Development of Salt Induced Liquid–Liquid Extraction Combined with Amine Based Deep Eutectic Solvent-Dispersive Liquid–Liquid Microextraction; An Efficient Analytical Method for Determination of Three Anti-Seizures in Urine Samples

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Abstract

Background: A simple, fast, efficient, and environmentally friendly microextraction technique named salt induced liquid-liquid extraction combined with amine based deep eutectic solvent-dispersive liquid-liquid microextraction method followed by gas chromatography-flame ionization detector was applied for the determination of some anti-seizure drugs in urine samples.

Methods: In this method, sodium chloride (30% w/v) was dissolved in the sample solution (adjusted at pH=10) as phase separation agent and iso-propanol was added to the solution. After manually shaking, the solution was centrifuged and the supernatant phase was removed and mixed with choline chloride: benzyl ethylenediamine (85 µL) and the mixture was rapidly dispersed into a deionized water containing 5.0 % w/v sodium chloride and adjusted at pH=10. The cloudy solution was centrifuged and the sedimented phase was removed and 1 µL of it was injected into the determination system.

Results: Under final conditions, the limits of detection and quantification at the ranges of 3.4-6.9, and 11.5-23.3 ng mL⁻¹ were obtained, respectively. The relative standard deviations for intra- and inter day assays were lower than 10.9%. The effect of exogenous and endogenous effect on the performance of the method was studied and the results showed that these factors were nearly effect less on the efficiency.

Conclusion: The introduced method was satisfactorily utilized to determine the selected benzodiazepines drugs in the patients’ urine samples.

Introduction

Epilepsy or seizure is one of the most known brain diseases which occurred suddenly by a hysterical electrical trouble in the brain. In this disease the brain activities were abnormal and it affects the behavior, feelings, movements, and awareness of someone who is involved with this disease. In a normal person electrical impulses were sent and received with nerve cells continuously and anything that disrupts these pathways can lead to a seizure. Different agents like high fever, insufficient sleep, hyponatremia, head trauma, brain tumor, illegal drugs and alcohol abuse can lead to the epilepsy. Seizure was classified to two different types consists of focal and generalized seizures. In focal seizures an irregular electrical activity was performed in one area of brain while all parts of the brain were involved in generalized seizures. A seizure can occur with different severities and last from a few seconds to more than five minutes. A seizure occurred longer than these times or more than two times within a few minutes is a medical emergency known as status epileptic. Since a seizure can be an incident there is no need to treatment until the person experiences more than once. However in epileptic cases medication should be performed with the use of anti-seizure medications. Anticonvulsants like carbamazepine, and benzodiazepines (BDZs) like midazolam, diazepam, and chlordiazepoxide, are the most common drugs used as anti-seizure. These drugs mainly affect the emotional...
reactions and awareness of the person who had taken them. As results some activities like driving were banned for these persons. Therefore, the development of an efficient and reliable method for the analysis of the drugs in different samples like pharmaceutical preparations, clinical or criminal examinations and biological fluids is crucial for the analysts.\textsuperscript{6} Up to now, diverse approaches such as capillary electrophoresis,\textsuperscript{7,8} gas chromatography (GC),\textsuperscript{9,10} and high performance liquid chromatography\textsuperscript{11–13} have been developed for the determination of these types of drugs. Among these methods, chromatographic techniques are preferred due to the possibility of simultaneous determination of several anti-seizures in different samples. Since the biological samples have relatively complex matrix and the drugs concentration is low, performing a consistent pretreatment procedure is needed before their analyses.\textsuperscript{16} An ideal pretreatment method must be capable to isolate and preconcentrate the studied compounds from the biological samples.\textsuperscript{17} Usually liquid–liquid extraction (LLE) and solid phase extraction (SPE) were used for treatment of real sample. However, solid phase microextraction\textsuperscript{18,19} and liquid–phase microextraction (LPME)\textsuperscript{20} methods have been proposed for miniaturization, simplification, and minimization of organic solvents utilization. Among LPME methods, dispersive liquid–liquid microextraction (DLLME) procedure attracts more attentions and applications for the extraction of different compounds in several samples.\textsuperscript{21} This method was introduced by Assadi and co–workers and a mixture of dispersive and extraction solvents are quickly injected into the sample solution. The extraction solvent is finely dispersed in the sample solution with the aid of dispersive solvent and the analytes are transferred into the droplets efficiently.\textsuperscript{22–24} Different liquids including organic solvents heavier or lighter than water,\textsuperscript{25} room temperature ionic liquids (RTILs),\textsuperscript{26} supramolecules,\textsuperscript{27} and deep eutectic solvents (DESs)\textsuperscript{28} were used as the possible extraction solvent in DLLME. Toxicity and availability of the solvent is important factor in its utilization as extraction solvent in DLLME and the use of green solvents as a substitute of organic solvents is preferred. From toxicity viewpoint, RTILs and DESs mainly used as the extraction solvents. However, the use of RTILs is restricted due to their high viscosity, boiling point, and price. DESs are formed from of biodegradable, safe, and cheap components and they are a good choice instead of RTILs.\textsuperscript{29} DESs were prepared by simple mixing of appropriate amounts of two or three components which they are acted as hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD). Hydrogen bonding interactions between the HBA and HBD leads to form a low-toxic liquid which can be used an acceptor phase in extraction techniques. The main goal of this study is the development of an efficient and rapid microextraction procedure for simultaneous isolation of three anti-seizures according to DES–based DLLME method from biological samples. In this work, firstly, the analytes was extracted into a water miscible extraction solvent (iso–propanol) which was used as the disperser solvent in the followed procedure. Then, the water–immiscible synthesized DESs were used as the extraction solvent in DLLME method. The main advantage of this work is the use of DES as a green and cheap extractant instead of classical organic solvents, which makes the method environmentally–friendly and green for human health.

