Research article

Drug Testing and Analysis

Received: 28 August 2010 Revised: 18 December 2010 Accepted: 18 December 2010 Published online in Wiley Online Library: 5 March 2011

(www.drugtestinganalysis.com) DOI 10.1002/dta.259

Applying cloud point extraction technique for the extraction of oxazepam from human urine as a colour or fluorescent derivative prior to spectroscopic analysis methods

Ahad Bavili Tabrizi[a]* and Maryam Harasi[b]

Two new methods based on cloud point extraction (CPE) technique were developed and optimized for the extraction and preconcentration of oxazepam from human urine, as an azo or fluorescent derivative. The first method is a spectrophotometric one, which is based on the acid hydrolysis of the oxazepam to a benzophenone, diazotization of the benzophenone, and then the coupling with oxine to form an azo dye. The second method is a spectrofluorimetric one, which involves reduction of the target compound using Zn°/HCl at room temperature with the formation of a highly fluorescent derivative. The main factors affecting the chemical reactions and CPE were investigated and optimized systematically. Under optimum experimental conditions, the calibration graphs were linear in the range of 0.1 to 1.5 (0.05 to 2.0) μg/ml with correlation coefficients of 0.9989 (0.9985), for the CPE-spectrophotometric (CPE-spectrofluorimetric) method. The limit of detection was found to be 0.034 (0.018) μg/ml and the relative standard deviation was calculated to be 1.35 (2.52)%. Recoveries in the spiked samples ranged from 87 to 94%. Finally, the proposed methods were applied to the determination of oxazepam in human urine.

Keywords: oxazepam determination; cloud point extraction; spectrophotometry; spectrofluorimetry; urine

Introduction

Benzodiazepines (BZPs) are among the most widely prescribed drugs used in the treatment of stress, anxiety, sleep disorders, muscle spasms, and seizures. Many patients develop a dependence on these drugs which are often involved in intoxications. Therefore, BZPs are frequently encountered both in clinical and forensic toxicological analysis.[1]

Oxazepam (OXZ), a 3–hydroxy–l, 4–benzodiazepine derivative, is a commonly prescribed BZP. After oral consumption, peak plasma level was reached between 1.7 and 2.8 h. The elimination half-life ($t_{1/2}$) and the plasma $t_{1/2}$ were 6.7h and 5.8 h, respectively. Absorption was almost complete, with a bioavailability of 92.8%. Urinary recovery of its glucuronide was 80.0% and 71.4% of the dose after intravenous and oral administration, respectively.[2] In addition, OXZ is the metabolite of other BZPs and is easily removed through the urine as glucuronic acid metabolite, thus its monitoring in urine is very important.[3,4]

Different analytical methods such as flow injection analysis,[5] high performance liquid chromatography (HPLC),[6–10] HPLC-mass spectrometry (MS),[11–13] gas chromatography-MS (GC-MS),[14–16] electrochemistry,[17,18] thin layer chromatography (TLC),[19] fluorimetry[20] and immunoassay techniques[12,15,21–23] have been reported in the literature for the determination of OXZ in various biological fluids. However, most of these methods are labour-intensive and time-consuming or require expensive equipment and expert technicians. Alternative techniques, such as enzymatic and immunoassay, have also been developed, but the used enzymes and antibodies are relatively expensive.

The analysis of BZPs in biological samples is preceded by sample preparation techniques such as liquid-liquid extraction (LLE),[2,7,17,20,24] solid-phase extraction (SPE) or solid-phase micro-extraction (SPME).[1,3–5,16,24] However, these techniques can suffer from lengthy extraction times, slow derivatization reactions, large sample volumes, and/or excess use of organic solvents.[4]

Simple, effective, and environmentally friendly extraction procedures are still in demand. The unique behaviour of surfactants at cloud point has attracted increasing attention in sample extraction and preconcentration. Thus, CPE has been recognized as an alternative to conventional extraction systems because of the following advantages: good capacity to dissolve compounds with different types and nature; the ability to concentrate solutes with high recoveries; safety and cost benefits; very small amounts of the relatively non-flammable and non-volatile surfactants are required; easy disposal of the surfactant; compatibility with micellar or hydro-organic HPLC mobile phases; and inhibition by the surfactants of adsorption of non-polar analytes to glass surfaces.

