effect of back-extraction time was studied in the range of 1–15 min. As Fig. 5 shows, after 11.0 min, the mass transfer into the acceptor phase was found to increase very slowly. It is obvious that under these conditions, thermodynamic equilibrium is not established because at higher agitation rates, higher extraction efficiencies may be obtained. Thus, 12 min was selected as optimum back-extraction time.

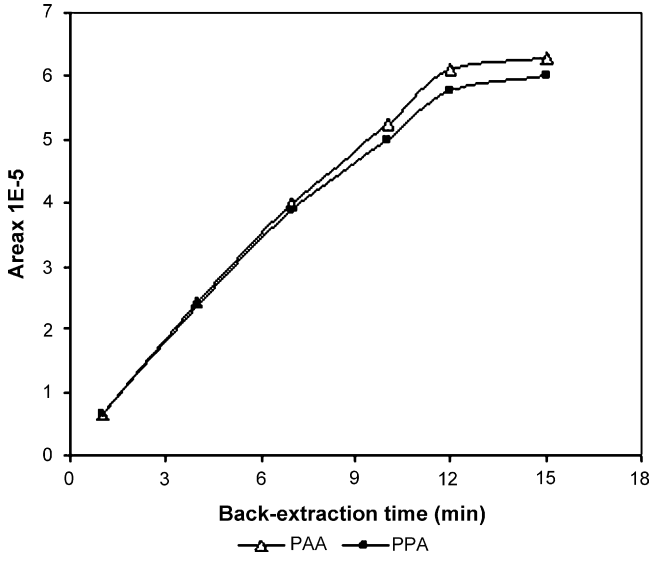

Fig. 5. Effect of the back-extraction time on the extraction of analytes: Extraction conditions: 0.2 mg/l of the analytes; donor phase: 3 ml of the solution containing 2.3 M Na_2SO_4 (pH in the range of 2.0–3.5); acceptor phase: 4 μl of the aqueous solution (0.1 M NH_3, pH 11.1); donor phase temperature: 45–50 °C; organic phase: 95 μl of 1-hexanol; extraction time: 20 min.

3.3. Method performance

To evaluate the practical applicability of the proposed three phase LPME method, calibration curves were plotted using 11 spiked levels in the range of 0.6–5000 μg/l. Each standard sample (contained 100 μg/l NAA, as internal standard) was extracted by the proposed method at optimum conditions. The calibration curves were obtained by plotting the relative peak areas against the concentration of the analytes in the donor phase. The limits of detection (LODs) were calculated based on a signal-to-noise ratio of 3. Also, the enrichment factors (EFs) at a given time were defined as the ratio of the analyte concentration in the acceptor phase to its initials concentration in the donor phase. The analytical performance of the method is summarized in Table 1. Both analytes exhibited good linearity with correlation coefficients more than 0.9988. The repeatability (intraday) and reproducibility (interday) were studied based on the relative peak areas at 20 and 200 μg/l concentration levels. Based on the reported results in Table 2, suitable intraday and interday repeatabilities with RSDs < 6.5% were obtained.

3.4. Determination of free PAA and PPA in human urine

In order to determine free PAA and PPA in human urine, 20 ml of human urine from two healthy persons was separately collected in disposable polyethylene containers and 0.1 g of NaF was added into each container as preservative. The samples were kept at 4 °C before analysis. Due to high concentration of PAA in urine, the urine samples were diluted with a dilution factor of 1:100. This process reduces matrix effect. For this purpose, aliquots (1 ml) of each urine were transferred into a 100 ml volumetric flask and after the addition of NAA (100 μg/l as internal standard), its pH was adjusted at a fixed value in the range of 2.0–3.5 and ionic strength was adjusted at 2.3 M using Na_2SO_4. Then the volume of the resulted solution was reached to the mark using deionized water. Then, 3.0 ml of this sample was transferred into the extraction cell and the analytes were extracted at optimum conditions. Fig. 6a shows a chromatogram of the

