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Research Article

Elevated Temperature Air-Assisted Liquid-Liquid Microextraction of Pseudoephedrine from Human Urine Samples Coupled with High-Performance Liquid Chromatography-Ultraviolet Detection

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Abstract

***Background*:** Pseudoephedrine is a broadly used drug for the clinical treatment of bronchitis, respiratory allergies, and cancer pains. However, the use of pseudoephedrine is restricted by several medicinal commissions like International Olympic Committee (IOC). In this study, a reliable, inexpensive, green, and sensitive microextraction method has been developed for extraction and preconcentration pseudoephedrine in urine samples.

Methods: It is based on an air-assisted liquid-liquid microextraction (AALLME) technique in which choline chloride: p-chlorophenol deep eutectic solvent (DES) was used as the extraction. The extracted analyte was determined by a high-performance liquid chromatography (HPLC)-ultraviolet detector. The effective factors on the efficiency of the method such as kind of extraction solvent, pH of sample solution, extraction time, and ionic strength were investigated.

Results: Under the optimized conditions, the extraction recovery (ER) and enrichment factors (EFs) were 89% and 222, respectively. The method detection limit, relative standard deviation (RSD), and linear range were 0.21 ng mL^{-1}, 4.9%, and 0.69-1000 ng mL^{-1}, respectively.

Conclusion: The validated method was successfully performed on different urines samples obtained from the persons who were under treatment with pseudoephedrine.

Introduction

Ephedrines especially pseudoephedrine is an extensively used drug for clinical treatment of common cold, bronchitis, respiratory allergies, sinusitis, and relieve cancer pains. Unfortunately, ephedrine alkaloids have amphetamine-like properties in high doses and can cause tachycardias, nervousness, seizures, hypertension, and psychosis. On the other hand, the medical commission of the IOC restricted their use by an athlete due to their stimulating properties on the central nervous system. Consequently, sensitive detection of these substances in biological samples is interesting for IOC.[1,2] Several analytical methods including high-performance liquid chromatography (HPLC)-variable wavelength detector, gas chromatography coupled to mass spectrometry (MS) and flame ionization detector, high-performance thin-layer chromatography, reversed-phase HPLC, liquid chromatography–ion trap-tandem mass spectrometry coupled with electrospray ionization, and liquid chromatography-tandem mass spectrometry have been developed for the determination of pseudoephedrine in different samples.[3-11] In all methods, the samples were not directly injected into the determination system because of sample matrices' complexity or low concentration of the analyte. As a result, a sample preparation step has been done before analysis. Liquid-liquid extraction and solid-phase extraction are the most famous methods for the pretreatment of a biological sample. In recent years, many attempts performed to replace these methods with other approaches with low consumption of extraction solvent.[12,13] As a result, miniaturized extraction methods have been developed and applied to different samples.[1415] Air-assisted liquid-liquid microextraction (AALLME) is an effective and facile microextraction procedure developed in 2012 for the extraction of phthalate esters from water samples.[16] In this method, the analytes were extracted into a few microliters of a solvent which was dispersed in the sample solution with the aid of a syringe. The dispersed solvent should be collected by centrifugation. This method is disperser-less and the extraction solvent was used at microliter level. Therefore it is a preferred method to use in sample preparation methods. However, the use

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of commercial organic solvents is the main drawback of this method, and in recent years, green solvents like deep eutectic solvents (DESs) are preferred to use as the extraction solvent. DESs are a new generation of solvents broadly prepared and used in different fields. They are mainly prepared by mixing a hydrogen bond donor and a hydrogen bond acceptor simply and its heating to form a homogenous and clear solution. Compared to organic solvents, DESs have less toxicity and high extraction capability. Up to now, different applications of DESs have been reported in the determination of diverse compounds.[17-22]

The main aim of this work is the application of a DES-based AALLME method for efficient extraction of pseudoephedrine from urine samples before HPLC-UV determination. In this approach, the analyte was extracted into a higher density than waster DES from urine samples obtained from patients who consumed the drug. The method was done at higher temperatures due to the driving force of temperature for the migration of the analytes into the extraction solvent. To the best of our knowledge, this is the first report on the application of DES-ALLME for extraction/preconcentration pseudoephedrine from a urine sample.