**Experimental**

**Materials and solutions**

Carbamazepine, diazepam, and chlordiazepoxide were kindly provided by Sobhan Darou (Rasht, Iran). Benzyl ethylenediamine, 2, 4–dichloro aniline, and p–chlorophenol were supplied from Sigma (Missouri, USA). Choline chloride (ChCl), hydrochloric acid (37%), ammonia, sodium chloride, tetrahydrofuran, acetonitrile, iso–propanol, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Deionized water was obtained from Ghazi Pharmaceutical Company (Tabriz, Iran). A 400 mg L$^{-1}$ (each analyte) mixture solution of the analytes was prepared in methanol and stored in a refrigerator at 4 °C during the extraction method. Working standard solutions at different concentrations were freshly prepared in deionized water.

**Instrumentation**

The analytes separation and determination was performed on Agilent 7890A gas chromatograph (Agilent Technologies, CA, USA) equipped with a flame ionization detector (FID). A constant flow rate of nitrogen (99.999%, Gulf Cryo, United Arab Emirates) was used as the carrier gas and make up gas and they were adjusted at 1.0 mL min$^{-1}$ and 30 mL min$^{-1}$ for carrier gas and make up gas, respectively. The oven temperature was held at 110 °C for 2 min and then increased at 11°C min$^{-1}$ to 300 °C. Finally it was held at 300 °C for 5 min. The injection port was adjusted at 280 °C and used in pulsed split mode (sampling time of 0.7 min). Data acquisition and processing was performed by ChemStation software. The injections were performed by a zero dead volume microsyringe (1 μL, Hamilton, Switzerland). Phase separation was accelerated using a Hettich centrifuge (Tuttlingen, Germany).

**Real samples**

Analytes–free urine samples were prepared from volunteers who had not consumed any drug for at least two months. Also, two urine samples were collected from patients who were under treatment with carbamazepine (tablet containing 200 mg of each drug, twice daily). These samples were collected within 15 h from the first oral administration and they were kept in polypropylene tubes at −20 °C before analysis. All sample donors have been informed on details of the drugs and signed a consent form which was confirmed by the Ethical Committee of Tabriz University of Medical Sciences and registered with the approval code of IR.TBZMED.REC.1397.492.
Synthesis of deep eutectic solvent

Synthesis of the DESs was performed by mixing ChCl as a HBA with three HBDs including benzyl ethylenediamine, 2,4–dichloro aniline, and p–chlorophenol molar ratios of 1:2 HBA: HBD in screw–cap test tubes. The tubes cap were blocked and heated at 90 °C for 45 min in a water bath while the mixture was vortexed for 10 min at two times. The obtained clear solvent was used in DLLME. It is remarkable that other molar ratio of HBA: HBD (1:0.5, 1:1, and 1:2) were investigated too and only in the case of mole ratio 1:2 of HBA: HBD the DESs were formed.

