Development of a Homogeneous Immunoassay for the Detection of Zolpidem in Urine

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Abstract

Sleep disorders are common conditions that affect about 40 million people in the U.S. every year, the most common of which is insomnia, which is characterized by difficulty falling or staying asleep. Zolpidem (Ambien®) is a non-benzodiazepine prescription drug that is used to treat insomnia and is often preferred over the commonly used benzodiazepines due to a lesser side effect profile. This is because the non-benzodiazepine binding is more selective to GABA-A receptors versus the non-selective binding of benzodiazepines. With the increasing popularity of non-benzodiazepines, drug abuse and driving-while-impaired cases involving sleep-inducing drugs have risen. Therefore, a highly sensitive and rapid homogeneous immunoassay (EMIT-type assay) has been developed for the detection of zolpidem in urine. The zolpidem antibody is highly specific and does not cross-react with other newer sleep aids such as zopiclone and zaleplon. This assay has a detection limit of 5 ng/mL for zolpidem in urine. Further evaluation of this assay using liquid chromatography–tandem mass spectrometry (LC–MS–MS) analysis of authentic urine samples demonstrated that the accuracy of the assay is greater than 90%. Because this assay is designed to measure the non-conjugated drug in urine, it resulted in simplification for gas chromatography–MS or LC–MS–MS confirmation methods that do not require urine hydrolysis before solid-phase extraction or liquid–liquid extraction.

Introduction

According to the U.S. Food and Drug Administration, about 40 million people in the United States suffer from some form of sleep disorder every year (1), of which insomnia is the most common in adults (2,3). Insomnia is characterized as trouble falling or staying asleep and early morning waking. Lack of sleep causes functional impairment while awake or other physiological effects such as blurred vision and muscular fatigue (3).

Zolpidem (Ambien) is a non-benzodiazepine sedative-hypnotic drug that is used as a short-term treatment for insomnia and was approved for use in the United States in 1993. Since the first launch of the product, more than 12 billion doses have been prescribed worldwide (2). This treatment is ideal due to its rapid absorption, short elimination half-life (T½ = 1.5–2.4 h), and its selectivity towards the sedative effect versus the muscle relaxant and anti-seizure effects. The short half-life ensures next-day carryover effect is not apparent when used as prescribed. Moreover, in healthy adults, it does not appear to affect next-day psychomotor and physical performance, which may further help increase its appeal as a sleep aid (4). In addition, zolpidem is sometimes preferred over the conventional, commonly used benzodiazepines due to a lesser side effect profile (4–6). This is due to more selective binding to GABA-A (gamma-amminobutyric acid-A) benzodiazepine receptor versus the non-selective binding of benzodiazepines. Despite the fact that zolpidem may reduce some side effects and have a shorter elimination half-life, it appears to cause both psychological and physical dependence (7,8). Several studies have concluded that zolpidem withdrawal symptoms are similar to those of benzodiazepines, such as tremor, agitation, anxiety, and seizure (2,8,9). From 2001 to 2006, the prescription rate of zolpidem in the United States increased from 18 million to 28 million (6). This increase in popularity has resulted in many cases of abuse or misuse of the drug while operating a motor vehicle, specifically in individuals taking more than its recommended dosage (10 mg) or not allowing at least an 8 h window after taking the drug before driving (10–12). The usual adult dose of zolpidem tartrate is 10 mg in conventional tablets or 12.5 mg in extended-release tablets; however, in geriatric or debilitated patients, an initial dose of 5 mg may be used. Low-dose sublingual zolpidem tartrate (3.5 mg) has also been reported as potentially suitable for the treatment of patients who have difficulty resuming sleep after awakening in the middle of the night (13). Because zolpidem is increasingly encountered in forensic specimens, a highly sensitive and rapid homogeneous immunoassay (EMIT-type assay) was developed for the detection of zolpidem in urine. The enzyme-multiplied immunoassay technique (EMIT) was developed in 1972 for the rapid detection of drugs or drug metabolites (analytes) in body fluids using specific antibodies raised against the target drug or analyte (14,15).
The homogenous EMIT-type assay (HEIA) technique is based on an immunoassay format in which both the antibody (RA) and enzyme-drug conjugate (RE) are in ready-to-use solutions. The assays are performed by simply mixing the sample with reagents RA and RE, and no separation step is required. Measurement of the resulting enzyme activity correlates with the concentration of the analyte. The measurement of the enzyme activity is based on the kinetic mode of detection, which minimizes endogenous interference and reduces the background. In the absence of the target analyte in the specimen, enzyme-labeled drug conjugate binds to the antibody resulting in a decrease of enzyme (G6PDH) activity; hence, lower absorbance at 340 nm is observed (15). If the target analyte is present in the specimen, it competes with the enzyme-labeled drug to bind to the antibody, resulting in more enzyme activity and yielding an increase in absorbance at 340 nm. As a result, the target drug concentration in the specimen can be correlated to enzyme activity that is measured spectrophotometrically at 340 nm. This HEIA is highly sensitive and has a low detection limit of 5 ng/mL for zolpidem in urine. Further characterization of the zolpidem antibody showed it to be highly specific with no cross-reactivity to other common sleep-aids such as zopiclone and zaleplon.

