Forensic Science International 257 (2015) 84–92

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Rapid determination of benzodiazepines, zolpidem and their metabolites in urine using direct injection liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 22 April 2015
Received in revised form 21 July 2015
Accepted 26 July 2015
Available online 3 August 2015

Keywords:
Benzodiazepines
Zolpidem
Metabolite
Urine
Direct injection
LC–MS/MS

ABSTRACT

Benzodiazepines and zolpidem are generally prescribed as sedative, hypnotics, anxiolytics or anticonvulsants. These drugs, however, are frequently misused in drug-facilitated crime. Therefore, a rapid and simple liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed for identification and quantification of benzodiazepines, zolpidem and their metabolites in urine using deuterium labeled internal standards (IS). Urine samples (120 μL) mixed with 80 μL of the IS solution were centrifuged. An aliquot (5 μL) of the sample solution was directly injected into the LC–MS/MS system for analysis. The mobile phases consisted of water and acetonitrile containing 2 mM ammonium trifluoroacetate and 0.2% acetic acid. The analytical column was a Zorbax SB-C18 (100 mm × 2.1 mm i.d., 3.5 μm, Agilent). The separation and detection of 18 analytes were achieved within 10 min. Calibration curves were linear over the concentration ranges of 0.5–20 ng/mL (zolpidem), 1.0–40 ng/mL (flurazepam and temazepam), 2.5–100 ng/mL (7-aminoclonazepam, 1-hydroxymidazolam, midazolam, flunitrazepam and alprazolam), 5.0–200 ng/mL (zolpidem phenyl-4-carboxylic acid, α-hydroxyalprazolam, oxazepam, nordiazepam, triazolam, diazepam and α-hydroxytriazolam), 10–400 ng/mL (lorazepam and desalkylflurazepam) and 10–100 ng/mL (N-desmethylflunitrazepam) with the coefficients of determination (r^2) above 0.9971. The dilution integrity of the analytes was examined for supplementation of short linear range. Dilution precision and accuracy were tested using two, four and ten-folds dilutions and they ranged from 3.7 to 14.4% and −12.8 to 12.5%, respectively. The process efficiency for this method was 63.0–104.6%. Intra- and inter-day precisions were less than 11.8% and 9.1%, while intra- and inter-day accuracies were less than −10.0 to 8.2%, respectively. The lower limits of quantification were lower than 10 ng/mL for each analyte. The applicability of the developed method was successfully verified with human urine samples from drug users ($n = 21$). Direct urine sample injection and optimized mobile phases were introduced for simple sample preparation and high-sensitivity with the desired separation.

1. Introduction

Benzodiazepines (BZD) are a group of central nervous system depressant drugs used as hypnotics, anxiolytics, anti-depressants, muscle relaxants and anticonvulsants [1]. There are four main types of BZD, 1,4-benzodiazepines, triazolobenzodiazepines, diazolobenzodiazepines and 7-nitrobenzodiazepines [2]. BDZ are metabolized primarily in the liver via several different microsomal enzymes. The pathways of the BDZ biotransformation involve hepatic microsomal oxidation, aliphatic hydroxylation or N-dealkylation, and glucuronide conjugation [3]. Many hydroxylated metabolites of BDZ are pharmacologically active. As many of these active metabolites have been circulating in the market, it may be not easy to confirm which substance was ingested simply based on the results of analysis. Therefore, caution must be taken in the interpretation of the positive findings. BDZ often had unexpected side effects including a high potential for abuse [4]. They were

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http://dx.doi.org/10.1016/j.forsciint.2015.07.043
0379-0738/

frequently involved with forensic cases, irrespective of their therapeutic applications.

Zolpidem (ZP) is a non-benzodiazepine hypnotic of the imidazopyridine type for the treatment of insomnia [5]. It binds to the BDZ site on the gamma amino butyric acid-1 receptor [6]. It was considered as a safer medicine than BDZ as there was no evidence of abuse potential in its early period. But over the past decade, numerous cases of zolpidem abuse have been reported in the United States and many other countries. It was revealed that zolpidem had a higher potential for abuse than previously reported [7].

Drug-facilitated crime (DFC) has become a significant problem. Alcohol is the drug most commonly connected with DFC, but recently the use of other drugs is on the rise. Aside from alcohol, BDZ and ZP are the most frequently observed compounds in DFC cases in Korea. According to a previous study, most common substances detected in postmortem samples were ZP, diazepam (DIZ), nordiazepam (NDZ), midzolam (MIZ), alprazolam (APZ), triazolam (TRZ), 7-aminoclonazepam (7-ACLZ), 7-aminoflunitrazepam (7-AFNZ), lorazepam (LRZ), flurazepam (FRZ), temazepam (TMZ), dealkylflurazepam (DA-FRZ), and oxazepam (OXZ) [8].

