Zolpidem Urine Excretion Profiles and Cross-Reactivity with ELISA® Kits in Subjects Using Zolpidem or Ambien® CR as a Prescription Sleep Aid

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Abstract

Zolpidem, a Schedule IV controlled substance under the Federal Controlled Substance Act, has a rapid onset of action and short elimination half-life, rendering it ideal as a sleep aid. The cross-reactivity of two zolpidem ELISA kits was investigated using patients taking a known administration of zolpidem. Subjects provided urine samples before, 30 min after their prescribed dose, and upon waking. Specimens were screened for zolpidem by ELISA (Immunalysis and Neogen) and then confirmed and quantitated for zolpidem using gas chromatography–mass spectrometry (GC–MS) confirmation in select ion monitoring mode. All samples were measured for creatinine and corrected accordingly. The ELISA screening results demonstrated that all samples, except one, screened positive by ELISA using both kits, even when the GC–MS data found no zolpidem in the patient's urine sample. The maximum concentrations of zolpidem ranged from 15 to 120 ng/mg creatinine. Two of the patients showed zolpidem concentrations of 10 ng/mg creatinine or above after 20 h post dose. The high variability and concentration range seen in these patients, all on similar doses, suggest wide variability in the metabolism of zolpidem.

Introduction

It is reported that approximately 35% of the population suffers from insomnia at some point in their lives with the majority of individuals using prescription medication to promote and/or sustain sleep (1). Zolpidem is an effective non-benzodiazepine sedative hypnotic for the short-term treatment of insomnia. Zolpidem, an imidazopyridine compound that acts as an agonist at the benzodiazepine binding site of GABA receptor (2), has a rapid onset of action and a short duration of action and elimination half-life, making it useful as a sleep aid. Zolpidem is currently a Schedule IV controlled substance under the Federal Controlled Substance Act. It is available in several preparations: zolpidem (5- and 10-mg tablets, 5 mg/spray oral spray), Ambien (5- and 10-mg tablets), and Ambien CR (6.25- and 12.5-mg tablets). Ambien CR is a two-layer zolpidem tablet preparation with a biphasic release profile. It is indicated for the treatment of insomnia characterized by difficulties with sleep onset and/or sleep maintenance. About 60% of the drug is released immediately, with the remaining 40% released over a longer duration (3).

Zolpidem has reported adverse side effects including residual somnolence (sleepiness), sleepwalking, and amnesia (4,5), so it is reasonable to assume that zolpidem, when not taken as directed, may have a role in drug-impaired driving investigations or could be used as a drug of choice in drug-facilitated sexual assaults. Zolpidem has also been shown to impair coordination, reactive, and cognitive skills and reduce motor skills (6,7). Several studies have documented zolpidem as causing residual sedative effects on an individual for up to 7 h post dose (8–10), which is consistent with the zolpidem pharmacokinetic profile. A study in which subjects were dosed (10 mg) in the middle of the night demonstrated that individuals showed significant impairment when simulator driving, comparable to a blood-alcohol concentration (BAC) of 0.08 g/dL when the time interval was around 7 h from dosing to driving (8). It has been shown that zolpidem has an additive impairing effect on driving when used in combination with alcohol and other central nervous system depressants (9–11). Zolpidem, when taken alone or in combination with other drugs, is capable of causing or contributing to drug-impaired driving (DUI) or could be used as a drug in sexual assaults. Consequently, it is important to include zolpidem into the menu of drugs screened for in a forensic toxicology environment.

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Detection of the parent zolpidem is particularly difficult because of its extensive oxidative metabolism via a variety of cytochrome P450 enzymes that results in at least six metabolites excreted in the urine (12). Literature suggests the involvement of CYP: 3A4, 2D6, 1A2, 2C19, and 2C9 (13,14), with zolpidem undergoing oxidation of the methyl group on the phenyl ring or on the imidazopyridine moiety to produce carboxylic acid (metabolites I and II) (Figure 1). The phenyl carboxylic acid metabolite (I) is the main urinary metabolite at ~50% of the dose.