Table 2
Intraday and interday precision

Analyte	Concentration (μg/l)	Intraday RSD (%) ($n = 5$)	Interday RSD (%) ($n = 4$)
PAA	20.0	3.3	6.5
	200.0	2.1	6.4
PPA	20.0	4.1	5.2
	200.0	1.3	4.4

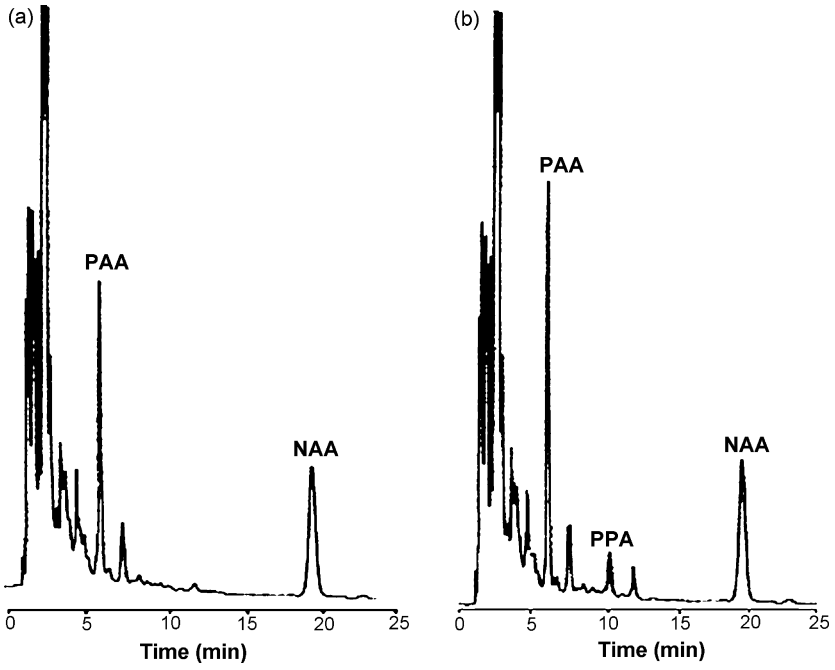

Fig. 6. HPLC chromatogram of human urine after extraction with the proposed method at optimum conditions: (a) non-spiked diluted urine sample; and (b) 40 μg/l of the spiked diluted urine sample.

diluted urine sample after extraction via the proposed method. As shown in Fig. 6a, no target PPA was found in the urine samples, but PAA was present at higher concentrations in both samples. To study the matrix effect on the extraction efficiency, a known amount of the analytes was spiked into the urine samples and extraction from the diluted spiked samples was performed. A chromatogram of the spiked urine samples after the extraction with the proposed method is shown in Fig. 6b. Relative recoveries of the spiked samples were higher than 93.0% and the results of the three repeated extractions of each urine sample are summarized in Table 3. Based on these data, satisfactory results can be obtained using the proposed method.

3.5. Determination of free PAA and PPA in human serum and plasma

The performance of the proposed method was also tested by extraction and determination of PAA and PPA in the serum and plasma samples. Frozen human serum and plasma samples were obtained from the Iranian Blood Transfusion Organization (Tehran, Iran), thawed and allowed to reach room temperature. Each sample of serum was diluted at 1:10 ratio and extracted using three phase LPME after the addition of the internal standard (100 μg/l of NAA) and pH adjustment. All the standard solutions for the calibration curves were extracted at similar conditions. Also, the plasma sample was diluted at 1:20 ratio, spiked at 50 μg/l concentration level of PAA and extracted via the proposed method. According to Table 3, unlike PAA, no PPA was found in the serum and plasma samples. A chromatogram of the non-spiked (a) and 50 μg/l spiked serum sample (b) is shown in Fig. 7. As Table 3 indicates, relative recoveries of the analytes from the spiked real samples were higher than 89%. The results indicated that our proposed method has high clean-up power, and that biological matrixes do not have any significant effect on the extraction efficiency of the method.

