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Development and validation of a practical analytical method for Zolpidem as a drug facilitated crime tool

Nurdan Kenan [1], Selda Mercan [1*] and Munevver Acikkol [1]

Istanbul University- Cerrahpasa, Institute of Forensic Sciences and Legal Medicine Buyukcekmece, 34500, Istanbul, Türkiye

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Abstract: Zolpidem, as a member of Z-drugs, is a non-benzodiazepine, imidazopyridine derivative chemical substance used in the treatment of insomnia. This substance is frequently reported as a tool of drug facilitated crimes such as date rape, robbery, extortion etc besides drugged driving. A Gas Chromatography-Mass Spectrometry (GC-MS) method by a one step extraction method with ethyl acetate from urine using HP-5MS capillary column was developed for the determination of zolpidem. Clozapine was used as internal standard. Linearity range was between 10-200 µg mL -1. Limit of detection was 0.28 µg mL -1 , limit of quantification was 0.35 µg mL -1, the mean range of recoveries was between 93.40-94.41% for three spiked concentrations (15, 20 and 50 µg mL -1). The difference of concentrations for same samples analyzed at 7-day intervals was found 0.02 CV% for stability study. The reported method was found cheap, sensitive, rapid, and suitable for the analysis of the spiked beverages and foods as well as urine as evidences of sexual assault, robbery or intentional/ non-intentional intoxication phenomena.

Keywords: Zolpidem; drug facilitated crimes; gas chromatography–mass spectrometry; forensic toxicology; Z-Drugs.

1. Introduction

The use of chemicals to change a person's behavior for criminal purposes is not a new phenomenon. Spiking of any psychoactive substances such as benzodiazepines, ketamine, barbiturates and Z-drugs to beverages and foods is a commonly encountered way among drug-facilitated crime cases [1-5]. These substances are often used to defuse the victim during crimes such as sexual assault and robbery [6,7] In addition, such substances are included in the field of forensic toxicology, causing intentional or unintentional poisoning and impaired driving under the influence of drugs as well [8-12].

Zolpidem, zopiclone and zaleplone, called Z-drugs, are commonly used as an alternative to benzodiazepines in the treatment of insomnia. Zolpidem is the most preferred Z-drugs by means of its minimal day after side effects [13]. In addition to the treatment of insomnia, it has anticonvulsant, anxiolytic and minor muscle relaxant properties [14]. Zolpidem is rapidly absorbed from the gastrointestinal tract after ingestion. Its effect is shown as gamma aminobutyric acid (GABA)-A agonist

* Corresponding author E-Mail: mercans@istanbul.edu.tr

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receptor via central nervous system (CNS) [15]. After ingestion, zolpidem is converted to two pharmacologically inactive metabolites by CYP3A4 and CYP2C9 enzymes in the liver [16]. Zolpidem, one of the most prescribed drugs in the treatment of insomnia [17] in Europe under the name Ambien [18], which has been approved by the American Food and Drug Administration (FDA) in 1993 and Stilnox since 1986 in Europe [19]. Zolpidem is also a controlled substance according to the FDA drug list and is placed in the 4th drug class used in the treatment of short-term insomnia [20].

Zolpidem does not make any physical or taste changes when it is spiked to beverages or foods. In this aspect, it is hard to notice the taste or smell of the drug by the victim in a drug facilitated crimes [13]. In addition, metabolizing of zolpidem up to 70% within several hours after ingestion is one of the features that makes its determination difficult [21]. Various analysis methods such as HPLC, LC-MS/MS, GC-MS, and electrophoresis are used for the determination of zolpidem from biological materials as well as beverages [20, 22-24]. Moreover, zolpidem is a substance that needs to be taken into consideration in antemortem and postmortem forensic toxicology because it has severe side effects causing coma and death in intentional or unintentional poisonings cases, especially when used with other drugs or alcohol together. To the best of our knowledge, there isn't any reported method developed by GC-MS as a universally used analytical technique in forensic toxicology laboratories neither for biological samples nor beverages in our country.

In this study, it was aimed to develop an easy, cheap, practical and reproducible method for the determination of zolpidem in urine. Linearity, precision, detection limit, extraction efficiency and other performance parameters were also considered in terms of the use of the validated method for the investigation of zolpidem in drug facilitated crimes when spiked to foods and beverages.

2. Experimental

2.1. Chemicals and Instrumentation

Methanol, ethyl acetate and sodium hydroxide (pellets pure) were obtained from Merck, (Darmstadt, Germany). Zolpidem and clozapine (internal standard) standards were obtained from Chiron AS (Trondheim, Norway). Urine samples were collected from a volunteer who does not use any medication. This study was approved by the ethical committee of Istanbul University, Cerrahpasa Medical Faculty with the decision dated 07th November 2017.

