Determining Zolpidem Compliance: Urinary Metabolite Detection and Prevalence in Chronic Pain Patients

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Zolpidem (Ambien®) is the most prescribed insomnia treatment in the USA; however, little is known about zolpidem metabolite excretion in chronic pain patients. As zolpidem is extensively metabolized in vivo to zolpidem 4-phenyl carboxylic acid (ZCA), metabolite detection may provide improved accuracy for compliance determinations, thereby improving clinical decisions. Zolpidem and ZCA were extracted from 1 mL human urine by mixed-mode solid-phase extraction. Samples were analyzed by LC–MS-MS using positive electrospray ionization with multiple reaction monitoring mode employed for detection and quantification. Gradient chromatographic separation was achieved with a reversed-phase column in a rapid 1.8 min analysis. The assay was linear from 4 to 1,000 μg/L for zolpidem and 4 to 10,000 μg/L for ZCA. Interday recovery (bias) and imprecision (n = 20) were 100–107% of target and 2.4–3.7% relative standard deviation, respectively. Extraction efficiencies were 78–90%. Pain compliance samples (n = 3,142) were de-identified and analyzed for zolpidem and ZCA. Zolpidem was detected greater than limit of quantification in 720 specimens (22.9%), while ZCA was detected in 1,579 specimens (50.3%). Only five specimens contained zolpidem alone. ZCA was observed without parent zolpidem in 864 specimens, thereby increasing population detection rates by 27.5%. Addition of a zolpidem metabolite to compliance determinations substantially improved detection for zolpidem intake and also should prove useful in clinical and forensic settings.

Introduction

Sleep disorders abound, with an insomnia disorder prevalence rate of 12–20% of the general adult population (1, 2). Currently, zolpidem (Ambien®) is the most prescribed sedative–hypnotic for short-term treatment of insomnia and the 15th most prescribed drug in the USA (3). It is an imidazopyridine derivative that binds to the gamma-aminobutyric acid–benzodiazepine receptor complex with relative selectivity for the Type-1 (omega-1) benzodiazepine receptor subtype and shares many similar pharmacological properties to benzodiazepines (4). Zolpidem is subject to abuse and may potentially lead to physical dependence (5, 6), substantiating Schedule IV placement under the Controlled Substance Act. Monitoring zolpidem use in pain patients may be warranted, particularly due to the increased risk of adverse effects with concomitant use of multiple other central nervous system depressants (4). In addition to its prevalence in clinical settings, zolpidem is often implicated in drug-facilitated crimes due to its rapid onset of action, rapid clearance from biological specimens and amnesic properties (7, 8).

Zolpidem is extensively metabolized by human cytochrome P450 (CYP) enzymes to inactive metabolites, primarily by CYP3A4 (61%). Additional CYP enzymes contribute to zolpidem metabolism, including CYP2C9 (22%), CYP1A2 (14%), CYP2D6 (2.5%) and CYP2C19 (0.5%) (9). Less than 1% of a dose is excreted in urine as unchanged drug (10). The major urinary metabolite is zolpidem 4-phenyl carboxylic acid (ZCA), also known as zolpidem metabolite I (Figure 1). This metabolite represents 33–48% of a dose in urine (11), although its abundance is subject to pharmacokinetic interactions with CYP3A4 substrates that often alter formation rates (12).

Rapid zolpidem elimination (t_{1/2} 2.6 h) complicates urinary compliance testing, as detection is limited to samples obtained soon after ingestion. Zolpidem is observed at low microgram per liter concentrations in urine, with peak concentrations of 5–25 μg/L reported 12 h following ingestion of a single 10-mg dose; urinary zolpidem concentrations approached detection limits following 36 h postadministration in this small cohort (7). Lewis and Vine (13) were unable to detect parent zolpidem even 8 h after ingestion of a single dose although ZCA was identified [2 μg/L limit of detection (LOD)] in urine for a period of 72 h following zolpidem administration. Strano Rossi et al. detected parent zolpidem for up to 36 h after ingestion [0.5 μg/L limit of quantification (LOQ)]; ZCA was again detectable for at least 72 h (unknown LOD) in this case (14).