Table 3
Performance of the proposed method for extraction of PAA and PPA from the biological samples

Sample	PAA				PPA			
	Determined (μg/l)	Spiked (μg/l)	Found (μg/l)	Relative recovery (%)	Determined (μg/l)	Spiked (μg/l)	Found (μg/l)	Relative recovery (%)
Urine A (1:100)[a]	236.9 ± 7.7[b]	40	280.5 ± 5.0	109	Non-detected	40	47.4 ± 1.0	118.5
Urine B (1:100)	94.8 ± 10.0	40	132.3 ± 10.1	93.6	Non-detected	40	45.8 ± 2.1	114.6
Serum A (1:10)	302.2 ± 12.3	50	353.3 ± 13.5	102.2	Non-detected	–	–	–
Serum B (1:10)	137.7 ± 4.1	50	182.6 ± 11.0	89.8	Non-detected	50	45.2 ± 4.2	90.4
Plasma (1:20)	65.1 ± 6.0	50	117.4 ± 1.7	104.6	Non-detected	–	–	–

[a] Dilution ratio.
[b] Mean of three replicate measurements ± standard deviation.

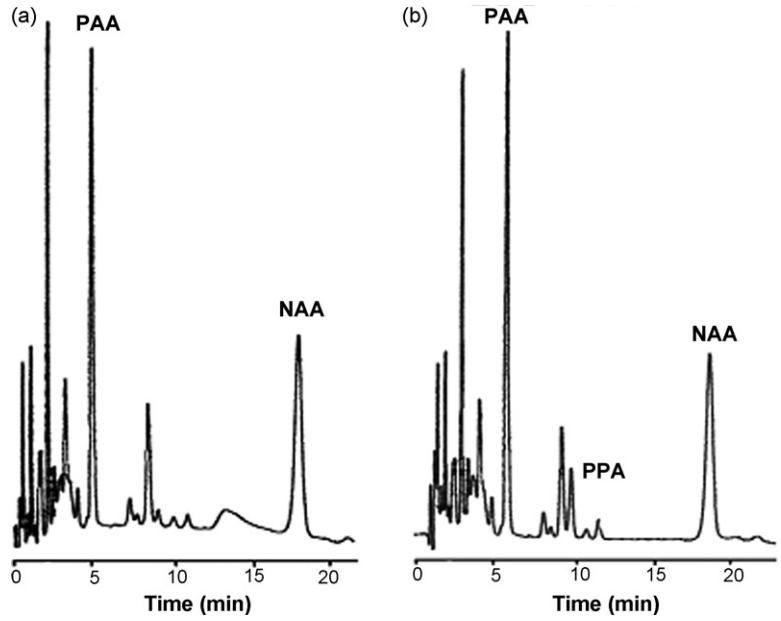

Fig. 7. HPLC chromatogram of human serum after extraction with the proposed method at optimum conditions: (a) non-spiked diluted serum sample; and (b) 50 μg/l spiked diluted serum sample.

4. Conclusion

In the present study, the potential of three phase LPME was demonstrated as a sample preparation technique prior to HPLC to determine the hydrophilic compounds such as phenyl derivative of carboxylic acids from biological matrixes. The proposed three phase LPME technique is attractive enough owing to its simplicity, analytical precision, low consumption of organic solvent, low cost and short sample preparation time. Comparing to the traditional methods, this method needs only one HPLC syringe for PAA and PPA determinations and the current design employs small sample volume which is compatible with the biological samples such as blood. Since a fresh acceptor phase is used for each extraction, there is no memory effect. Three phase LPME method has excellent clean-up and, in the present study, an enrichment factor up to 110-folds was obtained. Finally, low limits of detection make the three phase LPME as a method of choice for the measurement of target analytes in the complex matrixes such as the body fluids.

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