Saudi Pharmaceutical Journal 32 (2024) 101950

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

# Developing, validating, and comparing an analytical method to simultaneously detect z-drugs in urine samples using the QuEChERS approach with both liquid chromatography and gas chromatography-tandem mass spectrometry

Varsha Chauhan [a], Manu Sharma [a,*], Abhishek Tiwari [b], Varsha Tiwari [b], Manish Kumar [c], Ajay Sharma [d], Arya Lakshmi Marisetti [d], Ashok Kumar [e], Abdulsalam Alhalmi [f], Omar M. Noman [g], Ahmad Alahdab [h,*]

[a] National Forensic Science University, Delhi Campus, Ministry of Home Affairs, Sec-3, Rohini, Delhi 110085, India
[b] Pharmacy Academy, IFTM University, Lodhipur-Rajput, Moradabad, (U.P.) 244102, India
[c] Department of Pharmaceutics, ISF College of Pharmacy, Moga, Punjab, India
[d] School of Pharmaceutical Sciences, Delhi Pharmaceutical Sciences and Research University, Pushp Vihar, New Delhi 110017, India
[e] School of Pharmacy, Graphic Era Hill University, Dehradun 248002, India
[f] School Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India
[g] Department of Pharmacognosy, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia
[h] Institute of Pharmacy, Clinical Pharmacy, University of Greifswald, Friedrich-Ludwig-Jahn-Str. 17, 17489 Greifswald, Germany

ARTICLE INFO

*Keywords:*
Zolpidem
Zopiclone
Eszopiclone
LC–MS/MS
GC–MS/MS
QuEChERS

ABSTRACT

Detecting z-drugs, a sedative-hypnotic medication, is also misused for criminal activities. Therefore, the analysis of urine samples is crucial for clinical and forensic purposes. We conducted a study where we developed, validated, and compared an analytical method for simultaneously detecting z-drugs in urine samples. Our approach uses the QuEChERS method for sample preparation, combined with liquid chromatography (LC) and gas chromatography (GC) coupled with tandem mass spectrometry (MS/MS). We optimized the QuEChERS method to effectively extract z-drugs from urine samples while minimizing matrix effects and achieving high recovery rates. After extraction, we split the samples into two parts for analysis using LC-MS/MS and GC–MS/MS. We validated our methods, and the results showed good linearity over a broad concentration range (1–200 ng/mL) for each z-drug. The limits of detection and quantification were within clinically relevant ranges, ensuring sensitivity for detecting z-drugs in urine samples. We compared the two chromatographic techniques by analyzing a set of urine samples spiked with known concentrations of z-drugs using both LC-MS/MS and GC–MS/MS methods and then applied to the real samples. The results were statistically analyzed to assess any significant differences in accuracy and precision above 95 %, and both methods offered reliable and consistent results with the samples as well. In conclusion, our analytical method coupled with both LC-MS/MS and GC–MS/MS using the QuEChERS approach provides a comprehensive and robust solution for the simultaneous detection of z-drugs in urine samples. The choice between the two chromatographic techniques can be based on the specific z-drugs of interest and the required analytical performance. This method holds promise for applications in clinical toxicology, forensic analysis, and monitoring z-drug usage.

*Abbreviations:* CE, Capillary Electrophoresis; GC–MS/MS, Gas Chromatography-Tandem Mass Spectrometry; HPLC, High-Performance Liquid Chromatography; LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; QuEChERS, Quick Easy Cheap Effective Rugged Safe; DFSA, Drug-Facilitated Sexual Assault; DDD, Defined Daily Dose; LOQ, Limits of Quantification; LOD, Limit of Detection; MRM, Multiple-Reaction Monitoring; ESI, Electrospray Ionization; Z1, Zolpidem; Z2, Zopiclone; Z3, eszopiclone.

* Corresponding authors.
*E-mail addresses:* lantadene@hotmail.com (M. Sharma), onoman@ksu.edu.sa (O.M. Noman), ahmad.alahdab@uni-greifswald.de (A. Alahdab).

https://doi.org/10.1016/j.jsps.2023.101950
Received 24 September 2023; Accepted 31 December 2023
Available online 2 January 2024

## 1. Introduction

Zolpidem (Z1) and Zopiclone (Z2) are the most common and widely prescribed medications for insomnia, both short-acting sedative-hypnotics. These medications are becoming increasingly popular among people who suffer from short-term sleep disorders (Jones and Holmgren, 2012). With 30.3 defined daily doses per 1000 inhabitants per day, Z2 is the most commonly used z-hypnotic, while Z1 had much lower sales in 2012 with 5.1 defined daily doses. Z1 and Z2 have a short elimination half-life of 4–8 and 2–5 h, respectively. Due to the short half-life, these drugs have low blood concentration after 8 h of sleep (Brandt and Leong, 2017). Z1, Z2, and Z3′s wire frame structure are presented in Fig. 1.

## 2. Materials and methods

### 2.1. Chemicals and reagents

HPLC-Grade methanol, acetonitrile, analytical-grade ammonium formate, ethyl acetate and formic acid were procured from Sigma Aldrich, and QuEChERS EN salt was procured from Agilent. The reference standards of Z1 and Z2 were procured from the Indian Pharmacopoeia Commission and Z3 from Sigma Aldrich, India. The formulation of Z1, Z2, and Z3 was procured from Abbott's, Intas Pharmaceuticals, and Sun Pharma, India.

