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A rapid and accurate UPLC/MS/MS method for the simultaneous determination of zolpidem and its main metabolites in biological fluids and its application in a forensic context

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ABSTRACT

Zolpidem (ZPD) is an imidazopyridine derivative used as a new type of hypnotic and is commonly used in drug-facilitated crimes. A rapid, sensitive, and specific ultra-performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS) assay method for the simultaneous determination of zolpidem and its main metabolites zolpidem 6-carboxylic acid (ZCA) and zolpidem phenyl-4-carboxylic acid (ZPCA) in biological fluids was developed and validated. Aliquots of 0.1 mL blood or urine specimens were used for the analysis, and zolpidem and its metabolites were extracted in a single step using acetonitrile (containing 0.1% formic acid) precipitation. The supernatant was then dried, and 100 μL methanol was added. The separation was performed on an Acquity™ UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm) analytical column by API 4000Q ultra-performance liquid chromatography–tandem mass spectrometry. Positive ionisation tandem MS detection in the multiple reaction monitoring (MRM) mode was used. The mobile phases consisted of either acetonitrile or water containing 20 mmol/L ammonium acetate and 0.1% formic acid, and the flow rate was 0.5 mL/min. The chromatographic separation time was 4 min, and calibration curves for human blood were generated over the range of 0.1–300 ng/mL for ZPD, 0.1–500 ng/mL for ZPCA and 0.1–200 ng/mL for ZCA. For urine, the linear range was 0.1–600 ng/mL for ZPD and ZPCA, and 0.1–300 ng/mL for ZCA. The limit of detection was 0.05 ng/mL and the limit of quantitation was 0.1 ng/mL for ZPD, ZCA and ZPCA. The linear correlation coefficients were greater than 0.9995. Both the inter-day and intra-day precisions were less than 15%, the recoveries were in the range of 70.0–98.3%, the matrix effects were approximately 79.5–99.0%, and the process efficiency was between 60.7% and 94.4%. This method allowed for the determination of zolpidem and its metabolites in human blood and urine and may be applied to forensic toxicological analyses.

1. Introduction

Drug-facilitated crime (DFC) is a significant problem in China; the majority of these cases are robberies. In addition, there have been increasing reports of drug-facilitated sexual assaults (DFSA). Aside from alcohol and cannabis, benzodiazepines and hypnotics are the most frequently observed compounds in DFC cases. Many benzodiazepines act non-selectively at two central receptor sites, omega-1 and omega-2, which causes many adverse effects. Thus, new hypno-sedatives such as zolpidem are now preferred over conventional benzodiazepines to treat short-term insomnia. Zolpidem acts rapidly, and victims are less able to accurately recall the circumstances under which the offence occurred due to its amnesic properties. In recent years there has been a rising trend in the number of DFC cases involving zolpidem, and this trend has caused alarm among the general public [1–4].

Zolpidem (N,N,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide hemitartarate) is an imidazopyridine compound that is used for the treatment of insomnia (Fig. 1). It is selective for the gamma-amino butyric acid (GABA) α1 receptor because of its high affinity for this subtype. The α1 subtype is believed to be associated with sedation, whereas other subtypes are responsible for the different effects of GABAergic ligands such as muscle relaxation, anxiolysis and memory impairment [5–7]. Zolpidem undergoes extensive hepatic metabolism, and up to 96% of the dose is eliminated in bile, urine and faeces as metabolites. Its major metabolites are zolpidem 6-carboxylic acid (ZCA) and zolpidem phenyl-4-carboxylic acid (ZPCA). However, none of the metabolites of zolpidem appear to have any pharmacological activity [8–10].

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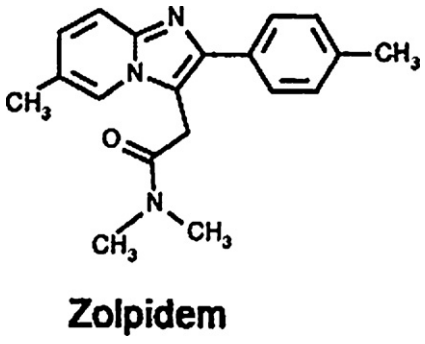

Fig. 1. Structure of zolpidem.

Blood and urine are the conventional specimens used for documenting drug exposure [11,12]. In most DFC cases, because of the amnesia caused by zolpidem, there is a 24 h or longer delay between a victim's report and the ingestion of the drug. In addition, zolpidem is quickly cleared from bodily fluids, whereas the metabolites of most drugs usually remain longer than the drug itself [3]. Therefore, under these circumstances, the simultaneous determination of zolpidem and its major metabolites has proven to be a solution to prolonging the window of detection.