Published methods and studies have often focused solely on detection of parent zolpidem, potentially yielding false-negative intake determinations at existing cutoff concentrations. This is problematic for compliance testing in clinical settings as non-compliance often leads to patient censure. Also, in drug-facilitated sexual assault (DFSA) casework victims may not immediately present for evaluation, resulting in undetectable parent zolpidem concentrations at the time of testing. Furthermore, immunoassays currently employed in some laboratories may not cross-react with ZCA, further obfuscating proper determinations (15). For these reasons, inclusion of the primary metabolite in mass spectrometry-based zolpidem analyses likely would yield improved accuracy in detecting intake.

To this end, we developed and validated a sensitive LC–MS-MS method for the simultaneous detection of zolpidem and ZCA in human urine. This method is specific and rapid, quantifying both analytes in 1.8 min. This method will be useful to toxicologists in clinical and forensic settings, where zolpidem intake determinations require a sensitive, rapid method for quantification of zolpidem over clinically relevant concentrations.

Experimental

Samples

Urine samples were obtained from chronic pain patients over the period of February–June 2012 from pain clinics located in...
20 states (AL, FL, GA, IL, KY, LA, MD, MS, NC, NJ, NV, NY, OH, PA, SC, TN, TX, UT, VA and WV). All patients were enrolled in pain management compliance monitoring that included drug testing for licit and illicit drugs. The study was approved by the Essex Institutional Review Board (Lebanon, NJ, USA). Urine specimens were collected into polypropylene containers that were capped, labeled and stored at room temperature until shipment to the laboratory for analysis. The specimens were analyzed at Aegis Sciences Corporation (Nashville, TN, USA).

**Instrumentation**

All experiments were performed on an AB Sciex API 3200 triple quadrupole mass spectrometer with a TurboV electrospray ionization (ESI) source (AB Sciex, Foster City, CA, USA). The mass spectrometer was interfaced with a Shimadzu LC-20ADXR high-performance liquid chromatograph (HPLC) (Shimadzu Corporation, Columbia, MD, USA). Evaporation under nitrogen was completed using a TurboVap LV evaporator from Biotage (Charlotte, NC, USA). Powder standards were measured using an XP-6 analytical balance (Mettler-Toledo, Columbus, OH, USA).

**Standards and reagents**

Zolpidem standards and deuterated internal standards were purchased from Cerilliant (Round Rock, TX, USA). ZCA and ZCA-d6 were from Toronto Research Chemicals (Toronto, ON, Canada). All reagents and solvents were from Sigma–Aldrich (St. Louis, MO, USA) and were HPLC grade or better. Clean-Screen ZSDAU020 solid-phase extraction (SPE) columns (United Chemical Technologies, Bristol, PA, USA) were used to prepare samples. Drug-free human urine was evaluated for the absence of zolpidem and ZCA prior to use.

**Urine analyses**

**Sample preparation**

Internal standard (zolpidem-d6 and ZCA-d6) was added to each sample to yield 100 μg/L in urine. Solid-phase extraction columns were conditioned with methanol and 0.1 M acetic acid. Specimens (diluted 1:1 with 0.1 M acetic acid) were loaded by gravity flow, followed by washing with deionized water, 200 mM hydrochloric acid, methanol and ethyl acetate. Columns were then dried under vacuum (SPE vacuum manifold) for 5 min, and samples were eluted with 5 mL dichloromethane:isopropanol:ammonium hydroxide (78:20:2). Samples were evaporated to dryness, reconstituted in mobile phase A and submitted for LC–MS-MS analysis.

**LC–MS–MS analysis**

Chromatographic separation was performed using a C18 HPLC column (Thermo Scientific Hypersil Gold, 3 μm, 100 × 2.1 mm). Mobile phase was 0.1% formic acid in HPLC water (A) and 0.1% formic acid in acetonitrile (B). Gradient conditions began at 20% B, increasing to 95% B by 0.3 min followed by a 0.5 min hold and re-equilibration. Chromatographic run time was 1.8 min; cycle time was 2.1 min (Figure 2). Mass spectrometric data were acquired with positive ESI in multiple reaction monitoring (MRM) mode. MS-MS parameter settings (Table I) were optimized via direct infusion of individual analytes (100 μg/L) in 50:50 mobile phase A:mobile phase B at 10 μL/min. Source parameters were as follows: curtain gas, 30 psi; collision-activated dissociation, 5 psi; IonSpray voltage, 1,500 V; source temperature, 450°C; gas 1 (zero air), 40 psi and gas 2 (zero air), 50 psi.