Several reviews and methods have been published for the identification and quantitation of the parent zolpidem in biological matrices (15,16); however, there is limited information on available screening techniques for zolpidem. When analyzing samples for drugs, screening is used routinely in areas of clinical chemistry and toxicology such as hospital clinics, the criminal justice system, and workplace drug testing. In these situations, samples are subjected to screening by rapid, inexpensive immunoassays with the criminal justice system requiring confirmation by an alternative method. One of the aims of this study was to analyze and compare the specificity and sensitivity of two different manufacturers of ELISA kits for the screening of zolpidem in urine samples.

As there is limited information on the urine excretion profile of zolpidem in patients using zolpidem as a long-term sleep aid, this study also seeks to identify and measure urine concentrations of zolpidem by gas chromatography–mass spectrometry (GC–MS) over time in subjects using zolpidem and analyze urine excretion profiles of these individuals. A urinary creatinine concentration was measured, and data are presented in nanograms-per-milliliter and nanograms-per-milligram creatinine.

Materials

Apparatus

A Dynex DS2 instrument was used for ELISA analysis of zolpidem and creatinine measurement in the urine samples, and data were analyzed with DSX/DS2 reporting software (ver: 10/21/2009, Lexington, KY).

GC–MS analysis was performed on an Agilent 5890 series II GC interfaced to a 5972 mass selective detector operated in electron ionization mode. The electron multiplier was set 200 volts relative to the autotune value. The GC was fitted with a Restek RXi-5MS® column (30 m × 0.32-mm i.d. × 0.25 µm df) operated in splitless mode (bottom gooseneck liner with no glass wool) with helium as the carrier gas at 1 mL/min and an injection volume of 2 µL. The injector and detector temperatures were 270°C and 300°C, respectively. The initial oven temperature was held at 70°C for 2 min, then ramped at 13°C/min to 230°C with no hold time, and then ramped at 12°C/min to 300°C with a final hold of 5 min. The MS was operated in selective ion mode using ions *m/z* 307, 219, and 235 (zolpidem) and 326, 243, and 256 (clozapine). Data were acquired and analyzed with Agilent Chemstation software version G1701AA (Santa Clara, CA).

Reagents and supplies

All reagents were purchased from J.T. Baker (Phillipsburg, NJ) and were reagent grade. Solvents, except for acetonitrile (Pierce Chemical, Rockford, IL), were purchased from Fisher (Thermo Fisher Scientific, Waltham, MA) and were high-performance liquid chromatography (HPLC) grade or equivalent.

Zolpidem ELISA kits were purchased from Immunalysis (Pomona, CA) and Neogen (Lexington, KY). The kits included 96-well plates, phosphate buffer, zolpidem conjugate, positive reference standard, synthetic negative urine, zolpidem TMB substrate, and an acid stop solution.

Creatinine colorimetric microplate assay kits were purchased from Neogen and included a 96-well plate, calibrators, and alkali and acidic reagents.

Standards and controls

Individual certified stock solutions of zolpidem and clozapine (at 1 mg/mL in methanol) were purchased from Cerilliant (Round Rock, TX). A working internal standard of clozapine at a concentration of 10 ng/µL was prepared in methanol by the dilution of stock 1 mg/mL standard. A working standard of zolpidem at a concentration of 10 ng/µL was prepared in methanol by the dilution of stock 1 mg/mL standard. All standard solutions were stored at –20°C when not in use.

Negative urine collected in house a non-drug-taking volunteer was certified as negative for zolpidem and other alkaline drugs by GC–MS and by an ELISA [Amphetamines, Metham-