Many methods and techniques have been developed for the analysis of zolpidem in biological fluids, including gas chromatography–mass spectrometry (GC–MS) [13], high-performance liquid chromatography (HPLC) [14,15], capillary electrophoresis (CE) [16] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [3,4,17,18]. However, no method especially LC–MS/MS method has been published for the simultaneous determination of zolpidem and its main metabolites to prolong the detection window of zolpidem in blood and urine. Recently, UPLC–MS/MS has been shown to be one of the most promising developments in the area of fast chromatographic separations [19]. Thus, the aim of the present study was to develop a rapid and accurate UPLC/MS/MS method for the simultaneous determination of zolpidem and its major metabolites in biological fluids and to apply it to forensic DFC cases involving zolpidem.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile were of HPLC grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water used for the UPLC–MS/MS analysis was prepared using a Milli Q water purification system (Millipore, MA, USA). Zolpidem (ZPD), zolpidem-d6, zolpidem 6-carboxylic acid (ZCA) and zolpidem phenyl-4-carboxylic acid (ZPCA) were purchased from Cerilliant (Round Rock, TX, USA). Ammonium acetate and formic acid were obtained from Fluka Chemical Co. (Buchs, Switzerland). Other reagents were all of analytical-reagent grade, and no further purifications were undertaken.

2.2. Sample collection

Blank blood was obtained from the Shanghai blood bank (China), and blank urine was donated by laboratory workers. Both the blank blood and urine were analysed for zolpidem and its metabolites, and negative results were obtained. Drug-positive blood samples from patients (one male and three females) were collected and stored in labelled K2 EDTA-vaccuettes at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0 and 24.0 h after administration of a single oral dose of 10 mg zolpidem tartarate tablets (Sanofi-Aventis Minsheng Pharmaceutical Co., Ltd. Hangzhou, China, Product lot: 9T034). Drug-positive urine samples were collected in plastic tubes at 1, 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 80 h after drug administration. Real case blood and urine specimens were obtained from a girl involved in a DFSA case 24 h after she was sexually assaulted. All samples were stored and frozen at −80 °C until analysis.

Table 1
Steps of gradient elution.

Step	Time (min)	Speed (mL/min)	*A* (%)	*B* (%)	Curve
1	0	0.5	80	20	
2	0.8	0.5	80	20	1
3	1.2	0.5	65	35	1
4	1.6	0.5	60	40	6
5	3.0	0.5	60	40	1
6	4.0	0.5	80	20	1

2.3. Sample preparation and optimisation

2.3.1. Blood samples

Zolpidem and its main metabolites (ZCA and ZPCA) were extracted from blood as follows: first, 0.1 mL blood was added to 10 ng ZPD-d6 as an internal standard, 0.1 mL mobile phase A (20 mmol/L ammonium acetate and 0.1% formic acid in water, pH 4.5) and 0.8 mL acetonitrile. Then, the above mixture was separated by centrifugation (10,000 × *g*, 3 min). The extract was dried under a stream of nitrogen, and the residue was reconstituted in 100 μL of methanol. Finally, 10 μL was injected into the UPLC–MS/MS system.

2.3.2. Urine samples

The urine samples were prepared in almost the same manner as the blood samples. First, 0.1 mL urine was added to 10 ng ZPD-d6 as an internal standard, followed by 0.1 mL mobile phase A (pH 4.5) and 0.8 mL acetonitrile. After centrifugation, the extract was dried under a stream of nitrogen, and the residue was reconstituted in 100 μL of methanol.

2.4. UPLC–MS/MS analysis

The UPLC–MS/MS analysis was performed on an Acquity™ Ultra Performance LC (Waters, USA) and an MDS Sciex API 4000 Qtrap MS/MS from Applied Biosystems (USA). An Acquity™ UPLC HSS T3 (100 mm × 2.1 mm,1.8 μm) analytical column was used and was guarded by a Van Guard™ HSS T3 column (Waters, USA). The column temperature was maintained at 20 °C, while the sample plate was maintained at 4 °C. The mobile phases consisted of either 20 mmol/L ammonium acetate and 0.1% formic acid in water (mobile phase A) or acetonitrile (mobile phase B). The gradient elution procedure is shown in Table 1. The total run time was 4 min.