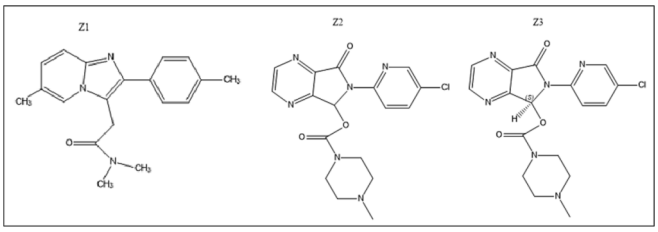


**Fig. 1.** Shows the structure of Z-drugs: Z1: zolpidem, Z2: Zopiclone, and Z3: Eszopiclone Over the past few years, there has been a growing concern about the harmful use of prescription medications, particularly hypnotic drugs that are commonly referred to as Z-drugs. These drugs have the potential to cause addiction, negative health effects, and societal problems. Z-drugs are non-benzodiazepine sedative-hypnotics that are often prescribed to manage short-term insomnia. However, their misuse is a major challenge to public health and safety. To ensure patient compliance, detect cases of misuse, and contribute to drug surveillance efforts, it is crucial to have accurate and efficient methods of detecting these substances in biological samples (Eliassen and Kristoffersen, 2014). One and three per cent of suspected impaired drivers were on Z1 and Z2, respectively. Nonetheless, the pervasiveness of these medications found by secured drivers has been moderately steady throughout recent years. Opioids and benzodiazepines are the most commonly diverted drugs in the UK, according to a 2022 report on "Diversion and Illicit Supply of Medicines" by the UK Advisory Council on Drug Misuse (Varsha et al., 2021). The most frequently illegally diverted medications from the regulated supply chain are Z-drugs and benzodiazepines (Hockenhull et al., 2021). Benzodiazepine and Z-drug exposure may be linked to adverse outcomes like infections, dementia, respiratory disease exacerbation, pancreatitis, and cancer. At the same time, along with cannabis and alcohol, the substances most frequently observed in alleged sexual assault are hypnotics and benzodiazepines. Due to their chemical instability, low-dose active products, amnesic properties, and rapid elimination from bodily fluids, these drugs can be challenging to identify (Sanna et al., 2002). Various studies to quantify racemic Z2 in biological matrices have been published over the past two decades using HPLC, GC–MS, GC, and LC-MS/MS techniques. On the other hand, only four studies on the separation or measurement of Z2 enantiomers in biological matrices were published (Halas, 2006; Meng et al., 2010). Numerous methods have been developed to analyze Z1 and Z2 in biological fluids, including HPLC, GC–MS, and LC-MS/MS. However, using LC-MS/MS, a method has yet to be developed to concurrently detect Z1, Z2, and Z3 in a biological matrix. In recent studies, LC-MS/MS and GC–MS/MS are one of the most promising developments in quick chromatographic separations (Hempel and Blaschke, 1996; Lewis and Vine, 2007; Varsha et al., 2020; Villain et al., 2004; Wang et al., 1999). This study addresses a critical gap in analytical methodology by proposing a comprehensive approach for the simultaneous detection of Z-drugs in urine samples. The integration of the QuEChERS technique with both LC and GC–MS/MS methodologies presents a promising solution to the challenges posed by Z-drug misuse and abuse.

### 2.2. Apparatus and equipment

Analytical balance, vortex, benchtop centrifuge, 50 mL centrifuge tubes, sonicator, adjustable pipettes: 10–100, 20–200, and 100–1000 µL with disposable tips, 10 mL volumetric flasks, LCMSMS: Agilent 6470-LC/TQ, GCMSMS: Agilent 7000D, Analytical Column: Poroshell EC-C18 2.7 µm (2.7 µm, 3.0 × 150 mm), HP-5MS UI 15 m, 0.25 µm, 25 mm ID Capillary column, mobile phase filtration assembly were used for the study.

### 2.3. Methodology

#### 2.3.1. Mobile phase preparation

The 5 mM ammonium formate with 0.1 % formic acid in water was prepared using a 1000-ml volumetric flask containing 1 mL of formic acid and 315.3 mg of ammonium formate and was diluted up to 1000 mL with water and was sonicated for approximately five minutes. Another solvent prepared was 0.1 % formic acid in methanol.

#### 2.3.2. Preparation of stock standard of drugs residues

A 10 mL volumetric flask containing 10 mg of standard equivalent was weighed and dissolved in HPLC-grade acetonitrile and labelled with the name of the standard, concentration, preparation date, and the expiry date. The solution was kept at −20 °C in a deep freezer.

#### 2.3.3. Preparation of working standard dilution for calibration curve

Acetonitrile was used to create the 10.0, 1.0, and 0.1 mg/L working standards. The calibration curve standard spiked was prepared in the different concentrations by using the working standard from 1 to 200 ng/mL.

#### 2.3.4. Samples collection

Urine samples were collected before the intake of medicine and 4 h after the dose. For analysis, 50 mL of the urine was collected, centrifuged, decanted, and kept at − 4 °C.

### 2.4. Extraction procedure

The optimization of the QuEChERS extraction method involved two approaches (Fig. 2). 5 mL of urine samples were placed in a 50 mL centrifuge tube and spiked with varying concentrations (1, 5, 10, 50, 100, and 200 ng/mL) of Z1, Z2, and Z3. To create a diluent, 10 mL of methanol and 10 mL of Milli Q water were added to the tube. The sample was then homogenized using a wrist action shaker for 12 min. After that, an EN QuEChERS salt pouch was introduced into the tube, and the mixture was further mixed using a vortex shaker for 2 min. Subsequently, the tube was centrifuged at 6000 rpm for 12 min at 5 °C. A d-SPE (dispersive-solid phase extraction) procedure was carried out on the obtained mixture. The supernatant was transferred to another 15 mL dispersive tube containing 100 mg of MgSO4, vortexed, and centrifuged for 5 min at 12,000 rpm. An aliquot of 200 uL from the final extract was transferred to a different vial for all clean-up procedures, and 10 uL from each sample was directly injected into the LC-ESI-MS/MS and GC–MS/MS apparatus. This methodology can be adjusted for smaller sample sizes and less solvent usage through a microscale strategy. Using this method, we can efficiently handle 1 mL of urine and 2 mL of methanol without sacrificing analytical efficiency. This adjustment not only tackles practical challenges encountered in forensic and clinical testing scenarios but also aligns with environmental concerns by notably decreasing the amount of reagents utilized during the validation of the methodology.