The principles, advantages and applications of CPE have been the subject of several review articles.[25,26] Moreover, the analytical potential of CPE has been demonstrated in many studies concerning extraction and preconcentration of both organic

* *Correspondence to: Dr Ahad Bavili Tabrizi, Associate Professor of Analytical Chemistry, Department of Medicinal Chemistry, Faculty of Pharmacy & Biotechnology Research Center, Tabriz University of Medical Sciences; Tabriz; Iran. E-mail: a.bavili@tbzmed.ac.ir*

a *Department of Medicinal Chemistry, Faculty of Pharmacy & Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

b *Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*

compounds and metal ions.[27–30] However, the use of aqueous surfactant solutions as alternative solvents in the extraction of drugs from biological fluids has rarely been reported in the literature.[31–35]

Thus the aim of this work was to develop new, sensitive, and simple CPE strategies for the extraction of OXZ derivatives from human urine and their subsequent determination by proper analytical methods. The spectrophotometric method uses oxine as a chromogenic reagent for diazotized benzophenone, which is obtained by acidic hydrolysis of OXZ, while spectrofluorimetric method is based on the reduction of the target compound with Zn°/HCl to form a highly fluorescent compound. The proposed methods permit the analysis of OXZ in urine samples in a simpler and cheaper way, compared to sophisticated and expensive methods, such as GC/MS or HPLC/MS, in addition to the other well-known advantages of CPE methodology.

Experimental

Apparatus

Spectral measurements were carried out with Shimadzu UV-visible Recording Spectrophotometer (UV-160 model) using 1-cm path length and 1.5-ml quartz cells. All fluorescence measurements were made on a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a 150 W Xenon lamp and using 700 µL quartz microcells. Instrument excitation and emission slits both were adjusted to 5 nm. A thermostated water bath (636 Friedberg/Hessen, Germany) was utilized throughout the work. A Hettich centrifuge (EBA 20 model/Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) with 15-ml calibrated centrifuge tubes (Hirschmann, EM techcolor, 74246 Eberstadt, Germany) was used to accelerate the phase separation process. A Corning M120 pH–meter (Halstead, Essex, UK) was used for checking the pH of solutions.

Reagents

The non-ionic surfactant Triton X-114 (TX-114) was obtained from Sigma (St Louis, MO, USA) and used without further purification. Zinc powder was obtained from Riedel-De Haën AG (Honeywell Riedel-de Haen, Germany). The β–glucuronidase, Type HP–2 from *Helix pomatia* (116,400 units/ml), was from Sigma-Aldrich. Inc., 3050 Spruce Street, St Louis, MO 63103 USA.

A stock solution of 500 µg/ml of OXZ was prepared by dissolving appropriate amounts of pure drug (obtained from Zahravi, Tabriz, Iran) in absolute ethanol (Merck KGaA, Darmstadt, Germany) and was kept away from the light in a refrigerator with a temperature of 4 °C. Working standard solutions were obtained by appropriate dilution of this stock standard solution.

A 1.25% (*w*/*v*) solution of oxine (Merck KGaA, Darmstadt, Germany) was prepared in absolute ethanol. Other solutions, i.e. 1% (*w*/*v*) $NaNO_2$, 1 and 6 M HCl (Merck KGaA, Darmstadt, Germany) and 1 and 5 M NaOH (Merck KGaA, Darmstadt, Germany) were prepared in doubly distilled water. The acetic acid/acetate buffer (1 M, pH 4.5) was prepared from sodium acetate trihydrate (Honeywell Riedel-de Haen, Germany) and acetic acid according to the literature with some modifications.[1] All other reagents were of analytical reagent-grade or higher.

Recommended procedures

Preparation of solutions for spectrophotometric analysis

From the 10 µg/ml OXZ standard solution aliquot volumes in the range of 0.1–1.5 ml were transferred into 15-ml centrifuge tubes and mixed with 0.75 ml of 6 M HCl solution. Then contents were diluted to 2.5 ml and heated for 1 h at 100 °C in a water bath. After hydrolysis, the tubes were immersed in an ice bath and 1 ml of 1% $NaNO_2$ solution was added. The contents were mixed and left to stand for 5 min. Then 0.3 ml of 1.25% oxine solution was added and coupling was performed in alkaline medium by adding 0.8 ml of 5 M NaOH solution.

Preparation of solutions for spectrofluorimetric analysis

From the 10 µg/ml OXZ standard solution aliquot volumes in the range of 0.05–2.0 ml were transferred into 15-ml centrifuge tubes then mixed with 0.025 g of zinc and 0.75 ml of 1 M HCl solution. The contents were diluted to 2.5 ml and left to stand for 15 min at room temperature. Then, the excess of acid was neutralized by adding 0.2 ml of 1 M NaOH and pH adjusted by using 1.5 ml of 1 M acetate buffer. After this process, clear solutions were separated from non-reacted zinc by centrifugation and transferred into other centrifuge tubes.

CPE and determination

An aliquot of 1.5 ml of 1% TX-114 solution was added to the solutions obtained from the above-mentioned steps and the volume was made up to 10 ml. The contents were mixed and kept at 40 °C for 10 min. Then by centrifuging for 6 min at 3800 rpm the surfactant-rich (SR) phase was completely separated from the dilute aqueous phase, so the supernatant aqueous phase was readily decanted with a Pasteur pipette. The remaining SR phase was diluted to 1.5 ml with ethanol–water (1 : 1, *v*/*v*) and its absorbance was measured at 516 ± 3 nm against a reagent blank prepared similarly, while the fluorescence intensity was read at 484 ± 3 nm with the excitation wavelength set at 334 ± 3 nm.