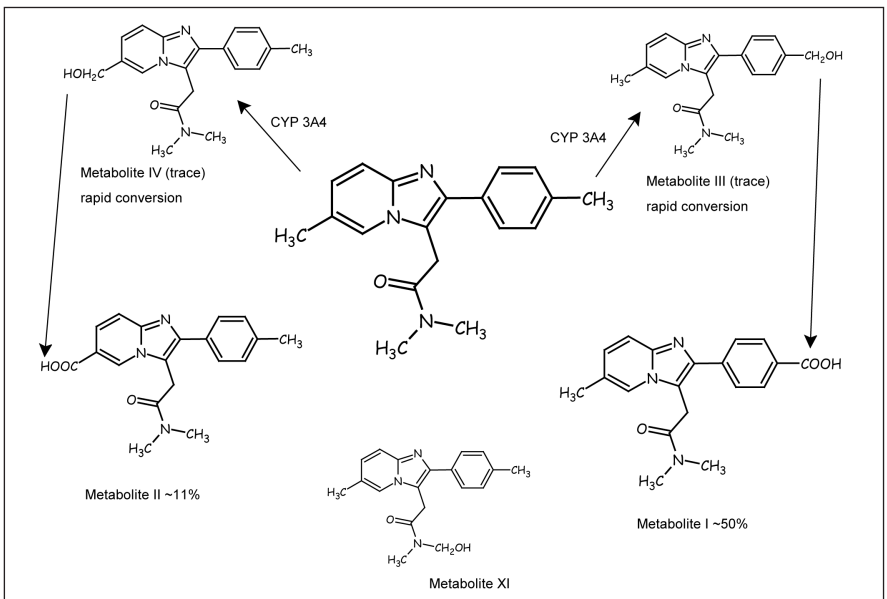

Figure 1. Metabolites of zolpidem identified in human urine samples. The values denote the percentage of administered zolpidem. [Adapted from Von Moltke et al. (13) and Vajta et al. (12).]

phetamine, Benzodiazepines, Opiates, Cannabis, Oxycodone, and Zolpidem (Neogen)] was used in this study.

Study Design

This study was carried out on subjects attending the sleep clinic at the University of Miami Sleep Center. The study protocol, informed consent forms, and all procedures were approved by the University of Miami Institutional Review Board (IRB: 20080607).

Subject selection

Men and women, between 18 and 64 years of age, who were treated at the clinic for sleep-related disorders and taking zolpidem as prescribed by their physician, were asked to participate in the study. The subjects entering the sleep center were asked to fill out a questionnaire and state the time they took their most recent dose of zolpidem. The questionnaire included demographic questions, other medications, and length of use.

Samples

Urine samples were obtained from the subjects enrolled in the study before taking their prescribed dose, after ~30 min of taking the dose or before retiring for the evening, and the following morning upon awakening. If the subject awoke during the night, they were asked to provide an additional urine sample if possible. All urine samples were collected into individual non-preserved urine sample cups and were stored at –4°C. Each urine sample was date and time stamped and analyzed independently.

In the morning, upon waking, subjects were asked to fill in the second questionnaire. Subjects were to document if they awoke in the night, and to note any side effects from taking the zolpidem (sleep eating, anxiety, amnesia, and morning drowsiness) and to describe their state of wakefulness on a scale of 1 (wide awake) to 10 (sleepy).

Methods

Zolpidem analysis by ELISA

The ELISA methods were carried out in accordance with the manufacturers' package inserts (17,18). All steps were carried out using the automated DS2 instrument. Briefly, urine specimens were prepared by diluting 1:10 with kit-supplied phosphate buffer (pH 7) on the day of analyses. Calibrators and controls were treated identically to specimens. To each appropriate well, 25 µL of diluted urine, calibrator, or control was added. All calibrators and controls were performed in duplicate with the placement of a negative and positive control every 10 samples. Next, 100 µL of enzyme conjugate was added and then incubated for 60 min. After incubation, the plate was washed six times with deionized water. A chromomeric substance (Zolpidem TMB) was added, and the plate then incubated for an additional 30 min in the dark (due to Zolpidem TMB reagent sensitivity to light). The reaction was stopped with a dilute acid, and absorbance measured at 450 nm. The yellow color intensity produced from the drug binding was inversely proportional to the amount of zolpidem in the specimen. Data reduction was accomplished using the supplied DSX/DS2 reporting software (ver: 10/21/2009).

A cutoff value of 25 ng/mL of zolpidem was used with a positive control set at three times the cutoff (75 ng/mL), and a low control at 12.5 ng/mL. The absorbance of the specimen well was compared to the mean absorbance of the cutoff wells, and if the specimen absorbance was equal to or lower than the cutoff absorbance, the sample was reported as presumptive positive. If the specimen absorbance was higher than the cutoff well, then the sample was reported as negative.

Accuracy, precision, and cross-reactivity data were provided by Immunalysis and Neogen (17,18). Additional intra-assay (intraplate) precision was determined using a fortified zolpidem urine control at 40 ng/mL, one positive and one negative specimen (determined by GC–MS) all analyzed five times in the same assay.