Materials and Methods

Chemicals and standards

Pseudoephedrine (with purity higher than 95%) was kindly provided from Drug Control Headquarters (Tehran, Iran). Choline chloride (ChCl), *p*-chlorophenol, alpha-naphthol, and beta-naphthol used in the preparation of the DESs were bought from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and water were provided from ChemLab (Zedelgem, Belgium). Sodium chloride, sodium hydroxide, and HCl solution (37% w/w) were purchased from Merck (Darmstadt, Germany). A stock solution of pseudoephedrine at a concentration of 100 mg L^{-1} was prepared in methanol and stored at 4°C. This solution was used for the preparation of model solutions.

Instrumentation

The analyte determination was carried out by an Agilent 1100 liquid chromatograph (Agilent Technologies, USA), equipped with an ultraviolet detector (UV). The analyte separation was done using a ZORBAX Eclipse XDB-C_{18} column (150 × 4.6 mm, particle size of 5µm). Combination acetonitrile and phosphate buffer (pH = 3.2 at a concentration of 0.01 M containing 0.3% diethylamine) at a ratio of 95:5 (v/v) were used as the mobile phase. The mobile phase flow rate was 1 mL min^{-1}. The detector was adjusted at 220 nm for monitoring of the analyte.

Preparation of real samples

Six urine samples were obtained from persons who consumed the drug. These samples were collected six hours after administration. Also, three blank urine samples were collected from volunteers from our laboratory (Tabriz, East Azarbaijan province, Iran) to use in optimization and validation steps. The samples were kept in the refrigerator before analysis. The sample's pH was adjusted at 8 using McIlvaine buffer.

ChCl: p-chlorophenol DES preparation

ChCl: *p*-chlorophenol DES was prepared according to the previously published method.[23] In this approach, 1.39 g ChCl was mixed with 1.58 g *p*-chlorophenol in a glass test tube, and the mixture was vortexed for 2 minutes. Then the tube was placed in a water bath at 75˚C for 10 minutes. The homogenous phase was used in the extraction procedure.

Microextraction procedure

A 5 mL McIlvaine buffer adjusted at pH=8 spiked with the analyte at a concentration of 20 ng mL^{-1} or urine sample was transferred into a glass test tube. After dissolving sodium chloride at a concentration of 2% w/v, the mixture was placed into a water bath to adjust its temperature at 60˚C. Then, 80 µL of ChCl: *p*-chlorophenol DES was placed at the bottom, and it was dispersed in the solution by performing aspiration/dispersion cycles five times. After centrifugation at 5000 rpm for 3 min, the sedimented phase was removed and diluted up to 20 µL with mobile phase, and the mixture was injected into the HPLC-UV system.

Results and Discussion

Selection of the extraction solvent

The extraction solvent selection is an essential factor in an ALLME method. The extraction solvent must form a two-phase system in the sample solution for its easy collection after centrifugation. In this study, three higher-density than water DESs including ChCl: *p*-chlorophenol, ChCl: alpha-naphthol, and ChCl: beta-naphthol were tested to extract the analyte. The experiments were done using 100 µL of each DES. The obtained extraction recoveries (ERs) for the analytes in the presence of each solvent are shown in Figure 1. The data showed that ChCl: *p*-chlorophenol DES is the

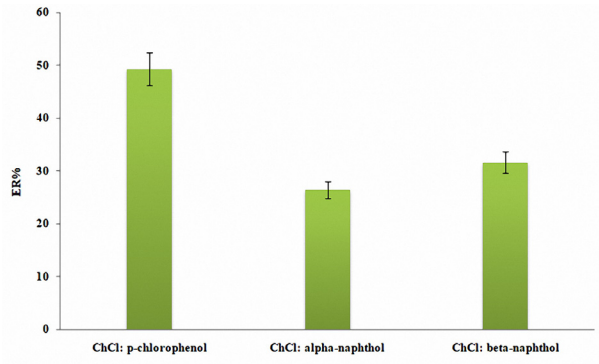

Figure 1. Selection of extraction solvent type. Extraction conditions: sample solution, 5 mL deionized water spiked with pseudoephedrine at a concentration of 20 ng mL1-; extraction solvent volume, 100 µL; sample solution pH, 10; extraction number, 4 times; sample solution temperature, 22˚C; centrifuge rate, 4000 rpm; and centrifuge time, 5 minutes. The error bars indicate the minimum and maximum of three determinations.

most appropriate for the analyte and was selected for the next steps.