Procedure for urine sample

The samples must be occasionally hydrolyzed to ensure appropriate detection of some BZPs, thus urine is usually hydrolyzed with the enzyme glucuronidase in order to obtain the free drugs/metabolites.[1–5,12,16,17,21] The previous hydrolysis of these glucuronides allows to obtain more reliable results, independently of the technique of determination.[17]

Urine sample was obtained from healthy male volunteer who took single oral dose of 10 mg OXZ tablet (Pursina Pharm. Co., Tehran, Iran). After administration, the samples were collected between 0 h and 48 h and frozen at −20 °C until analysis. For enzymatic deconjugation, 2 ml of urine was transferred into 10-ml centrifuge tube and 0.9 ml of sodium acetate buffer (0.2 M, pH 4.5) and 100 µL of β–glucuronidase/aryl sulfatase (116 400–1015 IU/ml) were added. The tubes were mixed vigorously and incubated at 56 °C for 2 h. Then tubes were centrifuged at 3000 rpm for 15 min and 0.2 ml aliquots of the supernatant solutions were subjected to the above-mentioned procedures.

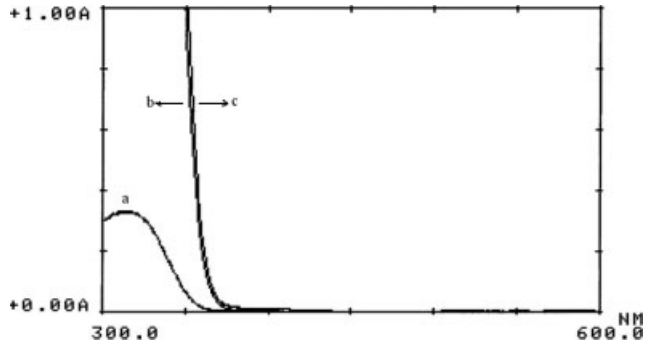

Figure 1A. Absorption spectra of: (a) standard solution of OXZ (0.4 μg/ml), (b) oxine, (c) OXZ-oxine without chemical reactions and CPE.

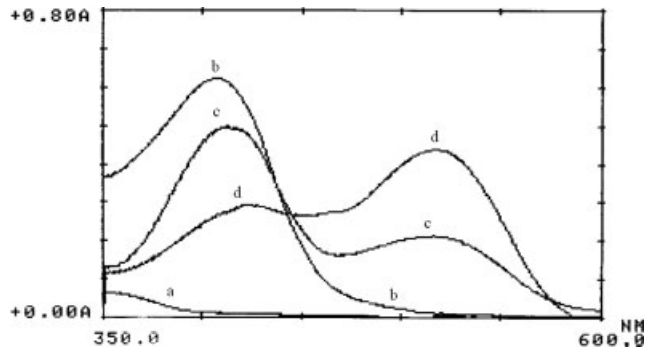

Figure 1B. Absorption spectra of: (a) TX-114, (b) oxine after CPE, (c) standard solution of OXZ (0.4 μg/ml) after chemical reactions and CPE, (d) urine spiked with OXZ (0.8 μg/ml) after chemical reactions and CPE. Other conditions: 0.75 ml of 1 M HCl, 1 ml of 1% $NaNO_2$ and 1.5 ml of 1.0% TX-114.

Results and discussion

Absorption spectra of OXZ, oxine, and OXZ-oxine with and without preconcentration have been shown in Figure 1. As can be seen in Figure 1A, there isn't any interaction between OXZ and oxine; moreover the reagents don't show any absorption peak in the analytical wavelength (Figure 1B). On the other hand, by taking into account that OXZ doesn't show any significant fluorescence in the studied conditions, thus chemical reactions based on the conversion of OXZ to a sensitive colour or fluorescent derivative were considered.

The spectrophotometric methods used for determining individual BZPs are generally based on their acid hydrolysis followed by a ring opening reaction and the subsequent determination of the obtained benzophenone. The benzophenone is diazotized with nitrous acid and then coupled with different chromogenic reagents.[5,36] These reactions take place at different pH values; in fact, the diazotized benzophenone is formed at very low pH (<1), whereas the coupled product requires an alkaline medium (pH > 12).[5] In this work, some of these agents such as 2-naphthol, salicylate and oxine were tried as a coupling agent and the results showed that sensitive colour reaction occurred when oxine was used. Figure 1B shows the absorption spectrum of the formed azo dye after CPE, which exhibits an absorption peak at 516 $\pm$ 3 nm. The recording of absorption spectrum for different agents used showed that the first band is due to oxine and this reagent does not show any significant absorbance at the analytical wavelength.