Immunalysis and Neogen provided information on cross-reactivity studies using a wide variety of drugs (17,18). The Neogen ELISA kits state cross-reactivity with zolpidem metabolite I as 0.02%. However, Immunalysis does not include any cross-reactivity data for any of zolpidem's metabolites. The cross-reactivity of Immunalysis zolpidem assay was assessed previously by Reidy et al. (19), and the kit demonstrated no cross-reactivity with zolpidem metabolite I at 1000 ng/mL.

Creatinine colorimetric assay

This assay is based on a modified Jaffe reaction wherein a yellow/orange color forms when the urinary creatinine is treated with picric acid under alkaline conditions. The color derived is then destroyed in acidic conditions. The difference in color intensity, before and after the addition of acid, is the direct estimate of creatinine concentration. The sample creatinine concentration is determined using a standard curve. A calibration curve of 0, 1, 3, and 10 mg/dL was included in each batch by the addition of 0.025 mL of each calibrator pipetted in duplicate into a 96-well plate. The subjects' samples were diluted 1:25 with deionized water according to manufacturer guidelines, and then 0.025 mL of the diluted sample was added in duplicate to the 96-well plate. To initiate the reaction, 0.18 mL of the alkaline picrate mixture was added and the plate shaken gently to mix. The plate was then incubated for 10 min at room temperature. After 10 min, the first reading is taken at 490 nm. Next, 0.015 mL of the acid reagent was added, and the plate was gently shaken to mix and incubated for 5 min at room temperature. The plate is read a second time at 490 nm, the second absorbance is subtracted from the first reading, and a calibration curve produced. Subsequent determination of the unknowns was calculated from the standard curve.

GC–MS analysis

GC–MS screening for zolpidem in urine was conducted with an alkaline liquid–liquid extraction with modifications to the

published procedure by Foerster et al. (20). Briefly, urine aliquots (2 mL) were dispensed into 17-mL silanized screw-cap tubes in duplicate. The internal standard (clozapine) was added to each tube of urine to give a final concentration of 50 ng/mL. To each tube, 9 mL of *n*-butyl chloride/methylene chloride (9:1) was added and briefly vortex mixed. One milliliter of concentrated ammonium hydroxide (33%) was added to each tube, and the tubes were sealed with Teflon-lined caps. The samples were then extracted on a rotor rack for 15 min. Tubes were centrifuged at 3800 rpm for 5 min at –10°C to produce separation and the organic layer transferred to a silanized conical tube and evaporated to just dryness in a TurboVap® at 50°C under a stream of nitrogen. The dry urine extract was reconstituted with 50 µL iso-octane/methylene chloride/ethanol (7:2:1), transferred to limited volume autosampler vials, and analyzed via GC–MS in selective ion mode (SIM). Ions selected were *m/z* 235, 307, and 219 (Figure 2). A calibration curve was prepared by fortifying negative urine using the following calibration curve 5, 10, 25, 50, 100, and 300 ng/mL. Positive and negative controls were prepared in-house, and possible matrix and IS interferences were investigated by running blank and negative urine controls.

The validation of this method was previously reported by Reidy et al. (18) with a limit of detection (LOD) of 5 ng/mL and limit of quantification (LOQ) of 10 ng/mL in SIM.

Results

Demographics

Eight subjects enrolled in the study (5 males and 3 females), each completed the questionnaires, and submitted urine samples for analysis. The demographics of the subjects and pertinent information are given in Table I. Three of the subjects only provided two urine samples. Five out of the eight subjects reported taking Ambien CR (12.5 mg) with three taking 10 mg of generic zolpidem.

Six out of the eight subjects are currently taking other medications including antidepressants (citalopram) and beta-blockers (propanolol, atenolol, etc.), with two subjects taking zolpidem preparations only. Various investigations into the residual side effects of zolpidem have been carried out (8–10) with most stating that the following morning there is little, or no, residual effect. These studies of residual effects on healthy subjects may not give an accurate representation of the effects after several days of treatment or compared to insomniac subjects.