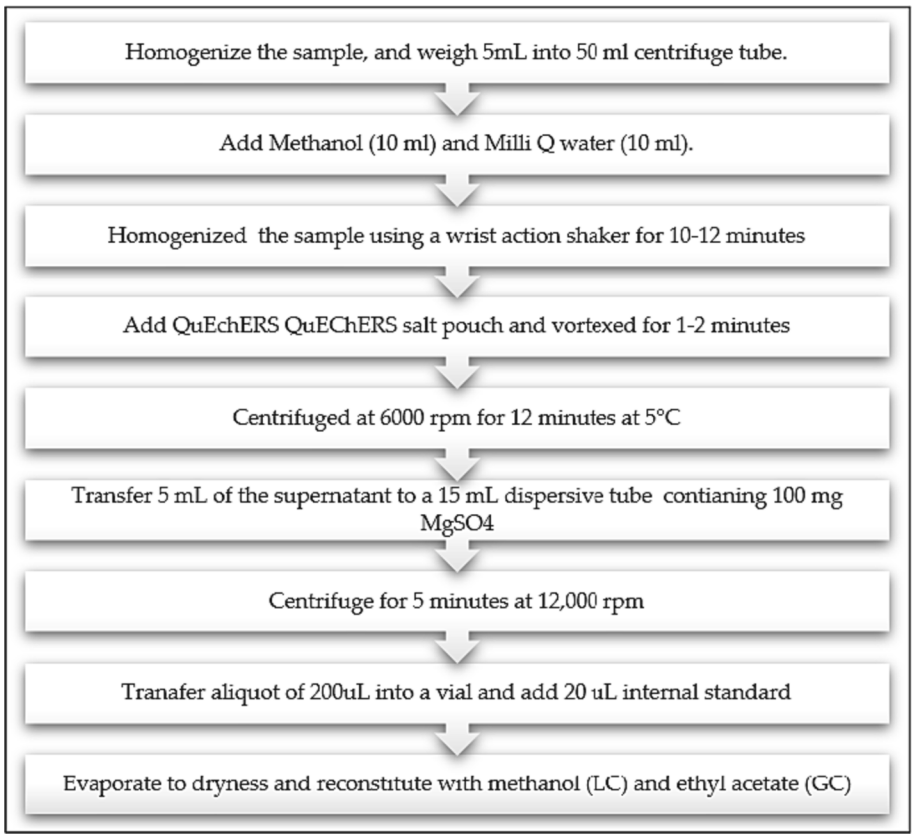


**Fig. 2.** Steps of applied QUEChERS procedure.

### 2.5. LC-MS/MS and GC–MS/MS conditions

An HPLC system from the Agilent 6470 series was used for the chromatography. For separation, a Poroshell EC-C18 column (50 × 2.0 mm with an internal diameter of 5 μm) was used. The mobile phase used to separate the components in the study was made up of 5 mM ammonium formate (mobile phase A) and 0.1 % formic acid methanol (mobile phase B). It flowed at a rate of 0.5 mL per minute. The strength of mobile phase B changed during the experiment: it started at 10 %, increased to 15 % after 0.50 min, then to 50 % after 3 min, and finally to 95 % after 4 min. This composition was maintained for 2 min before being changed back to the initial composition (10 % mobile phase B) for another minute to stabilize the system. The autosampler was operated at a temperature of 4 °C, and each sample analysis took 6 min to complete. The injection volume was 5 μL, and the column temperature was maintained at 65 °C.

An HPLC system from the 7000D series was used for the chromatography. For separation, an HP-5MS UI 15 m, 0.25 μm, 25 mm ID Capillary column was used. This analysis used Helium as the carrier gas and Argon as the collision gas. The column flow rate was set to 1.0 mL/min, and 1.5 μL of the sample was injected through an autosampler. The injector was set to high-pressure mode at 315oC. The ion source temperature was maintained at 230 °C while the injector port was set at 250 °C and 15.0216 psi. A 2.0 μL split-less injection was programmed into the system, and the total run time was 10.0 min.

Mass spectrometry was carried out with electrospray ionization. Conditions for MS were optimized, and the mass spectrometer worked in positive ion mode by doing full scans in positive ion detection mode. The multiple reaction monitoring mode was used to detect and quantify the sample. The quadrupoles Q1 and Q3 were set to unit resolution, and analytes were subjected to collision-induced dissociation to maximize the intensity of the protonated molecular ion and set the right conditions for multiple-reaction monitoring. To boost the signal for the two most common product ions, the collision energy (eV) was changed while the collision gas pressure of Argon was maintained. An Agilent Masshunter was used for data acquisition and quantitation. The ESI source parameters are mentioned in Table 1.

### 2.6. Method validation

The optimized methodology was validated according to ICH rules and USP general chapter 1225 for compendial operations. Characteristics, including selectivity, linearity, LOD, LOQ, accuracy, precision, and recovery, were evaluated to assess the validity of the developed methodology.

A test method must be selective to quantify an analyte in the presence of interference in the sample matrix. To determine selectivity, chromatographic blanks from a sample known to be analyte-free were analyzed within the expected time frame of the analyte peak. Six negative controls were used to screen the selectivity of the developed method using LC and GC. After injecting higher concentrations of Z1, Z2,

**Table 1**
ESI source parameters.

| ESI Source Parameters | |
|---|---|
| Drying Gas Flow | 7 L/min |
| Sheath Gas Flow | 9 L/min |
| Drying Gas Temperature | 350 °C |
| Ionization Mode | Positive |
| Sheath Gas Temperature | 350 °C |
| Nebulizer Gas | 40 psi |
| Capillary Voltage | 3600 V |
| | |
| MS Parameters | |
| Delta EMV | (+) 300 V |
| Scan Type | MRM |

and Z3, carryover peaks were determined. Blank samples were conducted after each injection of Z1, Z2, and Z3 at a higher ratio.

Over the 1–200 ng/mL concentration range, the calibration curves for the urine assay created with a peak-area ratio of Z1, Z2, and Z3 versus drug concentration were linear. Repeatability (intraday) and intermediate precision were used to evaluate the analytical method's level of precision (interday). Spiked blank urine QC samples were analyzed at three different concentrations six times daily to determine repeatability. The same urine samples were examined once per day for three days to determine the intermediate precision. Precision was determined using the RSD of the expected concentrations from the regression equation. The percentage relative error used to measure this analytical method's accuracy. In the calibration curve, the sensitivity was evaluated using the limit of quantification or the lowest urine concentration spiked with Z1, Z2, and Z3. The LOD for Z1, Z2, and Z3 was established to be the lowest concentration, resulting in a signal-to-noise (S/N) ratio of 3.

The three distinct batches of healthy human urine were used in this study for the blank urines, and five concentrations were evaluated (1, 5, 50, 100, and 200 ng/ml). The working solutions of Z1, Z2, and Z3 were spiked into the blank urine samples. After the samples had been extracted using the previously mentioned sample preparation, they were analyzed using MS coupled with gas and liquid chromatography.

## 3. Results

### 3.1. Method optimization

For Z1, Z2, and Z3, the conditions of mass ESI were evaluated using negative and positive ion detection modes. The specifications were then optimized using complete scans in the positive ion-detecting mode. Positive ionization mode proved to be more sensitive and produced less background noise for the drugs. Each drug's specific precursor/product ion mass transitions were measured, with Z1 measured at 308 → 236.1 *m/z*, Z2 at 389 → 244.9 → 217.1 *m/z*, and Z3 at 389 → 245.3 → 192.2 *m/z*. Figs. 3–6 demonstrate the simultaneous response and retention times for all three drugs in simulated urine samples at various concentrations (1, 5, 50, 100, and 200 ng/mL) indicating successful quantification and identification under the optimized conditions.

After that, several experiments were carried out to enhance chromatographic parameters, such as flow rate and mobile phase composition, so that the analyte signal, suitable peak shape, and shorter running time could be achieved. Methanol with 0.1 % formic acid and 5 mM ammonium formate buffer, modified with formic acid, served as the mobile phase. Ammonium formate was chiefly used to improve peak shape and boost source ionization.