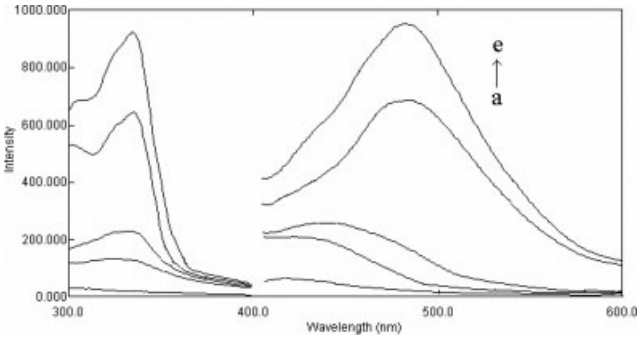

Figure 2. Excitation and emission spectra of (a) reagent blank, (b) urine blank, (c) collected urine sample after administration of OXZ to one volunteer (after chemical reactions and CPE of the product from urine), (d) standard solution of OXZ (1.0 μg/ml) after chemical reactions and CPE, (e) sample 'c' spiked with OXZ (1.2 μg/ml). Other conditions have been mentioned in the text.

The native fluorescence of BZPs is very low and a suitable signal intensity is only obtained either by cyclization to acridines after hydrolysis to benzophenones,[20] thermal heating in acidic solvent,[37] photochemical degradation,[38] derivatization with phthalaldehyde and fluorescamine,[39] or by reduction of the target compounds with Zn°/HCl to form highly fluorescent derivatives.[40] Native fluorescence of some BZPs in acidic or methanolic KOH solution was also reported.[4,41]

The reduction products of BZPs with Zn°/HCl which contain a hydroxyl group at C-3 are fluorescent.[40] Thus, the proposed spectrofluorimetric method involves reduction of the target compound using Zn/HCl at room temperature with the formation of a highly fluorescent derivative. The fluorescence intensity was measured at 484 $\pm$ 3 nm with the excitation wavelength set at 334 $\pm$ 3 nm against a reagents blank prepared similarly. Fluorescence excitation and emission spectra are given in Figure 2.

Optimization of experimental conditions

Optimization of chemical reactions for spectrophotometric analysis

The reaction conditions for the hydrolysis of BZPs vary considerably for individual compounds.[36] Therefore, in order to have optimal sensitivity, it is necessary to optimize the parameters affecting the hydrolysis and other chemical reactions. According to the literature, the hydrolysis temperature of 100 °C in acidic medium has been extensively used.[36] Thus, the temperature and time of the hydrolysis were fixed at 100 °C and 1 h, respectively, and the influence of HCl concentration was examined by taking varying volumes of 6 M HCl (in the range of 0.1–2.0 ml). As shown in Figure 3 (series 1) the absorbance rose with increasing volume of HCl reaching its constant value at 0.75–1 ml. After this optimum range further increase in volume of HCl caused slight increase in the absorbance. High HCl concentrations can favour the diazotization reaction but also decrease the stability of the coupled product.[5] Thus, benzophenone was prepared in 0.75 ml of 6 M HCl, where the diazotization rate and the stability of the coloured compound were optimal. The effect of hydrolysis time was investigated in the range of 15–60 min and according to the results the hydrolysis time of 60 min led to better hydrolysis.

The effect of $NaNO_2$ on the signal intensities was examined by using increasing volumes of 1% $NaNO_2$ from 0.1 to 2.5 ml. The results showed that the change of $NaNO_2$ concentration in the

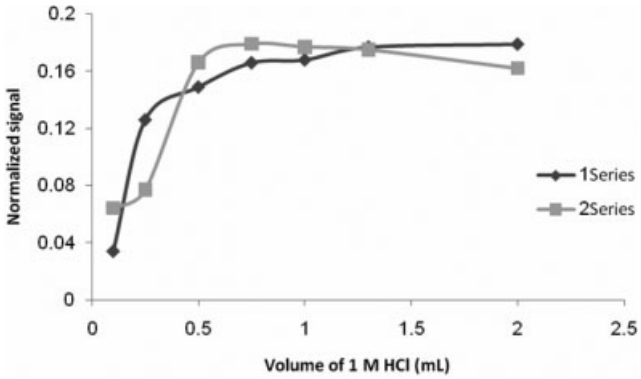

Figure 3. Effect of HCl concentration on the analytical responses, series 1 and 2 are referred to CPE-spectrophotometric and CPE-spectrofluorimetric methods, respectively, other conditions have been mentioned in the text.

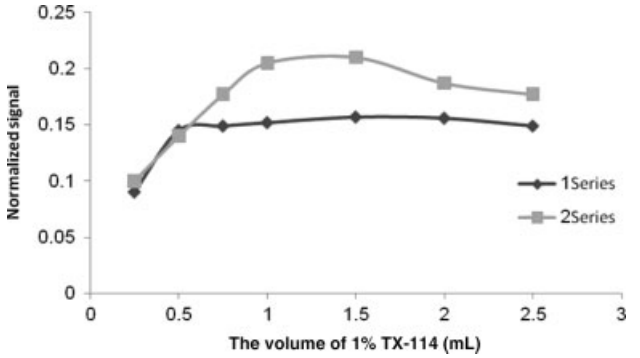

Figure 5. Effect of TX-114 concentration on the analytical responses, series 1 and 2 are referred to CPE-spectrophotometric and CPE-spectrofluorimetric methods, respectively, other conditions have been mentioned in the text.