Procedure

A 5.0 mL deionized water spiked with the analytes (2 mg L⁻¹, each drug) or urine sample adjusted at pH 10 with ammoniacal buffer (C=0.1 M) was added into a 10–mL glass test tube and 1.5 g NaCl (30%, w/v) was dissolved in the solution. Then 2.0 mL iso–propanol was added to it. The mixture was manually shaken for 2 min and the solution was centrifuged for 5 min at 4000 rpm. Thus, 0.9 mL of upper phase (iso–propanol) was removed and mixed with 85 µL of ChCl: benzyl ethylenediamine DES. The mixture was rapidly injected into 5–mL deionized water containing 5% w/v NaCl (adjusted at pH=10). After that, the formed cloudy solution was centrifuged at 5000 rpm for 7 min and 1 µL of the sedimented phase (10 ± 1 µL) was drawn and injected into the GC–FID system.

Results and Discussion

In the present work, a salt induced-LLE coupled with DES based-DLLME method, combined with GC-FID, was developed for the analysis of three anti-seizures in urine samples. The efficiency of the method is controlled with diverse parameters, including: (1) type and volume of extraction solvent in LLE and DLLME steps, (2) ionic strength, (3) the solution pH, and (4) centrifugation rate and time. These parameters were studied using the “one-variable-at-one-time” procedure.

Optimization of parameters in salt induced LLE step

Extraction solvent selection

The target analytes were extracted into a water-miscible organic solvent which is used as a dispersive solvent in the next step. The necessities for the selection of this solvent are its ability for extraction of analytes from the sample and its miscibility with aqueous phase and extraction solvent used in DLLME. According to these criteria three water-miscible organic solvents consist of THF, acetonitrile, and iso–propanol were examined as extraction solvents. For this purpose, 5.0 mL of deionized water spiked with the target analytes at concentration of 2 mg L⁻¹ (each analyte) was mixed with 1.25 g NaCl. Then, 2.0 mL of each solvent was added to the solution and after manually shaking and centrifugation, 0.9 mL of the upper collected phase was removed and mixed with 100 µL of ChCl: benzyl ethylenediamine and used in followed DLLME method. The obtained results in Figure 1 showed that the highest analytical signals were obtained in the presence of iso–propanol. Therefore, it was chosen as the extraction solvent in the next experiments.

Optimization of iso–propanol volume

The iso–propanol volume has main effect on the efficiency of the method. By increasing the volume of iso–propanol the ratio of organic phase to sample solution increases and it is expected that the ERs of the analytes increase. On the other hand, the collected phase volume was increased by increasing the iso–propanol volume which affects the dispersion of the extraction solvent in the followed DLLME procedure. To study iso–propanol volume several tests were performed with different volumes of iso–propanol at the range of 1.5–3.0 mL. The experiments showed that by increasing the volume of iso–propanol from 1.5 to 3.5 mL the upper phase volume alters from 0.7 to 2.0 mL. It is noted...
that when the upper phase volume was lower than 1.0 mL, it was diluted to 1.0 mL by iso-propanol. According to the results in Figure 2, the analytical signals increase up to 2.0 mL and then decrease. Therefore, 2.0 mL was selected for the next experiments.

Salt addition in LLE-DES step
Addition of salt is a crucial factor in the performance of the developed method since the LLE procedure in the first step was induced by addition of NaCl to the sample solution. Therefore, it was expected that without salt addition, the method was failed to work due to miscibility of iso-propanol with the solution. On the other hand, addition of NaCl decreases the analytes solubility in the aqueous phase and may increases the ERs of the method. To evaluate salt addition effect, different amounts of NaCl including 25, 27, and 30% w/v were added to the spiked ammoniacal buffer solution (pH=10) with the analytes (at concentration of 2 mg L⁻¹, each analyte) and the method was performed on the mixtures. The obtained results (Figure 3) showed that the ERs were relatively constant (p values > 0.05) for carbamazepine and chlordiazepoxide and increase in the case of diazepam by increasing NaCl amount. Therefore, 30%, w/v was chosen for the next experiments. It is obvious that at the salt amounts < 25%, w/v, the LLE step was not performable.