### 3.2. Method development

The interference test was evaluated using the acquired liquid chromatograms and mass spectra at the retention duration and *m/z* ratio. Monitoring the analytes using LC-MS and GC–MS analysis did not reveal any endogenous substance interference, nor was there any interference at the specific RT. Fig. 7 shows the chromatogram and selectivity of Z1, Z2, and Z3. After injections of Z1, Z2, and Z3 at high concentrations, no carryover peaks were noticed, even after repeated injections.

#### 3.2.1. Specificity

We tested the specificity of our method by comparing the chromatograms of blank urine samples to those with analytes and internal standard (IS: zolpidem d6, zopiclone d4) spiked at the LOQ level. We found no interfering peaks from endogenous substances at the retention times of both the analyte and IS. Fig. 7 displays the chromatograms, confirming the method's high specificity.

#### 3.2.2. Linearity

Based on a linear regression model with a 1/xˆ2 weighting factor, six calibration points were used to create calibration curves. The r-squared (R2) values obtained for urine samples were above 0.999 for LC and 0.998 for GC, within the 1–200 ng/mL concentration range (as shown in Fig. 8). The Limit of Detection (LOD) for Z2, Z3, and Z1 in urine samples using LC-MS/MS were 0.45, 0.27, and 0.09, respectively. The LOD for urine samples using GC–MS/MS were 0.95, 0.62, and 0.15, respectively. Table 2 provides an overview of the LOD, Lower Limit of Quantification (LLOQ), linearity, and calibration curve details for each analyte.

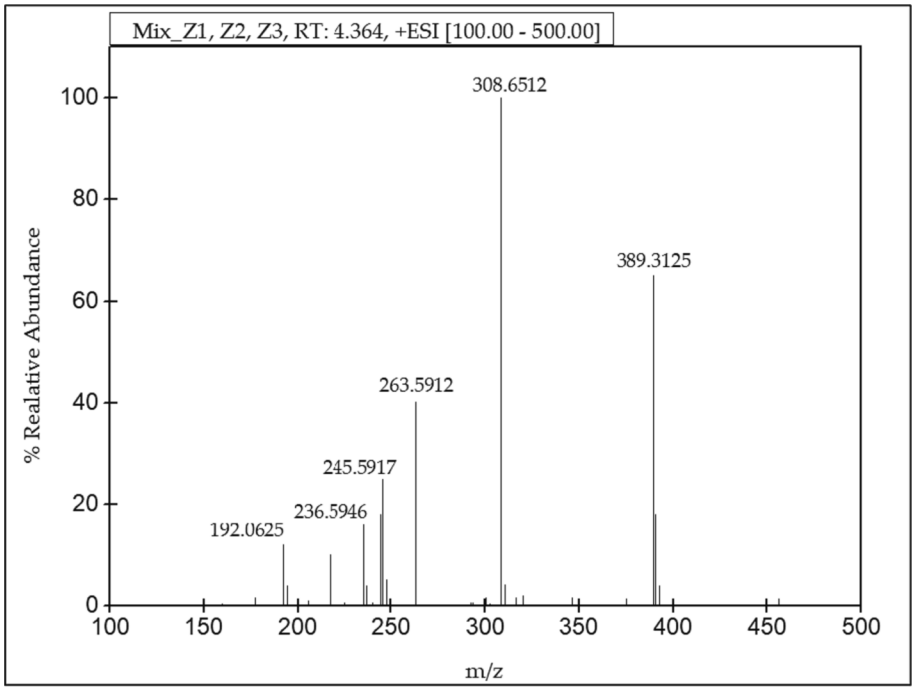


**Fig. 3.** Mass spectrum of urine extract at RT: 4.364, 4.711, and 4.950, *m/z* 308.6125, 245.5917, and 389.3125 corresponding to Mix_Z1_Z2_Z3.