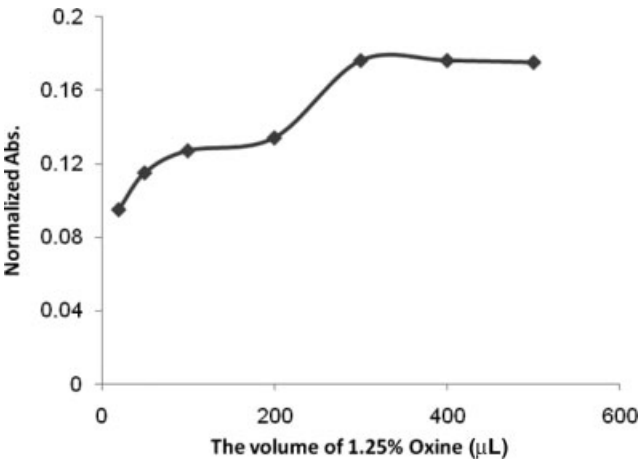

Figure 4. Effect of oxine concentration on the analytical responses; conditions have been mentioned in the text.

studied range has no significant effect on the analytical signals, thus the volume of 1 ml was used in other experiments.

The effect of oxine reagent was investigated over the range of 20–500 μL of 1.25% oxine. The absorbance rose with increasing concentration of oxine reaching its maximum value at 300–400 μL, which is sufficient for compete coupling of the diazonium salt. Larger volumes were found to have no significant effect on the absorbance (Figure 4), thus 300 μL of oxine was selected as an optimum value.

A high concentration of NaOH was required since the alkali must neutralize the acid present and provide the basic medium needed for the coupling reaction with oxine. Thus the optimum NaOH was determined by adding various volumes of 5 M NaOH (in the range of 0.6–2 ml). Optimum absorbance value was achieved at 0.8 ml of NaOH and the raising of NaOH concentration above this value caused a gradual decrease in the intensity, probably due to instability of the formed azo dye.

Optimization of chemical reactions for spectrofluorimetric analysis

The effect of zinc amounts on the fluorescence intensities was investigated in the range of 0.01–0.2 g. The emission signal was high at first and increasing the zinc amount above 0.025 g caused a gradual decrease in the emission intensity probably due to its quenching effect. Thus 0.02 g of zinc was selected.

The effect of HCl on the fluorescence intensities was examined by taking varying volumes of 1 M HCl (in the range of 0.1–2 ml). As shown in Figure 3 (series 2) the emission signal rose with increasing concentration of HCl reaching its constant value at 0.75–1.5 ml. After this optimum range further increase in volume of HCl caused slight decrease in emission signal. This is probably due to solutions with high acidity, so the subsequent buffer addition cannot adjust the optimum pH needed for the extraction. Thus 0.75 ml of 1 M HCl was used in other experiments.

The proposed method involves the rapid reaction between OXZ and zinc/HCl agent at ambient temperature without heating.[40] Thus the effect of equilibration time on the fluorescence intensity was investigated and the results indicated that a reaction time of 15 min is adequate to obtain the maximum fluorescence intensity. The produced fluorophore remained stable for more than 2 h in room temperature.

The influence of pH on the extraction of fluorophore was studied by adjusting the pH of solutions in the range of 1.5 to 5.5 by using HCl or acetate buffer, so the pHs below 2 were adjusted with dilute HCl and between 2 and 5.5 with 1 M acetate buffer. On the other hand, when the sample pH was higher than 5.5, the detection deteriorated due to hydrolysis of Zn^{2+} and its precipitation along with SR phase. The results showed that the pH range of 4.0–5.5 was optimum for the extraction of fluorophore and hence the pH was fixed at 4.5 by adding 0.5 ml of 1 M acetate buffer.

Optimization of CPE

The influence of TX-114 concentration on the analytical signals was examined when different volumes of 1% TX-114, ranging from 0.25 to 2.5 ml, were used. It can be seen from Figure 5 that maximum and constant absorbance and fluorescence intensities were observed by using 0.5–2.5 or 1.0–1.5 ml of TX-114, respectively. Thus, 1.5 ml of TX-114 (corresponding to the final concentration of 0.15%) was selected as optimum value for further studies.

Finally, the optimal incubation time and equilibration temperature are necessary to complete extraction and to achieve easy phase separation and efficient preconcentration. Thus the extractions were carried out at different temperatures (ranging from 30 to 60 °C) using a thermostated water bath for periods ranging from 10 to 30 min. Maximum and constant signals were obtained at 40–45 °C after 10 min. Higher temperatures can lead to a slight decrease in analytical signals probably due to instability of the formed products. Also, it was found that a centrifuge time of 6 min at 3800 rpm was optimal for the entire procedure.