Study of aqueous phase pH
Changing pH of the sample solution may affect the efficiency of an extraction method for the analytes containing acidic or basic groups. This can be attributed to the conversion of acidic or basic compounds to the deprotonated or protonated forms, respectively. To optimize the pH of sample solution different experiments were performed in the range of 6–12 by adding HCl and NaOH solutions and the results (data not shown here) showed that analytical signals were nearly constant for the analytes except for carbamazepine which decreases at pHs lower than 10. Subsequently, the sample solution was adjusted at pH=10 using ammoniacal buffer.

Optimization of DLLME step
Selection of the extraction solvent type and volume
Extraction solvent type had an important effect on the efficiency of DLLME procedure. The selected extraction solvents in the proposed method must meet several criteria including high extraction capability of the selected drugs, low solubility in water, environmentally friendly, good chromatographic behavior, and density higher than water. In this method, three different DESs consists of ChCl: benzyl ethylenediamine, ChCl: 2,4–dichloro aniline, and ChCl: p–chlorophenol were tested as the possible extraction solvents. All experiments were performed using 100 µL of each solvent. The results (Figure 4) reveal that the highest ERs were obtained in the case of ChCl: benzyl ethylenediamine except for carbamazepine which is a bit lower than ChCl: p-chlorophenol. Due to high differences of ERs for the other analytes in ChCl: benzyl ethylenediamine, it was selected for the next experiments. Suitability of the selected DES for GC–FID was studied by thermogravimetric analysis and the obtained results in Figure 5 showed that significant mass losses were occurred at 152, 208, and 283 °C in TGA curve. Evaporation of benzyl ethylenediamine and decomposition of ChCl were

Figure 3. Salt addition effect in LLE step
Extraction conditions: are the same as Figure 2 except NaCl concentration was changed at the range of 25-30% w/v.

Figure 4. Selection of extraction solvent in DLLME step.
Extraction conditions: are the same as Figure 3 in which three DESs consist of ChCl: benzyl ethylenediamine, ChCl: 2,4–dichloro aniline, and ChCl: p–chlorophenol were tested as possible extraction solvents in DLLME.

Figure 5. TGA curve of synthesized DES.
occurred in 152 and 283 °C, respectively. The mass loss at 208 °C attributed to pyrolysis of the DES. The ChCl: benzyl ethylenediamine volume was another significant factor affecting the ERs and EFs of the analytes and subsequently LODs of the present method. To examine this parameter, different volumes of ChCl: benzyl ethylenediamine (85, 90, 100, and 120 µL) were investigated. The data showed that the ERs were relatively constant at different volumes of ChCl: benzyl ethylenediamine DES whereas the volume of the settled phase was increased from 10 to 32 µL (Figure 6). Thereby, EFs of the present method was decreased. To reach high EFs and low LODs, 85 µL was chosen as the optimum volume of the extractant.

The solubility of the analytes can alter by salt addition in extraction solvent or the aqueous phase. This phenomenon increases the analytes transferring into the extraction solvent and the final volume of the sedimented phase. To investigate the salt addition effect on the performance of the developed method, different amounts of NaCl at the range of 0.0–10%, w/v, were added to enhance ionic strength of the aqueous solution. The obtained results (Figure 7) illustrated that the addition of salt has no significant effect on the performance of the method (p values > 0.05) for the analytes except for carbamazepine which was increased up to 5.0%, w/v, due to the salting–out effect of NaCl and then decreased gradually. Therefore, 5%, w/v, was selected for the next experiments.

**Optimization of other parameters**

Other important factors affect the method performance such as pH of aqueous phase used in DLLME step, and centrifugation rate and time were also optimized. To study of these parameters, the pH of the aqueous phase was adjusted at pHs in the range of 2–12 and centrifugation time and rate were changed at the ranges of 3–7 min and 2000–4000 rpm. The results indicated that the highest analytical signals were obtained at 10, 5 min, and 4000 rpm for pH, centrifugation time and speed, respectively.