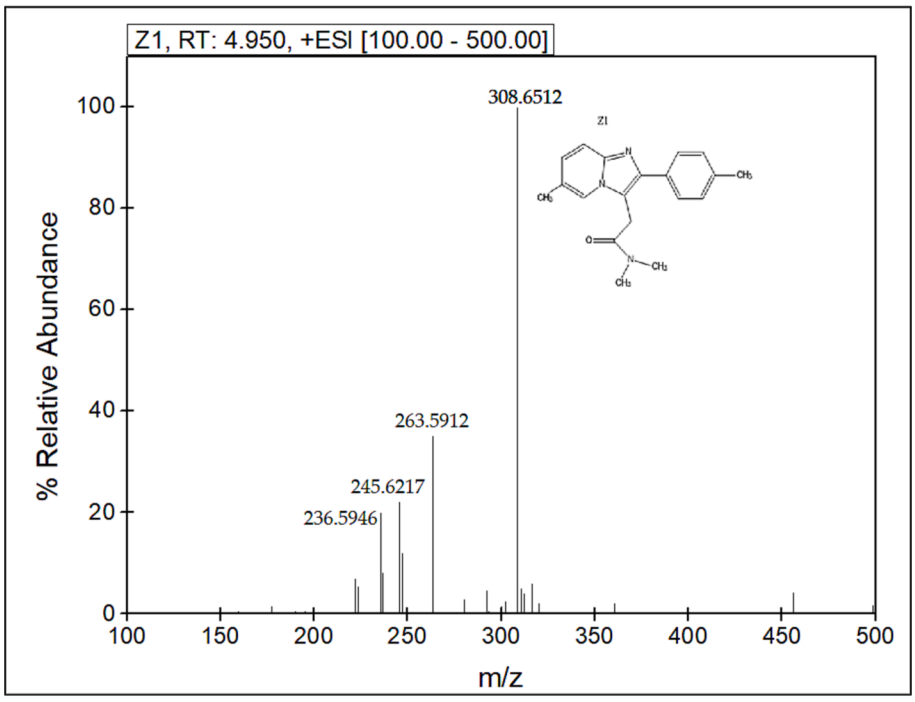


**Fig. 4.** Mass spectrum of urine extract at RT: 4.950, *m/z* 308.6125 corresponding to Z1.

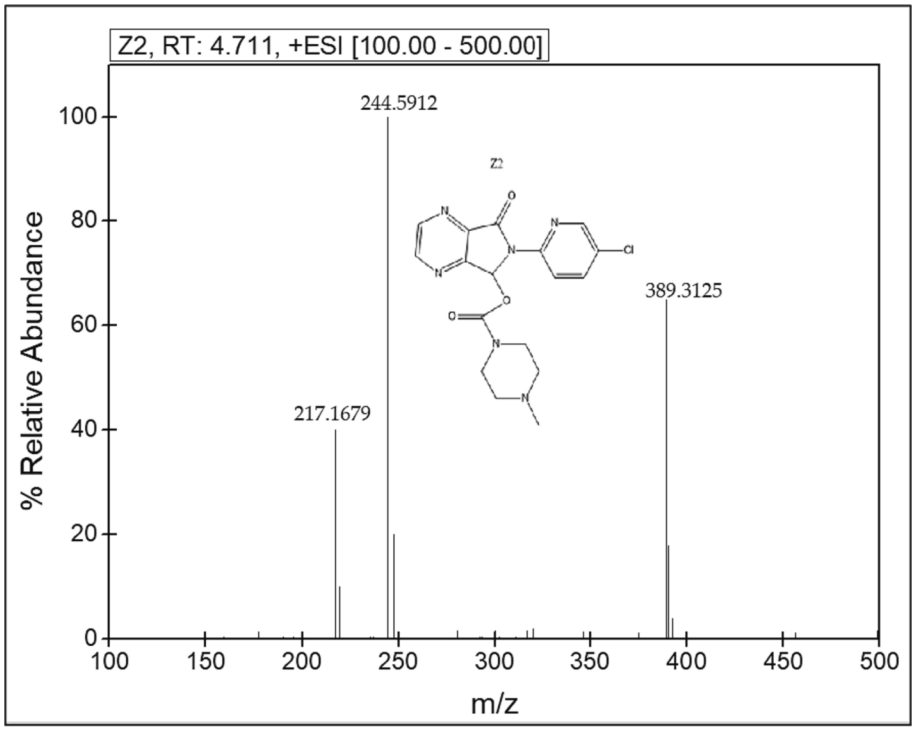


**Fig. 5.** Mass spectrum of urine extract at RT: 4.364, *m/z* 389.3125 corresponding to Z2.

### *3.2.3. Accuracy and precision*

To ensure accuracy and precision, the LQC (5 ng/mL), MQC (50 ng/mL), and HQC (200 ng/mL) were utilized with a permissible range of ±5 % for accuracy and a range of within 3 % for precision. Table 3 shows the intraday accuracy and precision of urine samples using LC-MS/MS, based on six replicates, which varied between 96.65 % and 101.25 % and within 3.29 %, respectively. Interday accuracy and precision, also based on six replicates, ranged from 95.56 % to 99.98 % and within 3.05 %, respectively. In contrast, GC–MS/MS was used for urine samples, and the intraday accuracy and precision, based on six replicates, ranged from 98.95 % to 93.65 % and within 3.97 %, respectively. Similarly, interday accuracy and precision, based on six replicates, ranged from 98.59 % to 92.56 % and within 4.52 %, respectively.

### *3.2.4. Stability, Recovery, and matrix effect*

In this study, we assessed the recovery rates of Z1, Z2, and Z3 at three

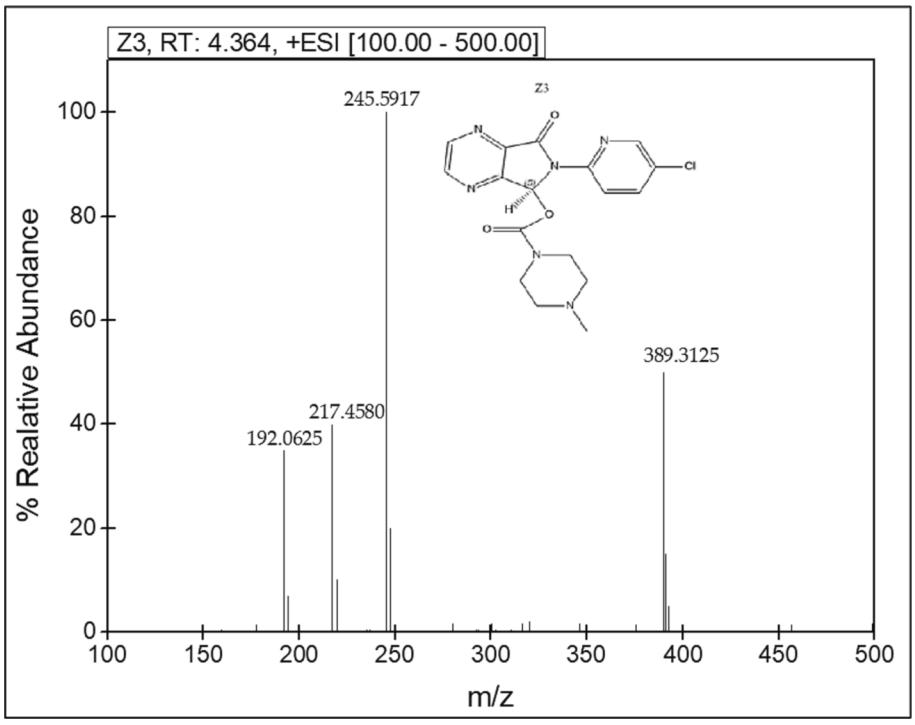


**Fig. 6.** Mass spectrum of urine extract at RT: 4.711, *m/z* 389.3125 corresponding to Z2.

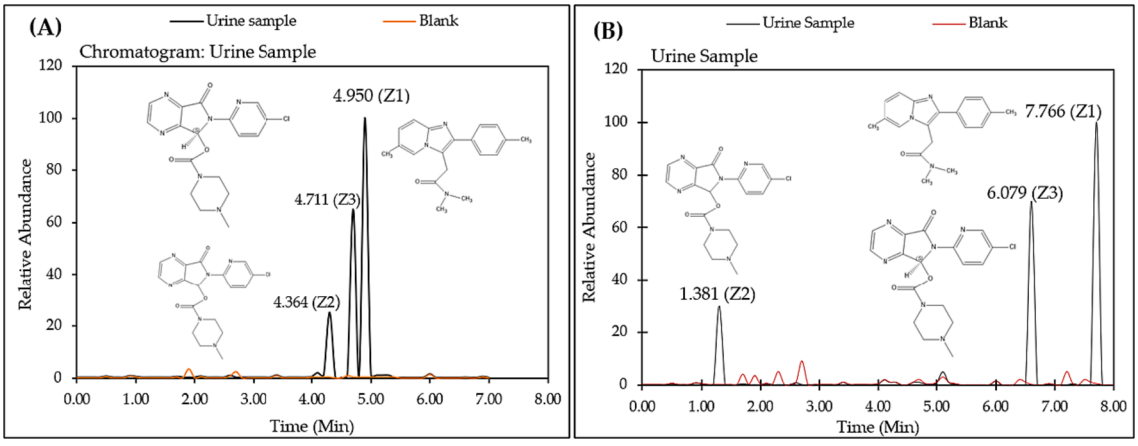


**Fig. 7.** Extracted ion chromatogram of analytes in urine sample using LC-MS/MS and GC–MS/MS at the 5 ng/mL listed in elution order: (A) LC-MS/MS – Z2 (4.364), Z3 (4.711), and Z1 (4.950); (B) GC–MS/MS – Z2 (1.381), Z3 (6.079), and Z1 (7.766).