Methods and Materials

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) reagents and supplies

Deuterated internal standard, zolpidem-d₆, and unlabeled zolpidem were purchased from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare (San Pedro, CA). All solvents were of HPLC-grade or better; all reagents were ACS-grade and purchased from Spectrum Chemical (Gardena, CA).

Immunoassay apparatus and reagents

The Olympus AU400e automatic chemical analyzer was used in the development of this assay and was provided by Immunalysis (Pomona, CA). Zolpidem, zaleplon, and zopiclone (1 mg/mL in methanol) drug standards were purchased from Cerilliant. Enzyme reagents and antibody/substrate reagents, zolpidem polyclonal antibody, and haptens were provided by Immunalysis. Glucose-6-phosphate dehydrogenase (G6PDH) was purchased from Oriental Yeast (Tokyo, Japan). Drug mixes for cross reactivity with unrelated drugs were all purchased from Alltech-Applied Science (State College, PA).

Preparation of antibody/substrate and enzyme conjugate reagents

RA reagent (substrate/antibody) was prepared from in-house raised polyclonal antibody diluted with 20 mM Tris-HCl buffer (pH 6.0) containing 0.05 M nicotinamide adenine dinucleotide (NAD) and 0.5% sodium chloride. RE reagent (zolpidem-enzyme) was prepared from in-house zolpidem-glucose-6-phosphate dehydrogenase (G6PDH) conjugate diluted with 0.1 M Tris-HCl buffer (pH 7.8) containing 0.2% bovine serum albumin (BSA) and 0.5 M glucose-6-phosphate (G6P).

HEIA protocol

The HEIA was performed on the Olympus AU400e automated chemical analyzer with a sample size of 10 µL, antibody reagent (100 µL RA), and enzyme reagent (70 µL RE). In the automated chemical analyzer, the sample and RA reagent were mixed and incubated for 5 min before the addition of the RE reagent. The absorbance of the enzyme activity was then measured at a wavelength of 340 nm.

Assay characterization

The detection limit (sensitivity) of zolpidem in urine for the HEIA was based on the minimum zolpidem concentration required to produce absorbance readings equivalent to the mean plus two standard deviations from the zero calibrator (n = 15). In semiquantitative mode, the tested concentrations were 5, 10, and 25 ng/mL and were analyzed using the Olympus AU400e automated chemical analyzer. The interday precision was determined by testing in replicates of 15. The interday precision was established over four days (n = 60). The accuracy was assessed by comparing a calibration curve prepared in synthetic urine (as is used in commercial calibration standards) with calibration curves prepared in authentic urine from zolpidem-free volunteers (n = 3). Both calibration sets were fortified at concentrations of 5, 10, 15, 20, and 25 ng/mL.

The cross-reactivity of the assay to related and non-related drugs was determined by introducing high concentrations into the system (10,000 ng/mL).

Finally, a study was performed to determine whether the enzyme conjugate performance was stable at 25°C. A 50-mL bottle of zolpidem G6PDH-conjugate reagent (RE) was stored at 25°C and unexposed to light for 35 days. The G6PDH-conjugate (RE) was tested at days 1, 3, 6, 18, and 35 to determine if 25°C storage affected the assay performance. A zolpidem G6PDH-conjugate reagent (RE) stored at 5°C (control) was tested along with the 25°C enzyme conjugate as a control for the assay performance.