The MS system was operated using electrospray ionisation (ESI) in positive mode. The optimum conditions were as follows: ion spray voltage (ISV), 5500 V; curtain gas, 172 kPa; collisionally activated dissociation (CAD), 48 kPa; heated nebuliser temperature (TEM), 550 °C; nebulising gas (GS1), 276 kPa; and heater gas (GS2), 345 kPa. Multiple reaction monitoring (MRM) mode was chosen for each analyte. The MRM transitions, retention times and conditions are shown in Table 2. The first parent/daughter ion pair (bold) was used for quantification. The data were processed using the Analyst 1.5.1 software.

2.5. Method validation

The analytical method was validated using spiked drug-free blood and urine as described in previous studies [20,21], and the following parameters were evaluated: selectivity, stability, linearity, the limit of detection (LOD), the limit of quantitation (LOQ),

Table 2
UPLC–MS/MS parameters of each compound.

Compounds	Parent ion (m/z)	Daughter ion (m/z)	DP (V)	CE (eV)	t_R (min)
Zolpidem	**308.1**	**235.2**	50	50	1.78
		263.2	50	35	
Zolpidem-d6	**314.4**	**235.2**	50	50	1.76
		263.2	50	50	
ZCA	**338.3**	**265.2**	80	52	0.86
		293.1	80	38	
ZPCA	**338.3**	**265.2**	80	52	1.49
		293.1	80	38	

matrix effect, recovery, intra- and inter-assay precision and accuracy. The selectivity of the method towards endogenous blood matrix components was assessed using twelve batches (K2 EDTA blood) of blank human blood. Stability experiments were carried out to determine the stability of the analyte in stock solutions and in biological samples under various conditions. The short-term stability at room temperature and the long-term stability of spiked solutions stored at −80 °C were assessed by comparing the area response of the stability-tested sample of analyte and IS with the area response of sample prepared from fresh stock solutions. According to Winek's, Musshoff's and the latest TIAFT (The International Association of Forensic Toxicologists) statistical data, the therapeutic levels of zolpidem in blood is 0.08–0.3 μg/mL and toxic levels of zolpidem in blood is 0.5 μg/mL [22,23]. So blood samples were prepared by adding 100 ng/mL ZPD-d6 and different concentrations of ZPD, ZCA and ZPCA (0.1 ng/mL, 0.5 ng/mL, 2 ng/mL, 10 ng/mL, 50 ng/mL, 200 ng/mL and 300 ng/mL for ZPD; 0.1 ng/mL, 0.5 ng/mL, 10 ng/mL, 50 ng/mL, 200 ng/mL and 500 ng/mL for ZPCA; and 0.1 ng/mL, 0.5 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL for ZCA) to investigate linearity. The ratio of area response for drug and IS was used for regression analysis. Each calibration curve was analysed individually by using least square weighed ($1/x^2$) linear regression. For the LOD and LOQ, drug-free blood samples spiked with each analyte at concentrations below 0.5 ng/mL were evaluated. The analyte concentration at which the signal-to-noise ratio was greater than 3 was chosen for the LOD and greater than 10 for the LOQ.

Method precision and accuracy were examined by analysing drug-free blood samples spiked with LOQ, low, medium or high concentrations of each analyte. The six replicates of each sample were analysed on four different days. The concentrations of the analytes were calculated using the calibration curves obtained on the day of analysis to account for possible daily variations in the curve. Accuracy was determined by comparing the mean calculated concentration of the spiked blood samples with the target concentration. Intra- and inter-day precision was assessed with one-way ANOVAs using Stata 7 software. The precision was expressed as the relative standard deviation (RSD%). For the method to be acceptable, the intra- and inter-day precisions were required to be below 15%, and the accuracy was required to be within ±15%.

The extraction recovery, matrix effects and process efficiency were assessed using the experimental design proposed by Matuszewski [24]. The following sets of samples were defined: set 1 consisted of blood sample spiked with QC and internal standard solutions prior to sample preparation; set 2 consisted of extracted blood sample with QC and internal standard solutions added to the solvent following protein precipitation extraction; set 3 samples consisted only of the prepared QC and internal standard solutions. Each sample set consisted of six replicates, and the experiment was repeated at each QC concentration level. The extraction recovery was calculated by dividing the set 1 mean analyte peak areas by the set 2 mean analyte peak areas. The matrix effects were determined by dividing the mean peak areas of set 2 by those of set 3. The process efficiency was calculated by dividing the mean analyte peak areas of set 1 by those of set 3.