Gas chromatography–mass spectrometry (GC-MS) analyzes were performed by Agilent (Palo Alto, CA USA) 7820A series gas chromatography and Agilent (Palo Alto, CA USA) 5977E series mass spectrometer. The GC column was the HP-5MS capillary column (30 mx 250 µm x 0.25 µm film thickness). Inlet temperature was set to 250 °C, aux temperature was set to 280 °C and flow rate was 1 mL min^{-1}. Helium gas was used as carrier gas. Oven temperature; 30 ^{o}C min^{-1} to 200 to 280 ^{o}C, 3 min at the initial temperature and 8 min at the end temperature was set to have a waiting time to prevent carry over and for conditioning the GC column. Total run time was set to 14 min. The mass detector operated in the scan mode in the 50-600 m/z ion range. Scanning (SCAN) and selected ion monitoring (SIM) mode, (for zolpidem 219, 235, 307; for clozapine 243, 256, 326) were used simultaneously.

2.2. *Method Validation and Sample Preparation*

Stock solutions were prepared with methanol at a concentration of 1000 µg mL^{-1} for the zolpidem standard and 1000 µg mL^{-1} for clozapine as internal standard (IS). Zolpidem stock solution was diluted with methanol to 10, 15, 20, 25, 50, 75, 100 and 200 µg mL^{-1} concentration including 25 µg mL^{-1} IS in each calibration solutions. Both stock and calibration solutions were kept at 4°C. Each calibration points were analyzed in six replicates in incremental amounts for linearity and linear range studies. Also blank solution including only IS was prepared and analyzed for six times to check out the carryover. The repeatability of each calibration point beside blank solution was observed by calculating standard deviation of six times replicated samples.

Calibration curve was plotted by the internal standard concentration ratio of the analyte concentrations. The limit of detection (LOD) was determined according to the standard deviation of ten

times replicated of the lowest calibration level spiked into urine by multiplying with 3.3 and with 10 for the limit of quantification (LOQ).

For recovery and reproducibility studies; 6 replicated results of the spiked urine sample at 15, 20 and 50 µg mL^{-1} concentrations were evaluated. Mean concentrations, standard deviation (SD) and relative standard deviation (RSD%) values were obtained from all three concentrations. Evaluation of extraction efficiency was made according to the RSD% value, which is accepted below 20%. For reproducibility, obtained results were compared to known concentrations and bias% values over 15 were rejected and repeated. In the stability study, the spiked urine samples (n=6) at 50 µg mL^{-1} concentration was analyzed for six times at 7-day intervals and the difference between injections was evaluated. To check out the matrix effect and interferences from urine, extracted blank urine samples (including IS) were used and analyzed six times.

Spiked urine samples including IS were extracted by liquid-liquid extraction (LLE) method using ethyl acetate from urine. Each levels of zolpidem standard solutions were spiked between 10-200 µg mL^{-1} with clozapine (internal standard) standard solution (25 µg mL^{-1}), to 1 mL urine sample. 120 µL 0.1 M NaOH was added followed by centrifugation by addition of 2 mL of ethyl acetate. The supernatant was removed and dried under nitrogen and the residue was reconstituted with 200 µL of ethyl acetate into a vial insert and each sample analyzed for three times by GC-MS system.

3. Results and discussion

In this study a GC-MS method has been developed for determination of zolpidem in urine for the evaluation of antemortem and postmortem intoxication cases. Urine samples were analyzed by the developed method and no interference was observed. Both total ion chromatograms and selected ion chromatograms of blank urine sample and the spiked urine sample were shown in Figure 1 and Figure 2, respectively. Peak shapes and resolutions of analytes were found good enough and there was no interference peak close to related retention times in urine for zolpidem and clozapine as well. It was observed that the sensitivity of the method was better in SIM mode of the method as it can be seen in figures. Quantitation was obtained by SIM mode of the study. Retention times (min±SD) and monitored ions (m/z) of zolpidem and clozapine standards were found 9.68±0.004 min. (219, 235, 307), 10.42±0.004 min. (243, 256, 326) respectively. Total sample preparation time was recorded as 45 min starting from extraction to analysis. Only in 60 min. a sample was treated and analyzed by GC-MS with the aid of one step extraction method. It was revealed that, this duration is very short for a quantitation method conducted by an advanced analytical technique. Thus, any rapid test does not need before quantitation such as immunoassay etc.