Urine specimens

Drug-free urine samples were collected from non-zolpidem using individuals. A time-dependent dosage study was carried out with volunteers, one naïve user and one frequent user, holding a valid prescription for the drug. All volunteers were adults and gave informed consent for specimen collection. Each volunteer ingested a single 10-mg dose of zolpidem, and urine specimens were collected over a period of 16 h.

The urine specimens were also analyzed by LC–MS–MS. Briefly, calibration standards and controls were prepared from drug-free urine fortified with zolpidem at concentrations of 1, 5, 10, 20, 50, and 200 ng/mL. For each batch, a six-point calibration curve was run; the internal standard concentration was 100 ng/mL.

Extraction procedure. One milliliter of 0.1 M acetic acid (pH 4.0) was added to each urine specimen (0.1 mL). The columns were conditioned with methanol (2 mL) and 0.1 M acetic acid (pH 4; 2 mL). The specimen was added and allowed to run through the column. The solid phase was washed with deionized water (2 mL), 0.1 M HCl (2 mL), methanol (1 mL), and ethyl acetate (1 mL). After drying the columns for 5–7 min,
the drugs were eluted with 2 mL ethyl acetate/ammonium hydroxide (98:2, v/v). The extract was evaporated to dryness and reconstituted in methanol (50 µL) for injection into the LC–MS–MS.

Analytical procedure. An Agilent Technologies 6410 LC coupled to a triple-quadrupole MS–MS operating in positive electrospray mode (ESI) was used for the analysis (Santa Clara, CA). The LC pump was an Agilent 1200 series and the column, also supplied by Agilent Technologies, was a Zorbax Eclipse XDB C$_{18}$ (4.6 mm x 50 mm x 1.8 µm). The column temperature was held at 40°C. The initial flow rate was 0.7 mL/min, and the injection volume was 5 µL. Solvent A consisted of 20 mM ammonium formate (pH 6.4); solvent B was acetonitrile. The mobile phase composition started at 85:15 (v/v) changing to 100% B after 4 min. At 5 min, the ratio returned to 85:15 (v/v). The MS operated at a capillary voltage of 4000 V; an initial nebulizer pressure of 35 psi; nitrogen gas flow of 10 L/min; and a gas temperature of 350°C. The MS was operated in multiple reaction monitoring (MRM) mode. The precursor and product ions for zolpidem-d$_6$ were 313.9 to 235.1 with a fragment voltage of 100 V and collision energy of 30 V. For unlabeled zolpidem, two transitions were monitored at 308.3 to 263.3 and 308.3 to 235.2 with fragment voltages optimized at 100 V and collision energies of 20 V. In order for specimens to be positive, the ratio of the transitions was required to be within 20% of that produced by the calibration standard at 10 ng/mL. The upper limit of linearity was 200 ng/mL; samples exceeding the limit were diluted into the linear range. The limit of quantitation (LOQ) was established by entering a signal-to-noise ratio (SNR) of 10 into the method analysis for the quantitative transition. If the response of the primary transition ion did not exceed an SNR of 10, it was not considered to be quantitative. The LOQ was 1 ng/mL, and the intra- and interday precisions at 7 ng/mL were 7.7% ($n = 6$) and 8.4% ($n = 5$), respectively. Inter- and intraday precision sat 30 ng/mL were 6% ($n = 6$) and 4.1% ($n = 5$), respectively. The zolpidem recovery from urine was 83%, and no ion suppression or enhancement due to matrix effect was observed.

Results and Discussion

Immunooassay

Evaluation of enzyme activity. A zolpidem calibration curve was generated using fortified zolpidem drug standard at calibration points of 5, 10, 25, and 50 ng/mL. Zolpidem drug concentration in the specimen was measured in terms of enzyme activity ($\Delta$mAU/min) in the assay. As shown on the dose response curve in Figure 1, with an increase in drug concentration in the specimen there was an increase in mAU/min or enzyme activity.

Sensitivity. The LOD for zolpidem in the HEIA format was determined to be 5 ng/mL, and the cut-off concentration was set to 10 ng/mL. More importantly, it was demonstrated that both qualitative and semiquantitative assays for zolpidem in urine could be performed.