Optimization of extraction solvent volume

The extraction solvent volume is another factor that could affect the extraction efficiency. The volume of extraction solvent should be selected as low as possible to obtain a high enrichment factor (EF) to obtain low detection limits. On the other hand, the extraction solvent volume must be sufficient for the extraction of the analyte. In this work, the extraction solvent volume was studied in the range of 60-120 µL and the results (Figure 2) showed that the method efficiency increases up to 80 µL and then reaches a constant value. It is remarkable that at volumes less than 60 µL, the sedimented was not formed, and the method was useless. Therefore, 80 µL was used in the other steps.

pH optimization

Due to the alkaline nature of the analyte, sample solution pH can be effective in the extraction of the analyte. To ensure the effective extraction of the analyte, the sample solution pH was investigated in the range of 5 to 11 by adding HCl or NaOH solution. The data (Figure 3) demonstrated that high ERs were obtained at high pH values (pHs ≥8). It can be attributed to the protonation of the analyte at acidic pHs. Therefore, pH=8 was chosen as the optimum pH for the following studies.

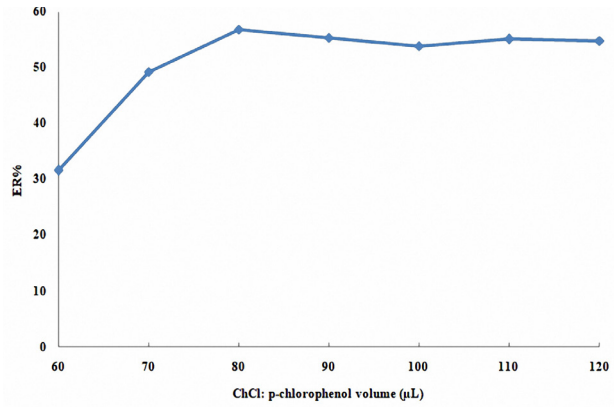

Figure 2. Optimization of extraction solvent volume. Extraction conditions: are the same as those used in Figure 1, except ChCl: p-chlorophenol DES was selected as the extraction solvent.

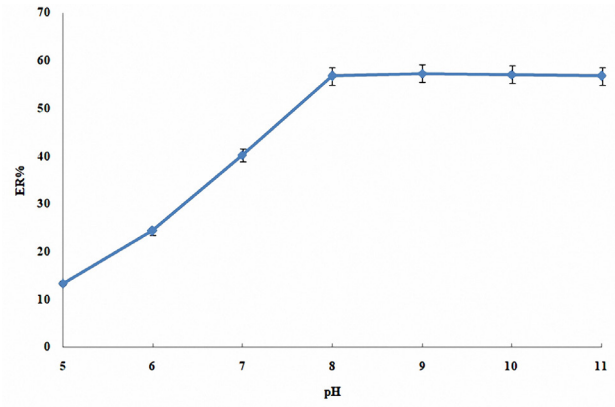

Figure 3. pH effect. Extraction conditions: are the same as those used in Figure 2, except 80 µL ChCl: p-chlorophenol DES was selected as the extraction solvent.

Ionic strength

The addition of salt to the aqueous sample solution may have several effects. Generally, salt addition decreases the solubility of the analytes in the aqueous phase and reinforces the analyte's partitioning coefficient into the organic phase. However, at a high salt concentration, the viscosity of the aqueous solution increases that can reduce the diffusion coefficient of the analyte. Therefore, the salt addition can induce two opposite effects. The effect of the aqueous phase ionic strength on the extraction efficiency of the method was evaluated by adding sodium chloride to the aqueous phase in the range of 0–8%, *w/v*. According to Figure 4, the analytical signals were increased up to 2% and then decreased gradually. It could be the increase in viscosity of the aqueous phase by adding NaCl which leads to the decrease in diffusion coefficients of the analytes. Therefore, 2%, *w/v*, NaCl selected for further experiments.

Optimization of extraction number

Performing aspiration/dispersion cycles is the main step of an AALLME method. This step leads to dispersion of the extraction solvent as very small droplets in the sample solution and increases its contact area extraordinarily. The extraction number should be optimized to obtain a high ER. In the present study, different experiments were done by extraction number in the range of 1-7 times, and the obtained ER for each data is presented in Figure 5. The results confirmed that the method efficiency increases up to 5 times and then the ERs reach constant values. Therefore, five times were selected as the optimum extraction times.