Table 1. Analytical characteristics of the different extractive methods

Method	Concentration range (μg/ml)	Slope	Intercept	r^2	RSD%[†]	LOD (μg/ml)	Mean recovery (%)	Reference
GC–MS	0.05–1.0	5.00×10^{-4}	−0.0137	0.9975	3.7[a]	0.030	59–65	[1]
GC–MS	0.05–8.0	–	–	–	1.4[b]	0.050 (LOQ)	62–87	[3]
Spectrophotometry	0.43–2.15	0.179	−0.0490	–	4.5[c]	0.14_3	99	[5]
GC	0.14–0.86	–	–	0.9910(r)	3.1[d]	0.13_9	105 (accuracy)	[16]
electrochemical	0.025–1.0	–	–	0.9986	1.8[e]	0.012	–	[17]
HPLC (with UV detection)	$0.5–18 \times 10^{-3}$	3.93	0.790	0.9990 (r)	4.3[f]	0.16	99	[42]
HPLC (with UV detection)	0.05–10.0	3.28×10^5	-2.32×10^4	0.9993	4.6[g]	0.025	>95	[43]
Spectrophotometry	0.1–1.5	0.493	0.0150	0.9980	1.4_5[h]	0.034	87–90	This work
Spectrofluorimetry	0.05–2.0	520.6	-64.3_8	0.9970	2.5_2[i]	0.018	91–94	This work

[†] Concentration values (as μg/ml) and their replications (n) related to these RSDs; [a] 0.02 (n = 6); [b] 0.3 (n = 6); [c] 0.86 (n = 11); [d] 0.3 (n = 6); [e] 0.5 (n = 6); [f] 1.5 (n = 9); [g] 0.5, [h] 0.4 (n = 10); [i] 1.2 (n = 5).

Analytical performance

Under the optimized experimental conditions, calibration curves were obtained by preconcentrating 10 ml of sample solutions. After dilution of the SR phase (≈0.2 ml) to 1.5 ml with ethanol-water, the absorbance or fluorescence was measured at desired wavelengths against a reagent blank. Thus, the theoretical and experimental preconcentration factors of 50 and ≈7 were obtained for the extracted samples.

Table 1 indicates the analytical characteristics of the proposed methods. Limits of detection (LODs) were calculated as $\frac{3\sigma_s}{R}$,[41], where σ_s is the standard deviation (SD) of the blank and R the slope of the calibration curve, and found to be 0.034 and 0.018 μg/ml in the case of spectrophotometric and spectrofluorimetric methods, respectively. These LODs were sufficiently low to be valuable for determination of OXZ in biological fluids. In addition, obtained linear ranges, LODs and RSDs were comparable or better than those reported in other extractive-analytical methods (Table 1).

The interferences

The hydrolysis and diazotization reactions are non-specific for BZPs; as a result, hydrolysis products of BZPs which contain a primary amino group in their structures, such as chlorazepate, diazepam, nitrazepam, and flurazepam can be interfered with spectrophotometric determination. Besides, the newer BZPs (alprazolam, triazolam among others) undergo no acid hydrolysis and so they form no BZPs. As a result, these BZPs could not be detected directly; however, most of them form metabolites that are hydrolyzed in this medium and give a primary amino group, such as prazepam, diazepam, and flurazepam, that allows their detection by the proposed spectrophotometric method.[5]

On the other hand, the reduction products of only BZPs which contain a hydroxyl group at C–3, such as lorazepam, cinolazepam, and temazepam are fluorescent and thus can be interfered with spectrofluorimetric detection.[40] But, it must be noticed that these drugs may not be prescribed or consumed simultaneously.

In short, OXZ is the form of elimination from the organism of many BZPs. For example, diazepam is metabolized in the organism to temazepam, amongst other compounds, and this, in its turn, is metabolized to OXZ.[17] Besides, the extensive biotransformation and/or distribution of these substances in the tissues give place to find either of the parent BZPs and intermediate metabolites in trace amounts in biological fluids.[17,20] Thus their interference effect can be neglected.

Table 2. Results of recoveries of spiked samples

Method	*OXZ added (μg/ml)	†OXZ found (μg/ml)	Recovery (%)
Spectrophotometry	0.4	0.360 ± 0.005	90
	0.8	0.696 ± 0.011	87
Spectrofluorimetry	0.4	0.364 ± 0.010	91
	0.8	0.752 ± 0.021	94

* A 0.2 ml portion of urine sample was used for recovery experiments.
† Average of three determinations ± standard deviation.

The validation and application of the method

A drug-free urine sample obtained from a healthy volunteer was prepared as mentioned in the section on 'Procedure for urine sample'. Aliquots of 0.2 ml portions were spiked with OXZ at concentrations of 0.4 and 0.8 μg/ml and subjected to the recovery experiments. The obtained recoveries ranged from 87 to 94%, as shown in Table 2, and seem to be satisfactory.