Fifty percent of the subjects in this study reported either feeling drowsy or having amnesia the following morning as a

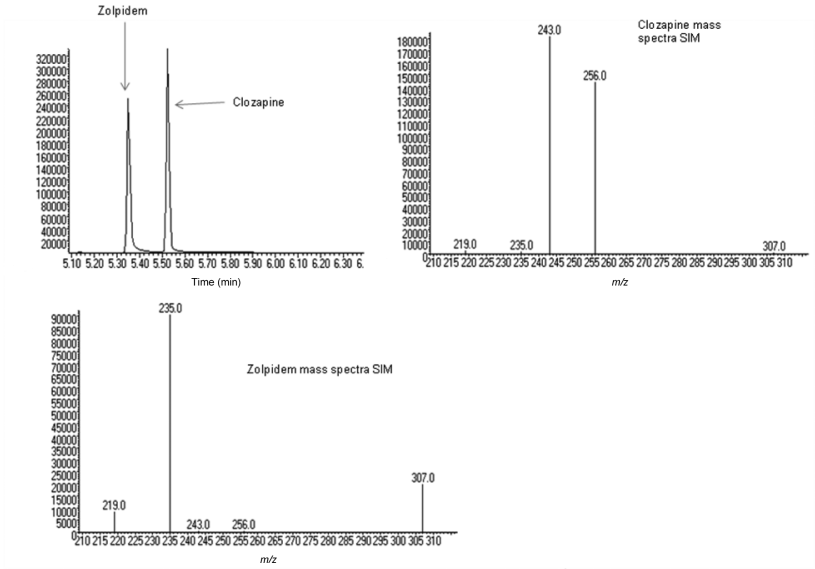

Figure 2. Gas chromatogram of zolpidem and IS (clozapine) and the mass spectra data used for SIM analysis. Ions 235, 219, and 307 (zolpidem) and 243 and 256 (clozapine). Data taken from extracted urine sample fortified with 50 ng/mL of each compound.

Table I. Subject Information

Subject No.	Sex	Age	Preparation	Dose (mg)	Length of Time Taking Dose	Side Effects
1	male	> 55	Zolpidem	10	1–2 years	Feeling tired in the morning
2	male	> 55	Ambien CR	12.5	> 1 year	None reported
3	male	> 55	Ambien CR	12.5	1–2 years	None reported
4	male	> 55	Ambien CR	12.5	2–5 years	None reported
5	female	45–55	Ambien CR	12.5	2–5 years	Amnesia, morning drowsiness
6	male	45–55	Zolpidem	10	> 1 year	None reported
7	female	35–45	Ambien CR	12.5	2–5 years	Morning drowsiness
8	female	45–55	Zolpidem	10	2–5 years	Sleep eating, amnesia

side effect of zolpidem use. One patient reported events of sleep eating while taking zolpidem, although this was not observed during the study. The effects on psychomotor and cognitive tests 8–9 h after ingestion are similar for both Ambien CR and zolpidem formulations (21). The results of this study corroborate this statement by showing no difference between reported residual effects of subjects using zolpidem or Ambien CR.

ELISA

One of the aims of this present study was to evaluate the cross-reactivity of zolpidem ELISA kits and urine excretion profiles of zolpidem in subjects using zolpidem for the long-term treatment of insomnia.

The performances of the ELISA kits were investigated by testing all 22 urine specimens in duplicate. Samples were first analyzed by ELISA assay and measured for creatinine (to correct for urine volume) with the results summarized in Table II.

The cross-reactivity of both ELISA kits to zolpidem provided some interesting results (Table II). Of the 22 urine samples tested for zolpidem, 21 tested positive for zolpidem after the ELISA screening test. Of these, 18 urine samples were confirmed positive by GC–MS, resulting in three urine samples unconfirmed (Table II). Four of the 18 samples were below the LOQ (10 ng/mL) but above the LOD (5 ng/mL) and therefore not reported as "false positive". There were no discrepancies between the two kits when comparing all samples, indicating that both cross-react either with the parent or with the metabolites equally. The specificity and sensitivity of the assay were calculated by applying Eq. 1, which requires the total number of true positive (TP), true negatives (TN), false positives (FP), and false negatives (FN) to be calculated (22).

$$\text{Sensitivity} = \frac{TP \times 100}{TP + FN} = 100\% \qquad \text{Eq. 1}$$

$$\text{Specificity} = \frac{TN \times 100}{TN + FP} = 25\%$$

Using Eq 1. the following criteria are used: a true positive produces both positive screening and confirmation results, true