Sample solution temperature

The temperature of the sample solution may enhance the method efficiency by increasing the analyte migration rate into the extraction solvent droplets. On the other hand, the sample solution and the extraction solvent viscosity are reduced by increasing the sample solution temperature

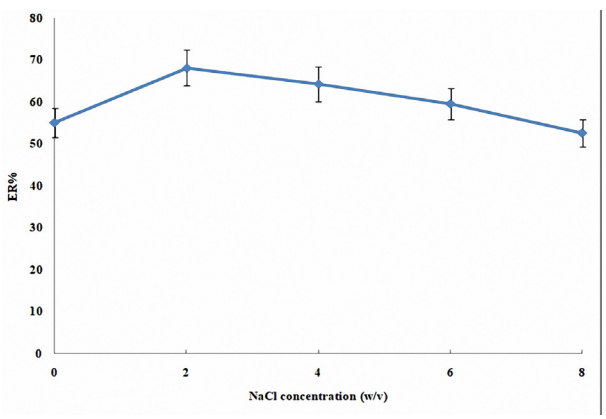

Figure 4. Ionic strength. Extraction conditions: are the same as those used in Figure 3, except the aqueous phase pH was adjusted at 8.

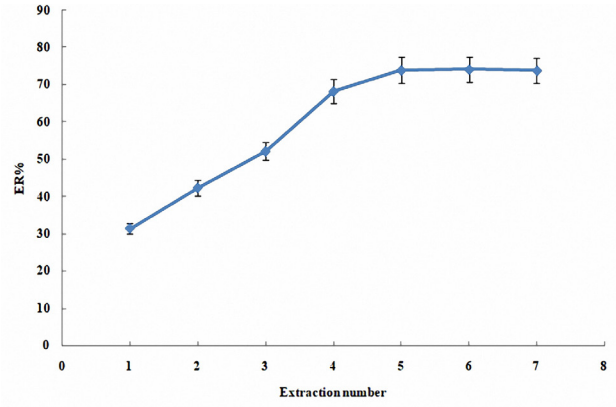

Figure 5. Optimization of extraction number. Extraction conditions: are the same as those used in Figure 4, except 2% w/v of sodium chloride was dissolved in the aqueous phase.

which facilitates their contacting and dispersion of the extraction solvent. Investigation of the sample solution temperature was done by adjusting it in the range of 22 (room temperature) to 80˚C. According to the results depicted in Figure 6, the ER% of the method increases up to 60 ˚C and then remains constant. Therefore, 60 ˚C opted for the next experiments.

Quantitative features of the method

Quantity parameters of the proposed method under optimized experimental conditions were evaluated by calculation of *limit of detection* (LOD), the limit of quantification (LOQ), linear range (LR), coefficient of determination (r^2), repeatability, EF, and ER. The obtained data are summarized in Table 1. Good linearity with r^2=0.9986 was obtained. LODs (calculated as three times of signal–to–noise) and LOQs (calculated as 10 times the signal–to–noise) were obtained equal to 0.21 and 0.69 ng mL^{-1}, respectively. Precision of the method was determined by analyzing standard solutions at a concentration of 1.0 ng mL^{-1}. Relative standard deviation (RSD)was 4.9% which indicates that the method is acceptably repeatable. EFs and ERs were 222 and 89%, respectively. The analyte stability in the urine sample was investigated by analyzing urine samples spiked at the concentrations of 1 and 5 ng mL^{-1} stored at 24°C for 24 hours and –22°C for 48 hours. In all cases, the analyte concentration was obtained after performing the method and it was compared with a freshly prepared sample. The obtained RSDs% were less than 12% which indicates good stability of the analyte in the urine sample. Selectivity of the method was followed by analyzing three urine samples obtained from the persons who did not consume the drug. The obvious response at the retention time of pseudoephedrine showed that there was an interfering peak in the retention time of the analyte.

Real sample analysis

Utility of the developed method in the analysis of pseudoephedrine in urine samples was tested by analyzing six samples obtained from the volunteers after their oral administration of the drug. The obtained results showed that pseudoephedrine concentration was in the range of 288-362 ng mL^{-1}. Figure 7 shows typical chromatograms of standards solutions of the drug after direct injection and one un-spiked urine sample after performing the method on it. It can be seen that the method is suitable for analysis of the analyte in the selected samples and there is no interfering peak in the retention time of the analyte. To study the matrix effect, an added-found method was performed on them and the mean relative recovery values are summarized in Table 2. The results illustrate that the samples have no significant effect on the proposed method.