The steady increase in the nonmedical uses of BZDs and ZP requires convenient and rapid methods in biological samples for forensic purposes. To date, several hyphenated mass spectrometric methods have been reported including gas chromatography–mass spectrometry (GC–MS) [9–13], capillary electrophoresis–mass spectrometry [14,15] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [16–20]. LC–MS/MS based technique is considered a powerful alternative to GC–MS. The main advantage of this technique is simplified sample preparation of direct measurement of hydrophilic analytes without hydrolysis and derivatization procedures. LC–MS/MS technique provides higher selectivity with less interference from co-elutes and matrices than single MS detection, resulting in less time consuming method development and faster analysis time [21]. Due to its usefulness, direct injection LC–MS/MS methods have been reported for measuring opiates, phenylethylamines, benzylpiperazine, and non-benzodiazepine hypnotics [22–24], while direct injection LC–MS method was reported for 10 benzodiazepines [25].

There have been continual demands for rapid drug tests for a large number of urine samples and multi-component analysis. LC–MS/MS-based technique is useful as a multi-component screening and is also thought to save the time of sample preparation and speed up the analysis [26,16]. The aim of the study was to develop a convenient and rapid method for simultaneous determination of 18 benzodiazepines, zolpidem and their metabolites using direct injection LC–MS/MS. The method was validated and its applicability was confirmed by analysis of authentic urine samples.

2. Materials and methods

2.1. Chemicals

The reference compounds of zolipem phenyl-4-carboxylic acid (ZPCA), 7-ACLZ, ZP, α-hydroxymidazolam (MIZ-OH), MIZ, FRZ, α-hydroxytriazolam (TRZ-OH), α-hydroxyalprazolam (APR-OH), OXZ, N-desmethylflunitrazepam (N-DFNZ), LRZ, DA-FRZ, NDZ, TMZ, flunirazepam (FNZ), APZ, TRZ, and DIZ were obtained from Cerilliant (Austin, TX, USA). The deuterated internal standards (IS) ZP-d_6, MIZ-OH-d_4, TRZ-OH-d_4, APZ-OH-d_5, OXZ-d_5, LRZ-d_4, NDZ-d_5, TMZ-d_5, TRZ-d_4, and DIZ-d_5 were also obtained from Cerilliant. HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). The water was purified using a Direct-Q water purification system (Millipore, Bedford, MA, USA). HPLC-grade acetic acid and ammonium trifluoroacetate were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of solutions

The stock standard solutions of each analyte were prepared in methanol at a concentration of 10 μg/mL or 100 μg/mL for each compound. Working standard solutions were prepared by mixing an appropriate amount of the stock solution with methanol. IS working solution was made by combining internal standard stock solutions of each IS at a concentration of 0.5 μg/mL (ZP-d6), 0.05 μg/mL (MIZ-OH-d_4), 2.0 μg/mL (TRZ-OH-d_4), 1.0 μg/mL (APZ-OH-d_5), 1.0 μg/mL (OXZ-d_5), 1.0 μg/mL (LRZ-d_4), 1.0 μg/mL (NDZ-d_5), 0.2 μg/mL (TMZ-d_5), 1.0 μg/mL (TRZ-d_4), and 0.5 μg/mL (DIZ-d_5). All of these solutions were stored at −20 °C before use.

2.3. Urine specimens

Blank urine samples were obtained from laboratory staff. Collected blank urine samples were pooled and used as blank urine for method validation. Twenty-one forensic urine samples collected from drug abusers were obtained from the Narcotics Departments at the District Prosecutors' Offices in the Seoul metropolitan area. Samples were stored in a Hetofrig CL 410 Deep Freezer (Heto, Denmark) at −55 °C until analysis.

2.4. Sample preparation

Urine specimens (1 mL) were centrifuged to obtain clear supernatants at 50,000 *g* for 3 min in a Sigma 3-30K centrifuge. Aliquots (120 μL) of the centrifugated urine samples were mixed with 80 μL of combined IS working solution. Five microliters of the sample solutions were directly injected into the LC–MS/MS system for analysis.

2.5. HPLC conditions

The HPLC system consisted of an Agilent 1200 series handheld control module, binary gradient pump, a vacuum degasser, an autosampler and a thermostatted column compartment (Palo Alto, CA, USA). The analytical column was a Zorbax SB-C18 (100 mm × 2.1 mm i.d., 3.5 μm, Agilent). Mobile phases were investigated at various contents and composition. 0.2% acetic acid and 2 mM ammonium trifluoroacetate in distilled water, and 0.2% acetic acid and 2 mM ammonium trifluoroacetate in acetonitrile were used as the mobile phase. Each solution was sonicated for 5 min using ultrasonic bath and then filtered through 0.45 μm membrane filters under vacuum.

The gradient started at 250 μL/min with 20% of mobile phase B for 0.5 min which was increased to 95% until 10 min, and then changed to an isocratic condition at 200 μL/min with 20% B for 4 min.

2.6. MS/MS conditions

The API 3200 Q Trap triple-quadrupole mass spectrometer (AB SCIEX, Foster city, CA, USA) was equipped with a Turbo Ion Spray source. Electrospray ionization (ESI) was carried out in the positive mode using nitrogen as the nebulizing, turbo spray and curtain gas, with the optimum values set to 30, 45 and 65 (arbitrary units). The turbo-gas temperature and the spraying needle voltage were set to 550 °C and 5000 V. The mass spectrometer was operated with unit (0.7 full width at half height) resolution for quadrupoles Q1 and Q3, respectively. Multiple reaction monitoring (MRM) detection was achieved using nitrogen as the collision gas (medium, arbitrary