**Method validation**

According to US Food and Drug Administration protocols, the developed method was validated considering several parameters consist of LODs, limit of quantification (LOQ), selectivity, linearity, accuracy, intra– and inter–day precisions, stability, EFs and ERs in analytes-free urine samples (Table 1). The method linearity was studied by preparing matrix–matched calibration and was linear at the range of 25–1000 ng mL\(^{-1}\) with R\(^2\) greater than 0.9965. The LODs, signal–to–noise ratio (S/N) of 3, and LOQs (S/N=10) were in the ranges of 3.4–6.9, and 11.5–23.3 ng mL\(^{-1}\), respectively. The method precision was studied by determining intra– (n=5) and inter–day (n=4) precision at the concentration of 125 ng mL\(^{-1}\) and they were lower than 11%. The method accuracy was evaluated by standard addition method. For this purpose analytes-free urine samples were spiked with the analytes at concentration of 125 ng mL\(^{-1}\) (each analyte) and the method was performed on them for five replicates. The found concentrations were calculated with calibration graph equations. The results showed that the deviations for found values were less than 10% from added concentrations for all analytes. The stability of the analytes in urine sample was studied by spiking the analytes to urine samples at the concentrations of 125 ng mL\(^{-1}\) in three different conditions including at room temperature (24 °C) for 24 h, and stored at −20 °C for two days. The stability of the analytes was determined after three freeze-thaw cycles (−20 to 24 °C) and the results were compared with the freshly prepared samples. The RSDs less than 9% for the analytes indicated good stability for the analytes. The EFs were calculated by dividing analytes concentrations in the DES to first concentrations of in urine samples and they were in the range of 305–430. The percentage of total amounts of analytes which were...
transferred into ChCl: benzyl ethylenediamine DES were considered as ERs and they were obtained in the range of 60-86%.

Matrix effect and real sample analysis
The developed method was performed for triplicates on two urine samples, obtained from healthy volunteers who had not taken the studied analytes, after spiking at three concentration levels including 125, 250, and 500 ng mL\(^{-1}\), each analyte. The results obtained for urine samples were compared with those obtained with spiked deionized water at the same concentrations. According to the results listed in Table 2, good mean relative recoveries at the range of 87-97% verified that the matrix of the samples had not significant effect on the determination of the analytes. On the other hand, to evaluate the matrix exogenous drugs that can potentially be available in the urine samples and can affect the results, the urine samples were spiked with antiarrhythmic drugs (propranolol, carvedilol, and verapamil) and antidepressants (nortriptyline, sertraline, clomipramine, and fluoxetine) at 125 ng mL\(^{-1}\) of each drug. The obtained results showed that no interference was observed at the studied analytes retention times and the method is selective for the studied analytes. Furthermore, two urine samples were obtained from two patients who were under treatment with tablet of carbamazepine (200 mg) and the method was performed. The samples were obtained 15 hours after taking the drug. After three determinations of each sample using added–found method, the carbamazepine concentrations in the urine samples were 643 ± 22 and 571 ± 19 ng mL\(^{-1}\). Typical GC–FID chromatograms of the urine samples after performing the developed method as well as a blank urine sample and the direct injection of a standard solution of the analytes were depicted Figure 8.

### Table 1. Quantitative features of the developed method for the selected analytes.

| Analyte          | LOD  | LOQ  | LR   | \(r^2\) | RSD% ^g | EF ± SD | ER ± SD |
|------------------|------|------|------|---------|---------|---------|---------|
| Carbamazepine    | 6.9  | 23.3 | 23.3–10000 | 0.9965 | 5.9     | 305 ± 30 | 61 ± 6  |
| Diazepam         | 3.4  | 11.5 | 11.5–10000 | 0.9974 | 3.6     | 430 ± 20 | 86 ± 4  |
| Chlordiazepoxide | 4.8  | 16.1 | 16.1–10000 | 0.9977 | 9.4     | 310 ± 40 | 62 ± 8  |

(a) Limit of detection (S/N=3) (ng mL\(^{-1}\)).
(b) Limit of quantification (S/N=10) (ng mL\(^{-1}\)).
(c) Linear range (ng mL\(^{-1}\)).
(d) Coefficient of determination.
(e) Relative standard deviation for intra– (n=5) and for inter–day (n=4) precisions at a concentration of 125 ng mL\(^{-1}\) of each analyte.
(f) Enrichment factor ± standard deviation (n=3).
(g) Extraction recovery ± standard deviation (n=3).