Table II. Summary of Concentrations of Zolpidem (ng/mL urine and ng/mg creatinine) Found in Subjects Using a Zolpidem Preparation as a Sleep Aid*

Subject No.	Sample	Immunalysis Estimated Result (ng/mL)	Neogen Estimated Result (ng/mL)	Zolpidem Concentration (ng/mL urine)	Creatinine Concentration (mg/mL)	Zolpidem Concentration (ng/mg creatinine)	Time Since Dose (min)
1	A	> 25	> 25	ND	1.50	N/A	20
	B	> 75	> 75	19	0.55	34	310
	C	> 25	> 25	< 10 ng/mL	0.55	N/A	1280
2	A	> 75	> 75	66	1.24	53	120
	B	> 75	> 75	66	1.65	40	320
	C	> 75	> 75	41	2.56	16	1220
3	A	> 25	> 25	11	1.83	6	193
	B	> 75	> 75	15	0.93	16	279
	C	> 25	> 25	ND	0.96	N/A	1455
4	A	> 25	> 25	71	0.87	81	70
	B	> 75	> 75	79	0.60	130	170
	C	> 75	> 75	59	0.65	90	240
	D	> 75	> 75	< 10 ng/mL	1.34	N/A	1410
5	A	> 25	> 25	78	2.29	34	488
	B	> 75	> 75	< 10 ng/mL	1.12	N/A	1440
6	A	> 75	> 75	58	0.89	65	135
	B	> 75	> 75	18	1.63	11	300
	C	> 75	> 75	< 10 ng/mL	0.81	N/A	690
7	A	> 75	> 75	78	0.42	183	465
	B	> 25	> 25	ND	0.68	N/A	1440
8	A	> 75	> 75	77	1.97	39	510
	B	Negative	Negative	ND	1.40	N/A	1430

* ELISA results estimated based on the cutoff and positive controls absorbance.
† Abbreviations: ND, not detected; N/A, not analyzed.

negative sample produces both negative screening and confirmation results, a false positive produces a positive screening and negative confirmation result, and a false negative produces a negative screening and positive confirmation result (22). Using these criteria, both Neogen and Immunalysis zolpidem ELISA kits produced 100% sensitivity and 25% specificity for the parent zolpidem. No false-negative results were obtained with the ELISA kits using the cutoff value of 25 ng/mL, thus leading to 100% sensitivity for these kits (Table III). The 25% specificity of these ELISA tests can be explained by the high number of apparent "false positives" that could not be confirmed by GC–MS. Three of the samples produced ELISA results equivalent to, or above, the 25 ng/mL cutoff, yet when subsequently analyzed by GC–MS, were negative for zolpidem. All three urine samples from Subject 1 (taking zolpidem 10 mg) tested positive for zolpidem in concentrations greater than 25 ng/mL; however, these results were not confirmed by the GC–MS data (Table II). In sample 1A, no zolpidem was detected, and 1C results were positive but below the LOQ using GC–MS confirmation. Samples 3C and 7B also tested positive for zolpidem using ELISA, yet no zolpidem was detected in the confirmatory analysis. This leads to the tentative hypothesis that the ELISA kits may be cross-reactive with another metabolite of zolpidem (Figure 1) found in urine and not just the parent alone.

Table III. Comparison of Results for ELISA Zolpidem Screening Test and GC–MS Confirmation for 22 Urine Samples*

	GC–MS	
	Positive	**Negative**
ELISA		
Positive	18 (81%)	3 (13%)
Negative	0	1 (4%)

* The Immunalysis and Neogen ELISA kits produced the same results. ELISA cutoff was 25 ng/mL, and the GC–MS LOD was 5 ng/mL.