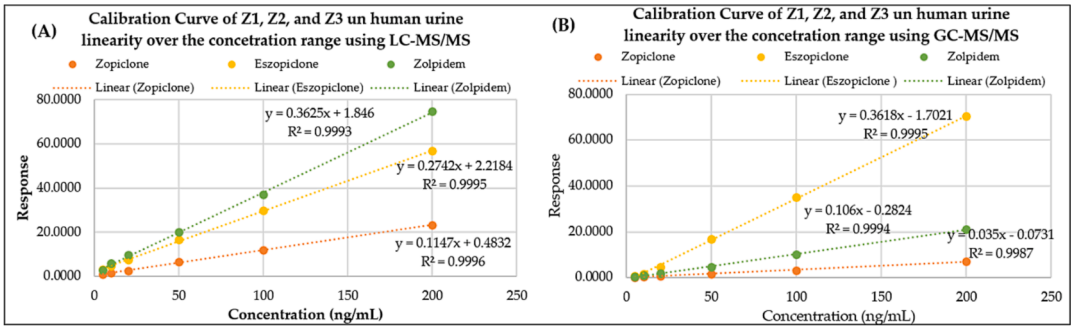


**Fig. 8.** Calibration curve of Z1, Z2, and Z3 in urine sample using LC-MS/MS (A) and GC–MS/MS (B).

**Table 2**
Limit of Detection (LOD), Lower Limit of Quantification (LLOQ), linearity, and calibration curve for each analyte in urine sample using GC–MS/MS and LC-MS/MS.

| Limit of detection (LOD), lower limit of quantification (LLOQ), linearity, and calibration curve | | | | | | | |
|---|---|---|---|---|---|---|---|
| | RT | Analyte | LOD | LLOQ | Calibration Range (ng/mL) | $R^2$ | Slope |
| URINE–LC-MS/MS | 4.364 | (±)-Zopiclone | 0.45 | 1.56 | 1–200 | 0.9996 | 0.1147 |
| | 4.711 | Eszopiclone | 0.27 | 1.39 | 1–200 | 0.9995 | 0.2742 |
| | 4.950 | Zolpidem | 0.09 | 1.06 | 1–200 | 0.9993 | 0.3625 |
| URINE–GC–MS/MS | 1.381 | (±)-Zopiclone | 0.95 | 2.05 | 1–200 | 0.9981 | 0.0301 |
| | 6.079 | Eszopiclone | 0.62 | 1.98 | 1–200 | 0.9995 | 0.3618 |
| | 7.766 | Zolpidem | 0.15 | 1.53 | 1–200 | 0.9994 | 0.1060 |

**Table 3**
Validation of the method using quality control (QC) samples yielded results for intra- and interday accuracy and precision, recovery, and matrix effect in urine samples using LC-MS/MS and GC–MS/MS.

| Urine Sample | Analyte | QC sample (ng/mL) | Intraday | | Interday | | Recovery % (n = 6) | Matrix Effect % (n = 6) |
|---|---|---|---|---|---|---|---|---|
| | | | Accuracy % | RSD % | Accuracy % | RSD % | | |
| LC-MS/MS | Z1 | 5 | 101.25 | 1.59 | 99.98 | 1.01 | 99.58 | 100.55 |
| | | 50 | 99.65 | 1.65 | 98.95 | 1.11 | 98.62 | 98.62 |
| | | 200 | 99.12 | 1.98 | 98.26 | 1.20 | 98.02 | 99.65 |
| | Z2 | 5 | 97.54 | 2.92 | 96.62 | 2.18 | 95.96 | 95.56 |
| | | 50 | 97.65 | 2.99 | 96.99 | 2.55 | 95.38 | 96.36 |
| | | 200 | 96.65 | 3.29 | 95.56 | 4.52 | 94.91 | 94.68 |
| | Z3 | 5 | 98.65 | 2.25 | 97.89 | 1.84 | 96.66 | 96.96 |
| | | 50 | 97.86 | 2.36 | 97.56 | 2.13 | 95.26 | 95.56 |
| | | 200 | 97.09 | 2.91 | 96.16 | 2.99 | 95.08 | 95.26 |
| GC–MS/MS | Z1 | 5 | 98.95 | 1.59 | 98.59 | 2.01 | 96.58 | 98.05 |
| | | 50 | 98.29 | 1.65 | 98.25 | 2.51 | 95.62 | 97.02 |
| | | 200 | 98.02 | 1.98 | 98.56 | 2.95 | 95.12 | 96.65 |
| | Z2 | 5 | 94.54 | 2.12 | 95.62 | 2.98 | 93.96 | 92.56 |
| | | 50 | 95.65 | 2.99 | 95.99 | 3.81 | 92.98 | 92.36 |
| | | 200 | 93.65 | 3.97 | 92.56 | 4.05 | 92.10 | 91.98 |
| | Z3 | 5 | 96.54 | 2.05 | 97.89 | 2.84 | 94.56 | 94.96 |
| | | 50 | 95.65 | 2.36 | 96.56 | 3.13 | 95.06 | 93.56 |
| | | 200 | 94.65 | 2.99 | 95.16 | 2.99 | 93.18 | 93.26 |

different concentration levels: LQC, MQC, and HQC. Using LC-MS/MS on urine samples, we found that the recovery rates ranged from 98.02 % to 99.56 % for Z1, 94.91 % to 95.96 % for Z2, and 95.08 % to 96.66 % for Z3, with relative standard deviations between 1.0 % and 2.9 %. For urine samples, using GC–MS/MS, the recovery rates were between 96.65 % and 98.55 % for Z1, 92.10 % and 93.96 % for Z2, and 93.18 % to 95.06 % for Z3, with relative standard deviations between 1.5 % and 3.5 % (Table 3).

The presence of matrix components in a sample can affect the accuracy and reproducibility of the assay, which is known as the matrix effect. Thus, we evaluated the matrix effect of our approach. Our results showed that the mean matrix effects ranged from 94.68 % to 100.55 % for urine samples using LC-MS/MS, with CVs between 1.02 % and 2.95 %. For urine samples using GC–MS/MS, the mean matrix effects ranged from 91.98 % to 98.05 %, with CVs between 2.92 % and 4.15 %. These results indicate that the ion suppression or enhancement from the samples was within acceptable limits under the experimental conditions.