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

| Analyte          | Spiked level (ng mL\(^{-1}\)) | Relative recovery ± SD |
|------------------|-----------------------------|------------------------|
| Carbamazepine    | 125                         | 93 ± 4 97 ± 5         |
|                  | 250                         | 97 ± 3 92 ± 6         |
|                  | 500                         | 92 ± 5 94 ± 4         |
| Diazepam         | 125                         | 87 ± 3 92 ± 5         |
|                  | 250                         | 94 ± 5 91 ± 4         |
|                  | 500                         | 96 ± 4 93 ± 3         |
| Chlordiazepoxide | 125                         | 95 ± 3 90 ± 4         |
|                  | 250                         | 96 ± 4 89 ± 6         |
|                  | 500                         | 93 ± 2 90 ± 5         |

Comparison of the method with other approaches
Analytical features of the present method were compared with other approaches reported in the literature and the LODs, LR, extraction time, extraction solvent and RSDs were listed in Table 3. The presented method has a good sensitivity, proper precision, wide LR, and better or comparable LODs with those obtained by other methods. On the other hand, the use of DES as an extraction solvent with lower toxicity and short extraction time of developed
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Table 3. Comparison of the proposed method with other methods in the extraction and determination of the selected analytes.

| Method               | Sample          | Extractive phase             | LOD^a (ng mL^-1) | LR^b (ng mL^-1) | RSD^c (%) | Extraction time (min) | Ref.  |
|----------------------|-----------------|------------------------------|------------------|----------------|------------|------------------------|-------|
| DNUM–HPLC–UV^d       | Urine and plasma | ZnS-AC nanoparticles        | 1.2-1.5          | 5-10000        | 3.5-5.8    | 23                     |       |
| UA-LDS-DLLME–GC–MS^e  | Urine and plasma | Ethyl acetate                | 1.0-3.0          | –              | 4.1-5.6    | 3                      | 50    |
| DLLME–HPLC–UV^f       | Plasma           | Chloroform                   | 3.5-8.1          | 8.1-5000       | 5.6-12.7   | 10                     | 32    |
| HT-BME-HPLC-DAD^g      | Urine            | Metahnol: acetomitrile (50:50)| 2-5              | 30-100         | <15        | >180                   | 15    |
| APCI-HPLC-MS/MS^h      | Urine            | 10 % Acetonitrile in water   | -                | 20-500         | 12-17      | ~120                   | 12    |
| SILLE–DES-DLLME–GC–FID^i | Urine           | Benzyl ethylenediamine       | 3.4-6.9          | 11.5-1000      | 5.9-9.4    | <20                    | This method |

a) Limit of detection.
b) Linear range.
c) Relative standard deviation.
d) Dispersive nanomaterial-ultrasound-assisted microextraction—high performance liquid chromatography–ultraviolet detector.
e) Ultrasonic assisted-low density solvent-dispersive microextraction–gas chromatography–mass spectrometry.
f) Dispersive liquid–liquid microextraction–high performance liquid chromatography–ultraviolet detector.
g) High throughput bar microextraction–high performance liquid chromatography–diode array detector.
h) Atmospheric pressure chemical ionization–high performance liquid chromatography–tandem mass spectrometry.
i) Salt induced liquid–liquid extraction–deep eutectic solvent based-dispersive liquid–liquid microextraction–gas chromatography–flame ionization detector.

method compared to the other works are other features of the presented method. These results are acceptable for an extraction method to be a rapid, efficient, sensitive, and reliable technique for the extraction and preconcentration of BZDs from urine samples.

Conclusion
A simple, easy, efficient, green, and sensitive DES based microextraction technique was developed for extraction of three anti-seizures drugs including carbamazepine, diazepam, and chlordiazepoxide from urine samples coupled to GC–FID. The procedure consists of two steps. In the first step, the analytes were extracted into a water-miscible organic solvent which was used as dispersive solvent in the followed DLLME and phase separation in LLE was induced by salt addition. In DLLME, amine-based DESs were used as extraction solvent to preconcentrate the extracted analytes. After optimization the method was validated and relatively low LODs and LOQs, high ERs and EFs, and acceptable precision and accuracy were obtained. The developed method was performed on urine samples obtained from patients who used the studied drugs and they were successfully determined in them.

Ethical Issues
This study was confirmed by the research ethics committee of Tabriz University of Medical Sciences with IR.TBZMED.REC.1397.492 ethic code. All sample donors have been informed on details of the drugs and signed a consent form.

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Conflict of Interests
The authors claim that there is no conflict of interest.

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