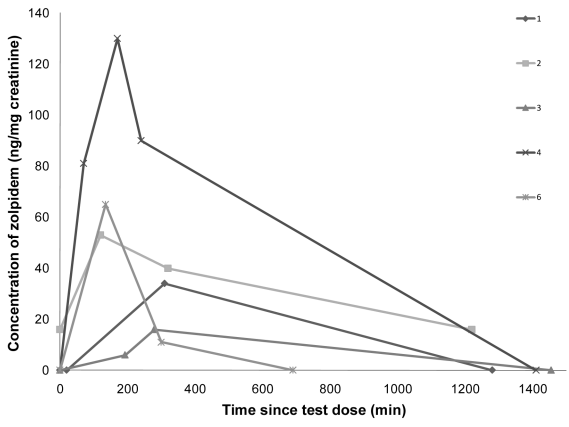

Figure 3. Urinary concentrations of patients 1–5 expressed as urine concentation per milligram of creatinine. Dose and preparation used by each subject is as follows: Subject 1, 10 mg zolpidem; Subjects 2–4, 12.5 mg Ambien CR; and Subject 6, 10 mg zolpidem.

GC–MS

A typical gas chromatogram of zolpidem obtained from extracted subject's samples is shown in Figure 2. The peaks detected were identified as zolpidem and clozapine using both retention time and GC–MS mass spectra. For quantitation, the MSD was operated in SIM mode using the ions *m/z* 307, 219, and 235 (zolpidem) and 326, 243, and 256 (clozapine). Zolpidem-d_6 was found to be unsuitable as an internal standard (IS) for GC–MS analysis because of the lack of unique fragmentation ions distinct from the non-deuterated zolpidem. Consequently, clozapine was chosen as an IS.

Linearity was observed from 10 to 300 ng/mL with an r^2 value of 0.990; all samples with concentrations above the curve were adequately diluted with certified negative urine to fall within this range. Ten matrix urine blanks and negatives were analyzed, and no interferences were noted from the biological matrix tested or from the IS. All the quality control samples gave quantitative results within ±20% of target values.

The urine excretion profiles of zolpidem depict intersubject variability in the concentration of zolpidem over time (Figure 3 and Table II). Literature suggests that zolpidem is excreted primarily as metabolites, and little, if any, is excreted as the parent drug at therapeutic doses (23–25). Urine concentrations in this study ranged from 6 to 183 ng/mg (11 to 79 ng/mL of urine). Subject 4 produced a maximum urinary concentration of 130 ng/mg (79 ng/mL) after taking a single 12.5-mg dose of Ambien CR at 170 min post dose (~3 h) but returned to less than 10 ng/mL (LOQ) in under 24 h . With the pharmacokinetic profile of Ambien CR, 60% of the dose is released immediately, accounting for the steep incline depicted in the graph for subjects 2 and 4. The trend for subject 4 shows a drop in urine concentration at 240 min (4 h) post dose with no plateau observed. However, this may be due to the lack of urine samples collected after this time until collection at 1410 min. Subject 7 yielded the highest level of zolpidem measured in the sample group (183 ng/mg), but with only two time points sampled for this subject, it is difficult to interpret the excretion profile.

Discussion

ELISA

A previous study (26) corroborated this study's findings. A homogeneous EMIT type assay (HEIA) for zolpidem using an $n = 2$ reported discrepancies between HEIA results and liquid chromatography (LC)–MS–MS data. The results of this study showed that HEIA semi-quantitative values were significantly higher than would be expected based on LC–MS–MS zolpidem quantitation values. It was hypothesized that this could be due to cross-reactivity with the main zolpidem urinary metabolite (26).

Excretion studies have concluded that between 0.2 and 1.3% is excreted as zolpidem and ~51%, ~11%, and 0.1–1% are

excreted as metabolites I, II, and IV, respectively (25,27). Therefore, it is reasonable to assume the urine samples will contain a large amount of zolpidem metabolite I. The Neogen package insert states that the assay is 0.02% cross-reactive with metabolite I. The main urinary metabolite accounts for about 32–43% of the dose (Figure 1). If the main metabolite is 0.02% cross-reactive then the urine samples should contain at least 125 µg/mL to produce an ELISA of > 25 ng/mL. This does not take into account other metabolites and their possible contribution to the ELISA result. There are no data available on the cross-reactivity of these metabolites, and because of the lack of commercially available standards of these metabolites, it was not possible to determine.