Precision. The results for the precision study are shown in Table I. The precision of the assay at three concentrations was less than 20% coefficient of variation (CV) for both intraday ($n = 15$) and interday (four days, $n = 60$).

Accuracy. Zolpidem-fortified synthetic urine samples were tested against a zolpidem standard calibration curve prepared in pooled authentic drug-free urine. Both were fortified at concentrations of 0, 5, 10, 15, 20, and 25 ng/mL. A percent drug recovery calculation was made for each known drug concentration, and zolpidem drug recovery in the semiquantitative range was within 80% (Table II).

Cross-reactivity with other sleep aids

The non-benzodiazepine sleep aids, zaleplon and zopiclone at 10,000 ng/mL drug concentration, showed no cross-reactivity towards the zolpidem antibody.

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Interference from unrelated drugs

Commonly used drugs that are structurally unrelated to zolpidem were assayed to determine if these unrelated drugs cross-react or interfere with the assay. Aliquots of unrelated drugs were fortified in synthetic urine at a concentration of 10,000 ng/mL. None of these compounds showed interference with the assay: alprazolam, amitriptyline, d-amphetamine, amobarbital, bromazepam, butalbital, caffeine, carbamazepine, chlorpromazine, clonazepam, cocaine, codeine, desipramine, diazepam, diphenhydramine, doxepin, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, ephedrine, ethylmorphine, ethosuximide, flunitrazepam, flurazepam, glutethimide, hexobarbital, hydrocodone, imipramine, ketamine, lidocaine, lorazepam, methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine, 3,4-methylenedioxy-N-methamphetamine, medazepam, meperidine, meprobamate, methadone, d-methamphetamine, methaqualone, metharbital, methocarbamol, 4-methoxyamphetamine, methylphenidate, 4-methylprimidone, methyprylon, morphine, nalorphine, nitrazepam, nordoxepin, nor-triptiline, normethsuximide, oxazepam, oxycodone, phenylethylmalonamide, pentazocine, phenylcyclohexene, phenobarbital, phenobsuffide, phentermine, phenylephrine, phenytoin, primidone, propoxyphene, protriptyline, secobarbital, temazepam, and trimipramine.

Stability

At the 10 ng/mL cutoff of the assay, there was no significant decrease in assay performance when enzyme conjugate was stored at 25°C for 35 days (Figure 2).

Authentic urine specimens

A time-dependent dosage study was performed using volunteers to determine the efficacy of the homogenous enzyme immunoassay. In addition, quantitation using LC–MS–MS confirmatory analysis was performed to determine accuracy of the HEIA. Prior to the ingestion of a 10-mg dose of zolpidem, the subjects provided initial pre-dose urine samples. Sample collections post-dose were taken hourly at 1, 4, 8, and 16 h. For the HEIA, all urine specimens were tested directly against the calibration curve, and no treatment or dilutions were needed for the authentic urine specimens. The enzyme HEIA assay was able to detect zolpidem in urine at least 16 h after ingestion of a 10-mg dose as shown in Table III. This is important because of individual misuse of prescription instructions and operating a motor vehicle within the 8 h window after the intake of zolpidem. In authentic specimens, the HEIA typically yielded a much higher concentration than LC–MS–MS, which may be ascribed to the high cross-reactivity of the HEIA assay toward zolpidem and the main carboxylic metabolites of zolpidem (16). Unfortunately, the metabolites were not available as drug standards, so they could not be tested for cross-reactivity in the assay. Overall, a total of 41 urine specimens were analyzed by both HEIA with cutoff set at 10 ng/mL and LC–MS–MS with cutoff set at 1 ng/mL. The HEIA displayed 100% specificity, 86% sensitivity, and 90% accuracy (Table IV).

Conclusions

This paper describes the development of a highly sensitive and rapid method of homogenous enzyme immunoassay (EMIT-type assay) for the detection of zolpidem in urine. The assay has a detection limit of 5 ng/mL and is a highly specific screening procedure for zolpidem in urine. With the increase usage of this prescription medication, a rapid and highly sensitive method is necessary to help with the investigation of forensic cases.

References