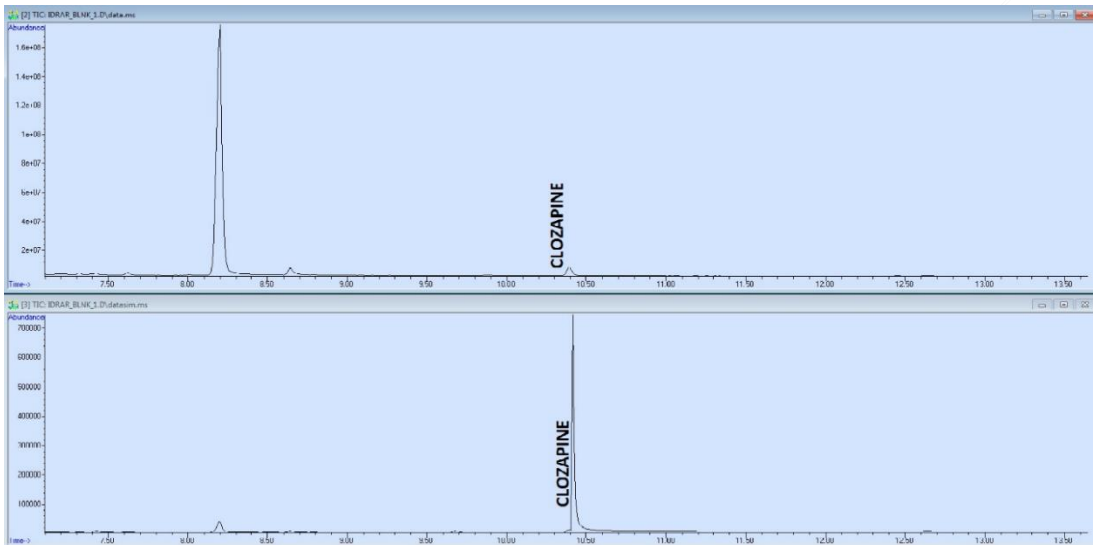

Figure 1. Chromatogram of the blank urine sample (SCAN mode on top, SIM mode on bottom)

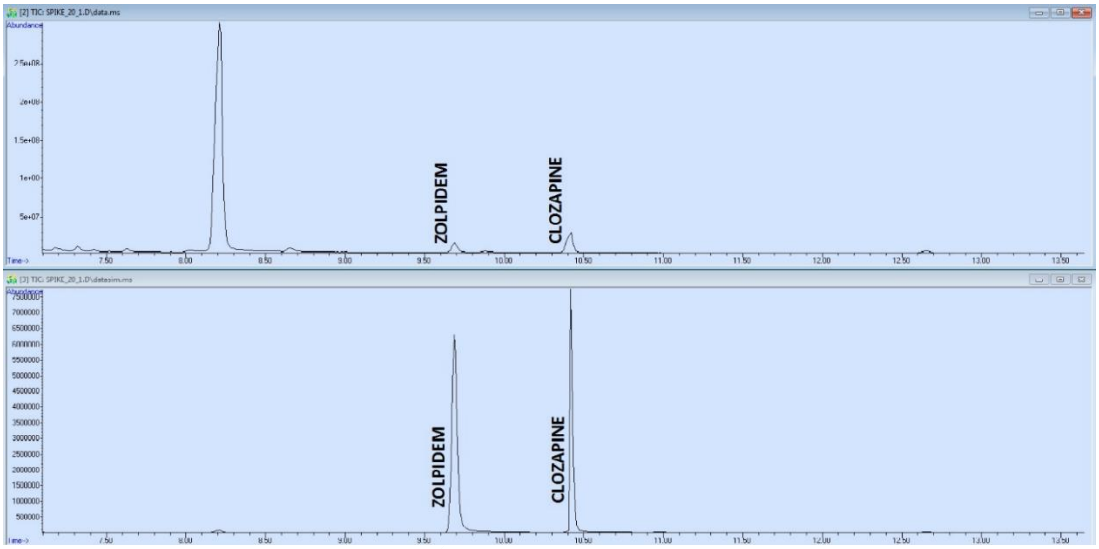

Figure 2. Chromatogram of the spiked urine sample (20 µg mL $^{-1}$) (SCAN mode on top, SIM mode on bottom)