Conclusion

In this study, a new, easy, and repeatable ET0AALLME method has been developed for the extraction and preconcentration of pseudoephedrine from urine samples before its analysis by HPLC–UV. In this method higher than water density DES was prepared from ChCl and p-chlorophenol and it was used as the extraction solvent in the extraction procedure. To enhance the method

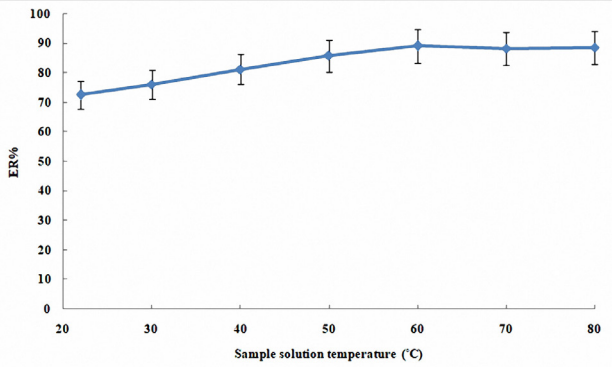

Figure 6. Optimization of sample solution temperature. Extraction conditions: are the same as those used in Figure 5, except five times was selected as the suitable extraction times.

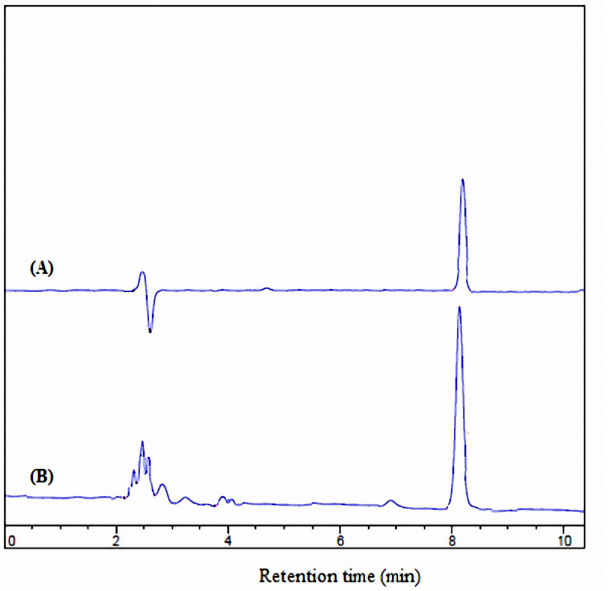

Figure 7. Typical HPLC-UV chromatograms of (A) standard solution of pseudoephedrine at a concentration of 25 mg L^{-1} (direct injection) and (B) un-spiked urine sample after performing the developed method.

Table 1. Quantitative features of the developed method

Analytes	Calibration curve equation	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LR (ng mL^{-1})	r^2	RSD %	EF ± SD[a]	ER ± SD[b]
Pseudoephedrine	y=3221x+116	0.21	0.69	0.69-1000	0.9986	4.9	222 ± 10	89 ± 4

LOD, Limit of detection; LOQ, Limit of quantification; LR, Linear range; RSD, Relative standard deviation (n=6, C =1.0 ng mL^{-1}); EF, Enrichment factor; r^2, Coefficient of determination

[a] Enrichment factor ± standard deviation (n = 3).

[b] Extraction recovery ± standard deviation (n = 3).

Table 2. Study of matrix effect in the proposed method in three analyte-free urine samples spiked at different concentrations

Analyte	Spiked level (ng mL^{-1})	Mean relative recovery ± standard deviation (n=3)		
		Urine #1	Urine #2	Urine #3
Pseudoephedrine	5	89 ± 6	97 ± 2	91 ± 4
	10	92 ± 3	92 ± 4	95 ± 6
	100	90 ± 4	96 ± 5	97 ± 5

efficiency, the extraction approach was done at elevated temperatures to accelerate the analyte migration rate. The optimized and validated method was performed on urine samples and the analyte was determined in them successfully. The results revealed that the developed method exhibits high ERs and EFs, low LODs and LOQs, short extraction time, simplicity, low cost, and good repeatability. This method can be used in routine analysis of urine. The excellent cleanup implies a great advantage over other sample treatment procedures.

Authors' Contributions

MN: Conceptualization; validation, writing original draft. MRAM: Methodology, writing original draft, formal analysis.

Conflict of Interest

The authors have no conflicts of interest to declare.

Ethical Issues

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research.

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Not Applicable.

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