## 4. Discussion

Z-drugs have been implicated in drug-facilitated crimes such as robbery and sexual assault. As the substance has a short half-life, it can quickly impair a person. Furthermore, because of its amnesic qualities, the victims cannot recollect the exact circumstances of the offence. Urine is the primary specimen used to document drug-facilitated crimes. Therefore, in this study, we used urine as a matrix to evaluate z-drugs. Many analyte methods have previously been developed using LC but very less using GC coupled with MS, but there is no report to analyze these analytes simultaneously where it incorporates enantiomeric z-drug (Z2 and Z3) separation along with Z1.

In the past, the Z1, Z2, and Z3 extraction recovery for the individual was less than 74.8 per cent, and the detection limit was 0.9 ng/mL (Ascalone et al., 1992; Bassan et al., 2011; Jones et al., 2012; Kazmierczak, 2019; Kintz et al., 2005; Lieberman, 2007; Shi et al., 2012; Stockham and Rohrig, 2010). A study was conducted to develop and verify a technique that utilizes GC–MS/MS for analyzing benzodiazepine derivatives and Z-drugs. The sample preparation involved liquid–liquid extraction. The intraday precision, expressed as CVs, varied from 0.2 % to 12.5 %, while interday precision ranged from 2.0 % to 14.5 %. The recovery rate was found to be between 83.8 % and 111.2 % (Banaszkiewicz et al., 2023). This study improved the QuEChERS extraction techniques to collectively extract Z1, Z2, and Z3 from urine efficiently.

When analyzing drugs in a toxicological setting, forensic and clinical toxicologists rely on biological samples such as blood, urine, and other biological matrices as they provide a clear connection between the amount of a substance and its effects (Kim et al., 2021). To detect z-drugs in urine, LC-MS/MS and GC–MS/MS are often used in forensic and toxicology laboratories. In this study, 5 samples were taken from volunteers and the concentration of each drug in the urine sample was evaluated. LC-MS/MS and GC–MS/MS are two powerful methods used to detect and quantify various compounds, including drugs. The choice between the two techniques depends on the specific characteristics of the compounds you are trying to detect and the advantages offered by each method. Z-drugs are a type of sedative or hypnotic medication that includes Z1, Z2, and Z3 (Roberts and Wood, 2010). The choice between LC-MS/MS and GC–MS/MS for detecting z-drugs depends on factors such as the physicochemical properties of the drugs, the presence of suitable chromatographic separation methods, and compatibility with

the mass spectrometry technique. GC–MS/MS is limited in its ability to analyze Z-drugs due to their low volatility and polarity. Derivatization may be necessary to improve their volatility and thermal stability for GC analysis. However, this can introduce variability and reduce sensitivity in some cases. Developing a GC–MS/MS method for Z-drugs can be complex and require optimization of derivatization conditions. LC-MS/MS is great for analyzing complex samples like biological fluids. It can handle a wide range of compounds and matrix interferences. This article showcases a reliable and verified analytical technique that can detect Z-drugs (Zolpidem, Zopiclone, and Eszopiclone) simultaneously in urine samples. The method is highly accurate, precise, and selective, which makes it an ideal tool for monitoring drug usage and identifying cases of misuse or abuse. By utilizing both LC-MS/MS and GC–MS/MS techniques, the approach presented in this article offers a strong and dependable way to detect these substances in biological matrices.

## 5. Application of methodology

Z-drugs are compatible with LC-MS/MS and it's sensitive enough to detect low concentrations. Developing an LC-MS/MS method for z-drugs is less challenging and doesn't require derivatization like GC–MS/MS. The method proved to be effective, producing results consistent with spiked samples. Developing a reliable analytical method using two significant techniques to detect z-drugs in urine samples is crucial for public health and safety concerns. Accurate detection is necessary for clinical diagnostics, forensic investigations, pharmacological studies, and monitoring drug trends. Validated methods are required for regulatory compliance and advancing the field of analytical chemistry. The developed method has the potential to impact healthcare, law enforcement, and scientific communities positively.

Both LC-MS/MS and GC–MS/MS methods appear to be reliable for analyzing the specified analytes in urine samples. LC-MS/MS generally shows slightly better performance in terms of matrix effect, LOD, LLOQ, and recovery, while both methods exhibit high accuracy and linearity. The choice between the two methods may depend on specific analytical requirements and the nature of the analytes being studied. The summary of the study is presented in Table 4.

## 6. Conclusions

Through our study, we have developed a GC–MS/MS method for detecting z-drugs without the need for derivatization. We have increased accuracy and recovery rate by optimizing the parameters. While z-drugs are polar and not highly volatile, LC-MS/MS may be a better choice for detection. However, other factors such as sample characteristics, instrument availability, and method development must also be considered.

## Author contributions

Conceptualization, V.C. and M.S.; methodology, V.C. and A.T.; software, A.T. and V.T.; validation, M.K., A.S., O.M.N. and A.L.M.; formal analysis, A.A. (Alhalmi); investigation, A.K. and A.A. (Alahdab); resources, V.C. and M.S.; data curation, A.T. and M.K.; writing—original draft preparation, V.C. M.S. and A.T.; writing—review and editing, A.T., V.T., M.K., A.S., A.L.M., A.K., A.A., O.M.N., and A.A (Alahdab).; visualization, M.K.; supervision, A.T. and M.K.; project administration, M.S.; funding acquisition, O.M.N, and A.A. (Alahdab). All authors have read and agreed to the published version of the manuscript."

## Funding

No External Funding.

**Table 4**
Comparision of the developed method using LC-MS/MS and GC–MS/MS.

| | Parameters | LC-MS/MS | GC–MS/MS |
|---|---|---|---|
| Analyte | Sample | Urine | Urine |
| | Calibration Range (ng/mL) | 1–200 | 1–200 |
| | Matrix Effect | 94.68 % to 100.55 % | 91.98 % to 98.05 % |
| | Accuracy | 96.65 % and 101.25 % | 95.56 % to 99.98 % |
| Z1 | R2 | 0.9993 | 0.9994 |
| | LOD | 0.09 | 0.15 |
| | LLOQ | 1.06 | 1.53 |
| | Recovery | 98.02 % to 99.56 % | 96.65 % and 98.55 % |
| Z2 | R2 | 0.9996 | 0.9981 |
| | LOD | 0.45 | 0.95 |
| | LLOQ | 1.56 | 2.05 |
| | Recovery | 94.91 % to 95.96 % | 92.10 % and 93.96 % |
| Z3 | R2 | 0.9995 | 0.9995 |
| | LOD | 0.27 | 0.62 |
| | LLOQ | 1.39 | 1.98 |
| | Recovery | 95.08 % to 96.66 % | 93.18 % to 95.06 % |

## Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethical committee of National Forensic Sciences University.

## Informed consent statement

Informed consent was provided by the participants for the study.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors extend their appreciation to Researchers Supporting Project number (RSPD2024R1087), King Saud University, Riyadh, Saudi Arabia. The authors also extend their appreciation to National Forensic Science University, Delhi Campus, India.