Typical spectra for the standard solution of OXZ, the urine blank, urine collected from one volunteer after administration of OXZ and the latter spiked with OXZ are presented in Figures 2 and 6. No additional picks due to interferences were observed at the analytical absorption or emission wavelengths (see Figures 2 and 6, respectively). Thus the coincidence of absorption or emission spectra along with reasonable recoveries indicated that no significant matrix effect was encountered in these two methods.

The proposed methods were applied successfully to the determination of OXZ in human urine. For this purpose, urine was collected for 48 h after a single oral administration of 10 mg of OXZ to one volunteer. The results of determination of OXZ by two proposed methods are shown in Table 3. A comparison using *t*-test at 95% confidence interval demonstrates that there isn't a significant difference between the achieved results using these two methods. The average concentration of OXZ in urine was found to be 4.81 μg/ml. The total OXZ excreted through urine was ≈70% of that taken in a total volume of 1.46 l

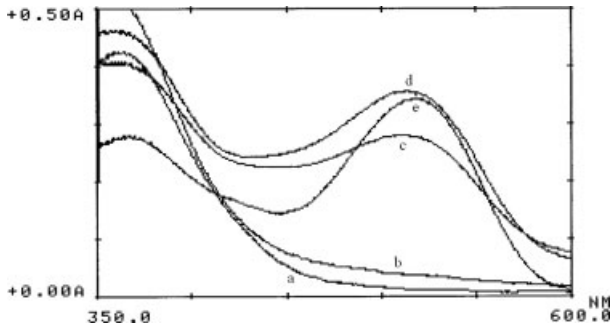

Figure 6. Absorption spectra of (a) reagent blank, (b) urine blank, (c) collected urine sample after administration of OXZ to one volunteer (after chemical reactions and CPE of the product from urine), (d) sample 'c' spiked with OXZ (0.2 μg/ml), (e) standard solution of OXZ (0.65 μg/ml) after chemical reactions and CPE. Other conditions have been mentioned in the text.

Table 3. Determination of OXZ in urine with the two proposed methods

*Method	♠OXZ concentration in the sample (μg/ml)	OXZ excreted through urine (%)	
Spectrophotometry	4.76 ± 0.07	69.5	†t = 1.12 (2.78)
Spectrofluorimetry	4.85 ± 0.12	70.8	†F = 2.94 (39)

* A 0.2 ml of collected urine sample (1.46 l) was used for OXZ determination.
♠ Average of three determinations ± standard deviation.
† Figures between parenthesis are the tabulated *t* and F values at p = 0.05 [44].

urine which is in accordance with values reported in other works.[2,3]

Conclusions

The feasibility of employing CPE as a simple and effective tool for the extraction of OXZ derivatives from human urine has been studied. The spectrophotometric method is based on the diazotization of the benzophenone in a prehydrolyzed sample at pH < 1, followed by the formation of azo dye with oxine at pH > 12, while the spectrofluorimetric method involves reduction of the target compound using Zn°/HCl at room temperature with the formation of a highly fluorescent derivative.

Compared to HPLC and GC, the proposed methods allow analysis of OXZ with low operational costs, simplicity of instrumentation, and without further sample clean-up steps. Thus, the time and cost of analysis can be significantly decreased in addition to other well-known advantages of CPE methodology. Because OXZ is the major metabolite of other BZPs in urine, thus the proposed methods can be used for screening in urine samples for the evidence of the other BZP drugs.

Acknowledgments

The author is grateful to the Research Office of Tabriz University of Medical Sciences for Financial support and to Dr H. Rezazadeh for his grammatical corrections.