The validation process of the method when using urine as the matrix component was the same as the validation process for blood.

3. Results and discussion

3.1. Optimisation of the method

3.1.1. Choosing the analytical column

To rapidly separate zolpidem and its main metabolites, it is important to choose an analytical column with high sensitivity, selectivity and pressure stability. In previously published studies, XTerra C18 columns [3,4], Inertsil ODS-3 columns [25], and Betabasic-8 columns [26] have been used to separate zolpidem. However, none of these columns have been used for the simultaneous determination of zolpidem and its major metabolites. It is difficult to separate these compounds in normal analytical columns because ZCA and ZPCA have the same parent/daughter ion pair, and their polarity is higher than that of zolpidem. Besides, ZCA and ZPCA have the similar properties. For LC–MS/MS analysis, five different chromatographic columns (The Acquity™ UPLC HSS T3 column, the Allure PFP Propyl column, the Capcell Pak C18 column, the Atlantis™ T3 column and the XBridge C18 column) were evaluated for their achievement of symmetric and nontailing peaks. It was tested at a medium concentration by comparing peak areas of the analyte. Furthermore, different mobile phases [methanol, acetonitrile, 0.1% formic acid, and ammonium acetate (10 or 20 mM)] adjusted to different pH values were tested in different combinations and ratios to achieve a good separation of the analytes within the shortest time of analysis. The Acquity™ UPLC HSS T3 column gave the best results for most of the analytes. The Acquity™ UPLC HSS T3 column is made from a new type of linkage technology that is especially suitable for polar compounds, and it has a high tolerance for pressure (18,000 psi), which allows it to be used for UPLC–MS/MS at a high flow rate. In the present study, four different analytical columns were used to investigate the separation results. It was found that the Allure PFP Propyl column (Restek, USA) and the Capcell Pak C18 column (Shiseido, Japan) were not suitable for UPLC analysis, and the Atlantis™ T3 column and the XBridge C18 column (Waters, USA) were unable to adequately separate ZCA and ZPCA. Thus, the Acquity™ UPLC HSS T3 column was chosen because it is able to satisfactorily separate zolpidem, ZCA and ZPCA within a short time period.

3.1.2. Optimisation of sample preparation

Sample preparation is the most important part of an analytical method. Liquid–liquid extraction, solid-phase extraction (SPE) and protein precipitation are the most common methods used for sample preparation. However, liquid–liquid extraction requires a large volume of organic solvent that is harmful to human health, and it usually requires a large number of specimens (specimens should be abstemiously used when dealing with forensic cases). Furthermore, emulsification happens so frequently during the extraction procedure that the repeatability is not satisfactory. SPE is usually complicated and requires much time and money, although the matrix effect and recovery of SPE are sufficient. Unlike the above two methods, protein precipitation is easy and rapid to perform, and it uses less sample and organic solvent. The recovery and repeatability is also good enough for the simultaneous extraction of zolpidem and its main metabolites in blood and urine. Sample preparation was performed as follows: 0.1 mL drug-positive blood (or urine) was added to ZPD-d6 (10 ng, used as an internal standard), followed by 0.1 mL mobile phase A (pH 4.5) and 0.1 mL

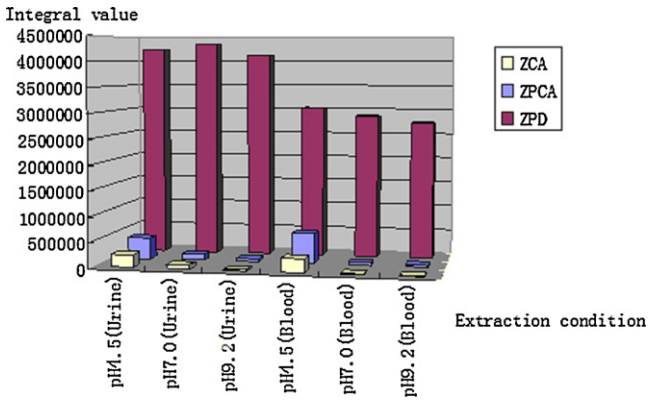

Fig. 2. The results of different extraction procedures.

deionised water (pH 7.0) or 0.1 mL borate buffer (pH 9.2); 0.8 mL acetonitrile was then added to deproteinate and to remove impurities. Six samples were investigated at the same time at each pH condition. Afterwards, each mixture was separated by centrifugation (10,000 × *g*, 3 min), and the extract was then dried under a stream of nitrogen. The residue was then reconstituted in 100 μL of methanol. Finally, 10 μL was injected into the UPLC–MS/MS system. Fig. 2 shows the results of the extractions at each condition. Protein precipitation at pH 4.5 was chosen after consideration of the results. Employing a single-step deproteinating extraction procedure minimises the chances of errors, saves considerable time and simplifies the sample preparation procedure. Because only a small volume of blood is required (0.1 mL), the volume of sample to be collected from the subjects at each time point is reduced significantly, allowing for the inclusion of additional data points.