There are limited studies published on the analysis of zolpidem metabolite I. Two published studies, using one 10-mg dose in naïve users, provided discrepancies between urine concentrations of zolpidem metabolite I. Lewis et al. (23) reported peak urine concentration at 10 h post dose of 29 µg/mL with detection for up to 72 h post dose. Ascalone et al. (24) reported 3.6 µg/mL of zolpidem metabolite I at t = 8.5 h with detection for up to 30 h. These studies were carried out on healthy volunteers and hence baseline levels (pre-dose) of zolpidem metabolite I should be zero. The subjects in this present study had been using zolpidem preparations on a routine basis for at least six months. One possible tentative hypothesis is that regular users of zolpidem may accumulate zolpidem metabolite I in their urine. If the ELISA kits are cross-reactive with the metabolite it may explain the high level of unconfirmed positive ELISA results presented in this study. To confirm this hypothesis, quantitation of the metabolite in these subjects' urine would be necessary, but it is outside the scope of this study.

GC–MS

This investigation compared urine excretion profiles of zolpidem in subject's using zolpidem as a treatment for sleep disturbance. This study was subject to several limitations. Firstly, there was no control as to when urine samples were provided, as they were asked on a voluntary basis, and hence it was difficult to collect sufficient urine time points for interpretation in some subjects. Secondly, observed administration of the drug was carried out, but information regarding illicit and other drug use was voluntary, and no confirmation of other drug use was carried out because of IRB limitations.

The urine zolpidem concentrations measured in these subjects do not substantiate concentrations reported in recently published literature (23,28,29). Urine concentrations between 5 and 25 ng/mL have been reported in healthy volunteers (29), and a concentration of 4100 ng/mL was recorded in an overdose in which a subject ingested 60 tablets (30). There are no data available on urinary concentration when zolpidem is used frequently as a sleep aid.

The detection window for zolpidem in this study also differed from previously published data. Villain et al. (29) demonstrated that zolpidem could be detected for 60 h after dosing from a 10-mg dose in a naïve user. In this study, three out of the eight subjects showed no zolpidem in the urine sample collected at 24 h post dose, and four subjects measured zolpidem at less than 10 ng/mL at this time point (Table II). Subject 2 (12.5 mg of Ambien CR), however, showed concentrations of 16 ng/mg 24 h post dose.

The difference in the data presented here, compared to previously published urinary excretion profiles, is that the subjects used in this study are long-term users of zolpidem. Previously data has relied heavily on healthy, naïve volunteers and hence does not always reflect urinary excretion profiles in the general therapeutic population. One study (26) investigated the urinary excretion profiles of zolpidem comparing one naïve user and one chronic user. The quantitation results showed a concentration of 283 ng/mL (chronic user) compared to 9 ng/mL (naïve user) showing a wide variation at t = 1 h post dose. These samples were not corrected for creatinine, so variation in concentration of the urine is not investigated.

There may be several possible interpretations drawn from these study data. One hypothesis put forward is that changes may occur in the metabolism and excretion profiles of long-term users of zolpidem preparations. Several of these subjects are also taking other preparations such as beta-blockers, anti-platelet medications, and calcium channel blockers, none of which, however, has been identified as causing interactions with zolpidem effects or metabolism (3,31). Zolpidem is metabolized primarily by the P450 CYP3A4, and many of these drugs listed by the subjects in this study are metabolized by CYP3A4. The consumption of grapefruit juice and star fruits may inhibit the metabolism of zolpidem by 3A4 (31); however, dietary information was not collected from these subjects in this study.

Conclusions

To conclude, the results obtained from the two manufacturers of ELISA kits used to detect zolpidem in the sleep subjects were indistinguishable. Both kits showed "false presumptive positives" and inaccurate quantitation values for zolpidem concentrations in the urine samples when compared to GC–MS data, and both kits produced a calculated assay specificity of 25%. One hypothesis for the high number of false positives is the possibility of the assay cross-reacting with zolpidem metabolites, although this has not been identified from the manufacturers (17,18). Unfortunately, the metabolites were not available as drug standards so they could not be tested for cross-reactivity in these assays. When interpreting and confirming these ELISA results the target for GC and LC–MS should be the parent zolpidem and the main metabolites, not solely the parent drug.

One limitation to this study was the number of subjects sampled. However, that said, there are very little data published on urinary excretion concentrations of zolpidem, and of the studies, found none examined urine samples from long-term users of the drug. As a preliminary conclusion, the concentrations of zolpidem measured in urine in this study were higher than in previously published data using healthy volunteers. This may be due to changes in the pharmacokinetic profile of zolpidem due to prolonged use.

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