The developed method demonstrated the easy detection of zolpidem. The findings of recovery, reproducibility, relative standard deviation, calibration coefficient, stability, LOD and LOQ values were given in Table 1. LOD was found 0.28 µg mL^{-1} while LOQ was found 0.35 µg mL $^{-1}$. The method was found linear between 10-200 µg mL^{-1} with 0.997 correlation coefficient values, which is satisfactorily successful. The powerful correlation coefficient and the wide linear range indicated that the presented method is reliable and applicable for wide ranged studies. The recovery and reproducibility rates were found to be over 80% for all three concentrations with a good compliance. As most of drug substances used in drug facilitated crimes except Gamma Hydroxybutyric Acid (GHB), zolpidem is extracted in base media as well by adding NaOH as the first extraction step [25, 26]. In consequence of this basic approach, high extraction efficiency values ranged from 93.40 to 94.41% for low, medium and high concentrations for zolpidem were found satisfactory. Using SIM mode for quantitation was also enhanced the performance of the method. In the study performed with gas chromatography with nitrogen phosphorus detector (GC-NPD by Stanke et al., the recovery results (89.8% ± 7.97) obtained were similar to the results of presented method [27]. In another study conducted by Shi et al. with LC-MS/MS, similar recovery rates (in the range of 88.7-95.6%) and stability results were found with the presented method [24]. Also reproducibility results were found quite close to exact concentrations for all three concentrations, which are 14.03 for 15, 18.68 for 20 and 47.21 for 50 µg mL $^{-1}$. A comparison table including studies conducted with different types of extraction reagents and analytical techniques was also present in Table 1. The parameters of the presented method, such as recovery and linearity, were similar to other studies using different matrices and methods. Lichtenwalner et al. conducted a study with GC/MS in a postmortem case; and found concentrations of zolpidem in blood as 7.9 µg mL^{-1} and in urine as 4.1 µg mL^{-1} which are also detectable amounts with the presented method [19].

In a study investigated the solubility of crushed and whole zolpidem tablets in pure water, cider and mineral water, Heide et al. used liquid chromatography-tandem mass spectrometry (LC-MS/MS) system and found the results within the LOD value with the linear range of the presented method. It is understood that the analysis method presented, according to these results, can be easily detect zolpidem in different matrices such as food and beverages [13].

In a study investigating of a fatal overdose of Ambien (zolpidem tablet), Winek et al. [18] revealed 4.1 µg mL^{-1} of zolpidem blood concentration by using GC/NPD. Meeker et al. [20] also used a dual-column GC with NPD and MS spectrometry to detect postmortem tissue concentrations of zolpidem.

Zolpidem was presented at concentrations of 2.91, 1.40, and 2.13 µg mL^{-1} in the heart blood, peripheral blood, and urine, respectively. In a postmortem case of ingestion of an unknown quantity of zolpidem containing tablets. Augsburger et al. reported zolpidem free-base concentrations in urine and gastric content which were reported with 6.9 and 14.5 µg mL^{-1} concentrations respectively [28]. In another study, Logan et al. investigated blood concentrations of zolpidem with 29 cases arrested for impaired driving. In 5 cases, zolpidem was detected in blood with a mean 0.65 µg mL^{-1} concentration [29]. Considering the LOQ level of the presented method, all above-mentioned results can be easily detected also by this validated method in several matrices such as blood, urine, foods and beverages.

Table 1. Validation results of zolpidem spiked into urine sample comparison of performance parameters with similar studies.

Reference	Method	r^2	Linear range	LOD	Recovery conc.	Recovery (%)	RSD (%)	Stability (%)
[27]	LLE, GC-NPD (plasma)	0.992	0.001-2.0 µg/mL	1 ng/mL	100 ng/mL	89.82	8.9	-
[24]	LLE, LC/MS/MS (urine)	0.999	0.16 ng/mL	0.05 ng/mL	0.1 ng/mL 0.5 ng/mL 10 ng/mL 200 ng/mL	90.20 88.70 94.80 95.60	15	0.15
Presented method	LLE, GC/MS (urine)	0.997	10-200 µg/mL	0.28 µg/mL	15 µg/mL 20 µg/mL 50 µg/mL	93.50 93.40 94.41	10.41 8.91 10.42	1.7

4. Conclusions

In this study an inexpensive, fast and reliable GC/MS method was validated to determine zolpidem in urine while it is also suitable for detecting this substance in other matrices such as foods and beverages. Although the detection limit of the developed method in the presented study is not as sensitive as the studies carried out with other novel analytical systems coupled with different detectors such as MS/MS, it is thought that the method can easily determine the zolpidem in drug facilitated crimes and intoxication cases both in antemortem and postmortem toxicology by a GC/MS system which is still a gold standard for a forensic laboratory. In the developed method, it is also possible to decrease the determination limits of zolpidem concentration by reducing the split ratio and increasing the extraction and injection volume.

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ORCID
Nurdan Kenan: 0000-0001-5393-2969
Selda Mercan: 0000-0002-0431-6972
Munevver Acikkol: 0000-0003-0642-6574

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