3.2. Validation of the method

The results of the method validation are summarised as follows: no interferences were detected at the retention times of the analytes and the internal standard in ten different blank blood and urine samples. The short-term and long-term biological samples stored at different temperatures were considered stable (the deviation from the nominal value was within ±10%). Freeze–thaw stability was performed at low and high concentrations using six replicates at each concentration. The samples were considered to be stable (the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15%). The zolpidem calibration curves were linear between 0.1 and 300 ng/mL in human blood, while the ZPCA and ZCA calibration curves were linear between 0.1 and 500 ng/mL and between 0.1 and 200 ng/mL, respectively. The linear range for urine was 0.1–600 ng/mL for zolpidem and ZPCA and 0.1–300 ng/mL for ZCA. The regression coefficient for all calibration curves was higher than 0.9995 ($r \geq 0.9995$). The lower limit of detection (LOD) and the lower limit of quantitation (LOQ) for all analytes were 0.05 ng/mL and 0.1 ng/mL, respectively (Fig. 3). The intra- and inter-assay precision, accuracy, matrix effect and recovery in blood samples were satisfactory (Table 3). Furthermore, the accuracy and precision for the data from urine samples were within the required limits. Inter- and intra-day precisions were less than 15%. Recoveries obtained from spiked samples ranged from 70.0% to 98.3%, the matrix effect was between 79.5% and 99.0%, and the process efficiency was between 60.7% and 94.4%.

In simple terms, the method proposed here is an improvement over other methods reported previously [3,4,13–16], and it is the first to allow for the simultaneous determination of zolpidem and its major metabolites using UPLC–MS/MS. Furthermore, greater sensitivity is achieved (0.10 ng/mL) even with low blood and urine