## References

Ascalone, V., Flaminio, L., Guinebault, P., Thénot, J.P., Morselli, P.L., 1992. Determination of zolpidem, a new sleep-inducing agent, and its metabolites in biological fluids: pharmacokinetics, drug metabolism and overdosing investigations in humans. J. Chromatogr. 581, 237–250. https://doi.org/10.1016/0378-4347(92)80277-w.

Banaszkiewicz, L., Woźniak, M.K., Kaliszan, M., Kot-Wasik, A., 2023. Determination of benzodiazepines and Z-hypnotic drugs in whole blood samples by GC–MS/MS: method development, validation and application. Microchem. J. 193, 109115 https://doi.org/10.1016/j.microc.2023.109115.

Bassan, D.M., Erdmann, F., Krüll, R., 2011. Quantitative determination of 43 common drugs and drugs of abuse in human serum by HPLC-MS/MS. Anal. Bioanal. Chem. 400, 43–50. https://doi.org/10.1007/s00216-011-4784-y.

Brandt, J., Leong, C., 2017. Benzodiazepines and Z-drugs: An updated review of major adverse outcomes reported on in epidemiologic research. Drugs r. d. 17, 493–507. https://doi.org/10.1007/s40268-017-0207-7.

Eliassen, E., Kristoffersen, L., 2014. Quantitative determination of zopiclone and zolpidem in whole blood by liquid-liquid extraction and UHPLC-MS/MS. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 971, 72–80. https://doi.org/10.1016/j.jchromb.2014.09.018.

Halas, C.J., 2006. Eszopiclone. Am. J. Heal. Pharm. AJHP Off J. Am. Soc. Heal. Pharm. 63, 41–48. https://doi.org/10.2146/ajhp050357.

Hempel, G., Blaschke, G., 1996. Direct determination of zolpidem and its main metabolites in urine using capillary electrophoresis with laser-induced fluorescence detection. J. Chromatogr. B. Biomed. Appl. 675, 131–137. https://doi.org/10.1016/0378-4347(95)00342-8.

Hockenhull, J., Black, J.C., Haynes, C.M., Rockhill, K., Dargan, P.I., Dart, R.C., Wood, D. M., 2021. Nonmedical use of benzodiazepines and Z-drugs in the UK. Br. J. Clin. Pharmacol. 87, 1676–1683. https://doi.org/10.1111/bcp.14397.

Jones, A.W., Holmgren, A., 2012. Concentrations of zolpidem and zopiclone in venous blood samples from impaired drivers compared with femoral blood from forensic autopsies. Forensic Sci. Int. 222, 118–123. https://doi.org/10.1016/j.forsciint.2012.05.008.

Jones, A.W., Holmgren, A., Ahlner, J., 2012. Toxicological analysis of blood and urine samples from female victims of alleged sexual assault. Clin. Toxicol. (Phila) 50, 555–561. https://doi.org/10.3109/15563650.2012.702217.

Kazmierczak, S.C., 2019. Chapter 2 – Alcohol Analysis in Various Matrixes: Clinical Versus Forensic Testing, in Dasgupta, A.B.T.-C.I. in A. and D. of A.T. (Second E. (Ed.),. Academic Press, pp. 17–23. https://doi.org/https://doi.org/10.1016/B978-0-12-815607-0.00002-2.

Kim, S.Y., Kwon, N.H., Cheong, J.C., Kim, J.Y., 2021. LC-MS/MS method for determining picogram-level of zolpidem and its main metabolites in hair using a zirconia-based sorbent. Talanta 228, 122041. https://doi.org/10.1016/j.talanta.2020.122041.

Kintz, P., Villain, M., Dumestre-Toulet, V., Ludes, B., 2005. Drug-facilitated sexual assault and analytical toxicology: the role of LC-MS/MS A case involving zolpidem. J. Clin. Forensic Med. 12, 36–41. https://doi.org/10.1016/j.jcfm.2004.08.005.

Lewis, J.H., Vine, J.H., 2007. A simple and rapid method for the identification of zolpidem carboxylic acid in urine. J. Anal. Toxicol. 31, 195–199. https://doi.org/10.1093/jat/31.4.195.

Lieberman, J.A., 2007. Update on the safety considerations in the management of insomnia with hypnotics: incorporating modified-release formulations into primary care. Prim. Care Companion. J. Clin. Psychiatry 09 (01), 25–31.

Meng, M., Rohde, L., Cápka, V., Carter, S.J., Bennett, P.K., 2010. Fast chiral chromatographic method development and validation for the quantitation of eszopiclone in human plasma using LC/MS/MS. J. Pharm. Biomed. Anal. 53, 973–982. https://doi.org/10.1016/j.jpba.2010.05.006.

Roberts, M., Wood, M., 2010. Quantitative analysis of 21 benzodiazepine drugs, Zolpidem and Zopiclone in serum using LC/MS/MS. Waters Corp. Printed/Produced U.S.A. Sanna, E., Busonero, F., Talani, G., Carta, M., Massa, F., Peis, M., Maciocco, E., Biggio, G., 2002.

Sanna, E., Busonero, F., Talani, G., Carta, M., Massa, F., Peis, M., Maciocco, E., Biggio, G., 2002. Comparison of the effects of zaleplon, zolpidem, and triazolam at various GABAA receptor subtypes. Eur. J. Pharmacol. 451 (2), 103–110.

Shi, Y., Xiang, P., Shen, B., Shen, M., 2012. 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. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 911, 140–146. https://doi.org/10.1016/j.jchromb.2012.10.018.

Stockham, T.L., Rohrig, T.P., 2010. The use of Z-drugs to facilitate sexual assault. Forensic Sci. Rev. 22, 61–73.

Varsha, C., S.K., S., G.P., S., 2020. Abuse of Z-drugs and its challenges to society. Int. J. Med. Toxicol. Leg. Med. 23, 191–205.

Varsha, C., K., S.S., P., S.G., 2021. Z-drugs for drug-facilitated sexual assaults. Int. J. Med. Toxicol. Leg. Med. 24, 97–107.

Villain, M., Chèze, M., Tracqui, A., Ludes, B., Kintz, P., 2004. Windows of detection of zolpidem in urine and hair: application to two drug facilitated sexual assaults. Forensic Sci. Int. 143, 157–161. https://doi.org/10.1016/j.forsciint.2004.04.019.

Wang, Q., Sun, L., Lau, C.E., 1999. Determination of zolpidem in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats. J. Chromatogr. B. Biomed. Sci. Appl. 734, 299–305. https://doi.org/10.1016/s0378-4347(99)00376-x.