References

[1] D. Borrey, E. Meyer, W. Lambert, C. Van Peteghem, A. P. De Leenheer, *J. Chromatogr. B* **2001**, *765*, 187.
[2] J. Sonne, S. Loft, M. Dossing, A. Vollmer-Larsen, K. L. Olesen, M. Victor, F. Andreasen, P. B. Andreasen, *Eur. J. Clin. Pharmacol.* **1988**, *35*, 385.
[3] J. G. Langner, B. K. Gan, R. H. Liu, L. D. Baugh, P. Chand, J.-L. Weng, C. Edwards, A. S. Walia, *Clin. Chem.* **1991**, *37*, 1595.
[4] L. A. Berrueta, B. Gallo, F. Vicente, *J. Chromatogr.* **1993**, *616*, 344.
[5] D. Gambart, S. Cárdenas, M. Gallego, M. Valcárcel, *Anal. Chim. Acta* **1998**, *366*, 93.
[6] A. C. Mehta, *Talanta* **1984**, *31*, 1.
[7] A. Bugey, C. Staub, *J. Pharmaceut. Biomed.* **2004**, *35*, 555.
[8] L. Mercolini, R. Mandrioli, M. Amore, M. A. Raggi, *J. Sep. Sci.* **2008**, *31*, 2619.
[9] A. G. Kazemifard, A. Dabirsiaghi, K. Gholami, N. Javadzadeh, *Acta Pol. Pharm.* **2007**, *64*, 287.
[10] F. E. Dussy, C. Hamberg, T. A. Briellmann, *Int. J. Legal Med.* **2006**, *120*, 323.
[11] A. Bugey, C. Staub, *J. Sep. Sci.* **2007**, *30*, 2967.
[12] B. E. Smink, M. P. M. Mathijssen, K. J. Lusthof, J. J. de Gier, A. C. G. Egberts, D. R. A. Uges, *J. Anal. Toxicol.* **2006**, *30*, 478.
[13] S. Hegstad, E. L. Øiestad, U. Johansen, A. S. Christophersen, *J. Anal. Toxicol.* **2006**, *30*, 31.
[14] T. Gunnar, K. Ariniemi, P. Lillsunde, *J. Chromatogr. B* **2005**, *818*, 175.
[15] M. Augsburger, L. Rivier, P. Mangin, *J. Pharmaceut. Biomed.* **1998**, *18*, 681.
[16] K. J. Reubsaet, H. R. Norli, P. Hemmersbach, K. E. Rasmussen, *J. Pharmaceut. Biomed.* **1998**, *18*, 667.
[17] M. E. Lozano-Chaves, J. M. Palacios-Santander, L. M. Cubillana-Aguilera, I. Naranjo-Rodríguez, J. L. Hidalgo-Hidalgo-de-Cisneros, *Sensor. Actuat. B* **2006**, *115*, 575.
[18] M. M. Correia dos Santos, V. Famila, M. L. Simões Gonçalves, *Anal. Biochem.* **2002**, *303*, 111.
[19] P. K. Salo, S. Vilmunen, H. Salomies, R. A. Ketola, R. Kostiainen, *Anal. Chem.* **2007**, *79*, 2101.
[20] A. M. Gil Tejedor, P. Fernández Hernando, J. S. Durand Alegría, *Anal. Chim. Acta* **2007**, *591*, 112.
[21] I. Rasanen, M. Neuvonen, I. Ojanperä, E. Vuori, *Forensic Sci. Int.* **2000**, *112*, 191.
[22] D. Felscher, G. Gastmeier, J. Dressler, *J. Forensic Sci.* **1998**, *43*, 619.
[23] T. Nishikawa, H. Ohtani, D. A. Herold, R. L. Fitzgerald, *Am. J. Clin. Pathol.* **1997**, *107*, 345.
[24] A. El Mahjoub, C. Staub, *J. Pharmaceut. Biomed.* **2000**, *23*, 447.
[25] E. K. Paleologos, D. L. Giokas, M. I. Karayannis, *Trends Anal. Chem.* **2005**, *24*, 426.
[26] Z. Sosa Ferrera, C. Padro'n Sanz, C. Mahugo Santana, J. J. Santana Rodri'guez, *Trends Anal. Chem.* **2004**, *23*, 469.
[27] H. Filik, Z. Yanaz, R. Apak, *Anal. Chim. Acta* **2008**, *620*, 27.
[28] F. Shemirani, N. Shokoufi, *Anal. Chim. Acta* **2006**, *577*, 238.
[29] A. B. Tabrizi, *J. Hazard. Mater.* **2007**, *139*, 260.
[30] A. Bavili Tabrizi, *Food Chem.* **2007**, *100*, 1698.
[31] H. Zhang, H.-K. Choi, *Anal. Bioanal. Chem.* **2008**, *392*, 947.
[32] X. Y. Qin, J. Meng, X. Y. Li, J. Zhou, X. L. Sun, A. D. Wen, *J. Chromatogr. B* **2008**, *872*, 38.
[33] A. B. Tabrizi, *Chem. Anal. (Warsaw)* **2007**, *52*, 823.
[34] A. B. Tabrizi, *B. Kor. Chem. Soc.* **2006**, *27*, 1780.
[35] A. B. Tabrizi, *B. Kor. Chem. Soc.* **2006**, *27*, 1604.
[36] J. Gasparič, J. Zímak, *J. Pharmaceut. Biomed.* **1983**, *1*, 259.
[37] G. Caille, J. Braun, D. Gravel, R. Plourde, *Can. J. Pharm. Sci.* **1973**, *8*, 42.
[38] M. A. Schwartz, E. Postma, *J. Pharm. Sci.* **1966**, *55*, 1358.
[39] J. Trochnetz, *Arch. Pharm.* **1981**, *314*, 204.
[40] M. I. Walash, F. Belal, M. E. Metwally, M. M. Hefnawy, *J. Pharmaceut. Biomed.* **1994**, *12*, 1417.
[41] A. A. Salem, B. N. Barsoum, E. L. Izake, *Spectrochim. Acta A* **2004**, *60*, 771.
[42] C. Pistos, J. T. Stewart, *J. Pharmaceut. Biomed.* **2003**, *33*, 1135.
[43] W. M. Mullett, J. Pawliszyn, *J. Pharmaceut. Biomed.* **2001**, *26*, 899.
[44] J. C. Miller, J. N. Miller, *Statistics for Analytical Chemistry*, John Wiley & Sons Ltd: New York, **1984**.