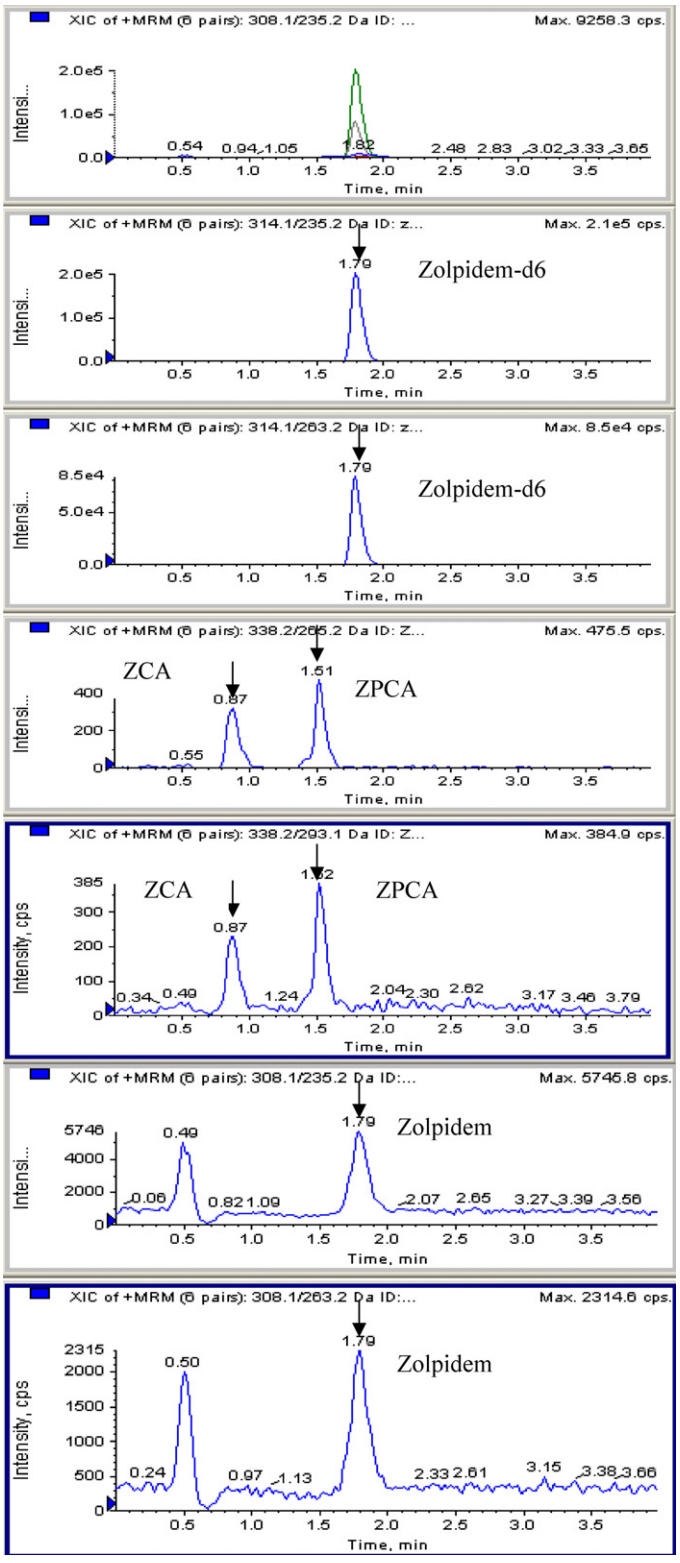

Fig. 3. Extracted ion chromatograms of a blank blood sample spike at LOQ concentration.

sample volumes, making this method well suited for forensic toxicology analyses. Moreover, the LOQ of zolpidem is the same as Villain's result (the LOQ of zolpidem in urine sample is 0.1 ng/mL), and better than Bassan's (the LOQ of zolpidem in plasma sample is 40 ng/mL) and Bjørk's results (the LOQ of zolpidem in blood sample is 10 ng/mL) [17,18]. In addition, the rapid sample analysis

Table 3
Accuracy, intra- and inter-assay precision, matrix effect, recovery and process efficiency of the method.

Compound	Spiked concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)	Matrix effect (%)	Recovery (%)	Process efficiency (%)
ZPD	0.1	89.2	7.09	8.14	94.7	90.2	85.4
	0.5	92.3	4.78	5.25	93.4	88.7	82.8
	10	91.5	3.52	5.68	98.7	94.8	93.6
	200	92.9	5.77	5.89	95.6	95.6	91.4
ZPCA	0.1	90.2	6.34	9.18	88.5	83.2	73.6
	0.5	86.1	5.17	10.12	87.3	85.4	74.5
	10	95.1	6.20	7.42	92.5	84.5	78.2
	400	94.5	7.95	6.44	95.4	85.1	81.1
ZCA	0.1	88.6	3.11	9.47	86.7	79.6	69.0
	0.5	88.7	8.91	6.24	96.5	86.3	83.3
	10	90.4	8.48	10.23	90.1	80.3	72.4
	150	93.5	6.13	5.46	93.6	83.5	78.2

ZPD: zolpidem; ZPCA: zolpidem phenyl-4-carboxylic acid; ZCA: zolpidem 6-carboxylic acid.

turnaround time of 4.00 min makes this an attractive method for high-throughput bioanalysis of zolpidem and its major metabolites (ZPCA and ZCA) in human blood and urine samples. The chromatographic conditions were optimised, and the results of the method validation in terms of specificity, linearity, precision, accuracy, matrix effect and recovery have been provided and were found to be satisfactory. The proposed method is thus suitable for both forensic toxicology and clinical analyses.

3.3. Windows of detection in blood and urine

The validated method was successfully applied for the assay of zolpidem and its major metabolites in four volunteers. The average blood (or urine) concentration vs. time profile of the four subjects after oral administration of zolpidem tartarate tablets (10 mg) is shown in Fig. 4. The method was sensitive enough to monitor the blood and urine concentration of zolpidem for up to 72 h. Fig. 4 shows that the maximum blood concentration of zolpidem occurred after approximately 1 h. Zolpidem was undetectable in the blood and urine after 16 h and 40 h, respectively (the drug concentration in urine was close to zero 40 h after drug administration). Zolpidem phenyl-4-carboxylic acid (ZPCA) was found to be the major metabolite of zolpidem in vivo [8,9], and its detection window was much longer than that of zolpidem. The results showed that ZPCA could be detected in blood and urine for 24 h and 72 h, respectively, with peak concentrations occurring at 1.5 h in blood and 8 h in urine after administration of a single dose.

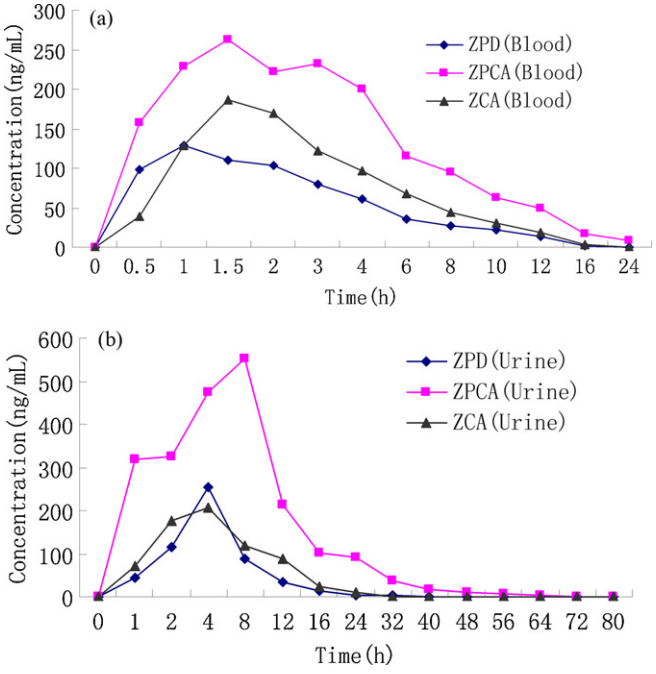

Fig. 4. Mean concentration–time curve of four subjects (A: blood sample; B: urine sample).

Villain et al. [3] have also studied the windows of detection of zolpidem in urine; they showed that zolpidem was detectable for up to 60 h, with the peak concentration occurring at 12 h, in contrast to the results obtained from our study. We were unable to detect zolpidem in the urine after 40 h, and the peak concentration occurred at 4 h. Comparison of these differing results leads to the conclusion that the Chinese population may have a different metabolic profile of zolpidem than the Caucasian population. Thus, racial factors should be taken into account when considering the metabolism of zolpidem. Our study also investigated the blood and urine concentration of ZPCA; the window of detection of ZPCA in blood and urine was much longer than that of zolpidem. Determination of ZPCA when zolpidem is undetectable could assist in the investigation of robbery and sexual assault cases involving zolpidem for nearly 1–2 days after zolpidem ingestion.

3.4. Pharmacokinetic analysis

The pharmacokinetic analysis was performed using the DAS 1.0 software. The main pharmacokinetic parameters are shown in Table 4. Previous studies have examined the pharmacokinetics of the Caucasian population after the oral administration of 10 mg zolpidem. Weinling et al. reported that the C_{max} of zolpidem in blood was 167 ± 34 ng/mL and the T_{max} was 0.8–2.0 h. Similarly, Drover et al. showed that the C_{max} of zolpidem in blood was 167 ± 81 ng/mL and the T_{max} 0.9–2.1 h [27,28]. These data suggest that the pharmacokinetics are different in the Caucasian and Chinese Han populations; our studies found a lower C_{max} and a shorter T_{max} compared with the reported pharmacokinetics in the

Table 4
Pharmacokinetic parameters of zolpidem after a single oral dose of 10 mg drug.

Parameters	ZPD	ZPCA
T_{max} (h)	0.85 ± 0.22	1.68 ± 0.23
$T_{1/2}$ (h)	3.23 ± 0.39	5.63 ± 0.21
C_{max} (μg L^{-1})	135.6 ± 46.1	262.8 ± 61.9
AUC (μg L^{-1} h)	558.5 ± 127.6	945.2 ± 118.0
CL (L/h)	16.7 ± 3.8	–
MR	2.9 ± 0.7	–

CL: oral clearance; MR: metabolic ratio.

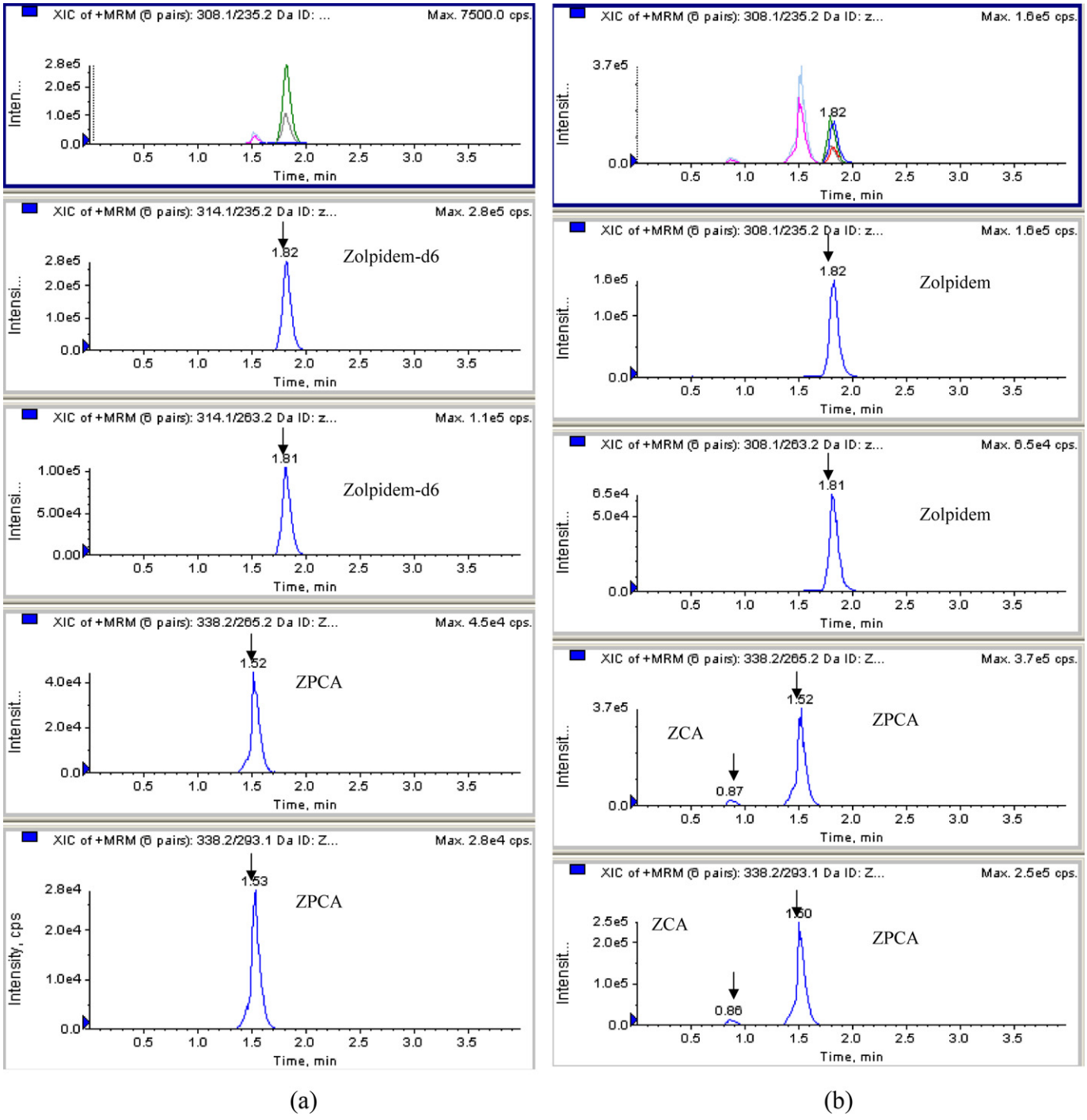

Fig. 5. Extracted ion chromatograms of a real case (a: blood sample; b: urine sample).

Caucasian population. When considering the metabolism of zolpidem, racial factors should be considered.

3.5. DFSA case

A girl was invited to a party at a bar. After drinking a soft drink offered by a young man, she fell unconscious and woke up 16 h later. She realised that she had been sexually assaulted and went to the police to report the case. At the medico-legal unit of the hospital, blood and urine samples were collected approximately 20 h after the victim had been assaulted. Forensic toxicology tests were carried out, and positive results were found using the developed method. In the blood samples, ZPCA was detected (10.5 ng/mL), but ZPD and ZCA were not. ZPD, ZPCA and ZCA were all found in urine at concentrations of 8.9, 94.2, and 13.6 ng/mL, respectively (Fig. 5). These results revealed the presence of zolpidem, confirming the girl's statement.

4. Conclusion

In summary, we have developed and validated a rapid and accurate UPLC/MS/MS method for the simultaneous determination of zolpidem and its major metabolites in biological fluids. This method allows for the determination of zolpidem in human blood and urine,

as well as its metabolites (which allows for a prolonged window of detection). The devised method is suitable for both clinical analyses and forensic toxicology, especially DFC cases.

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