**Data analyses**

Peak area ratios of target analytes and their respective internal standards were calculated for each concentration. Urine concentrations were measured in microgram per liter and were normalized to refractometer-determined urine specific gravity (16). Statistical calculations were completed with Microsoft Excel 2007 for Windows (Microsoft Corporation, Redmond, WA, USA) and JMP 9.0 for Windows (SAS Software, Cary, NC, USA).

**Validation**

The method was validated for bias, inter- and intrabatch imprecision, specificity, sensitivity, linearity, extraction efficiency, matrix effect, carryover, interference (endogenous and exogenous) and analyte instability.

Bias and imprecision were evaluated at three concentrations within the linear dynamic range of each analyte. Intrabatch imprecision (% relative standard deviation [RSD]) ranged from 0.3 to 2.7% for all analytes at all concentrations (n = 5); interbatch imprecision ranged from 2.4 to 4.2% (n = 20). Bias calculated as the percent of target concentrations at low, mid and high quality control (QC) concentrations for each analyte (50, 100 and 250 ng/mL, respectively), ranged from 100 to 107% of target concentrations (n = 20) and was considered clinically insignificant.

Specificity was based on precursor mass/fragment ion and relative retention time. Transition peak area ratios for QC and authentic specimens were required to be within 20% of the calibrator for each respective analyte. Relative retention time (analyte to internal standard) difference for QC and authentic specimens...
was required to be within ± 0.01 min compared with the relative retention time of the analyte to internal standard of the calibrator. Alternatively, retention time for each analyte could be within 3% of its respective retention time in the calibrator.

Sensitivity was evaluated by determining LOD and LOQ. A series of decreasing concentrations of fortified urine were analyzed to empirically determine LOD and LOQ. LOD was determined as the concentrations with acceptable chromatographic retention time and peak shape, a signal-to-noise ratio of at least 10:1, transition peak area ratios within 20% of the calibrator ratio and acceptable bias and imprecision (within at least 20% of target concentration and RSD within at least 10%, n = 6).

Analyte linearity was determined with 10 concentrations on 3 separate days. Linearity of the method was investigated by calculation of the least-squares regression line (1/x weighting) and expressed by the squared correlation coefficient ($R^2$). Linear ranges were 4–1,000 µg/L for zolpidem and 4–10,000 µg/L for ZCA; these ranges should prove useful for clinical and forensic casework. $R^2$ values (1/x weighting) were deemed acceptable ($R^2 > 0.995$) for all analytes. Following initial linearity determination, batches were analyzed using a single-point calibrator at a concentration of 100 µg/L (line forced through zero) due to efficiency with respect to time and resources (17). Precision and accuracy fulfilled a priori requirements across the linear range with the chosen calibrator concentration.

The extraction efficiency (percent) for each analyte was determined at two concentrations (4 and 40 ng/mL) and ranged from 90 to 94% for zolpidem and from 64 to 77% for ZCA. Similar results were obtained for the corresponding deuterated analog, and quantification was not adversely affected. Matrix effect was required to be within ± 0.01 min compared with the relative retention time of the analyte to internal standard of the calibrator. Alternatively, retention time for each analyte could be within 3% of its respective retention time in the calibrator.

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investigated through a postcolumn infusion (‘T’ connector) of the analytes while injecting an extracted blank urine sample. No significant ion suppression or enhancement was observed at the retention time for ZCA. Significant ion enhancement was observed at the retention time of parent zolpidem. However, similar ion enhancement for the deuterated internal standard minimized impact on quantification. In all cases, ion ratios met a priori specifications and QC samples all quantified within acceptable limits.

Carryover in a negative specimen following a specimen containing the upper limit of quantification (ULOQ) was assessed. No quantifiable carryover was observed for any analyte. In some cases, traces of zolpidem signal were observed in carryover specimens; however, ion transition ratios were not within 20% of the calibrator and any signal present was less than LOD.

For assessment of possible matrix interferences, negative urine samples were collected from six different sources and tested for potential endogenous interferences; no interference was observed in any pool for any analyte. In addition, interferences from over 55 illicit and common therapeutic drugs, metabolites and related compounds were evaluated by adding potential interferents at various concentrations into urine aliquots fortified at the LOQ. Compounds examined included several classes of common licit and illicit drugs, including amphetamines, barbiturates, cocaine, delta-9-tetrahydrocannabinol, benzodiazepines, opioids (natural, semi-synthetic and synthetic compounds and metabolites), muscle relaxants, zopiclone and zaleplon. A compound did not interfere if the LOQ quantified within 20% of target and had stable retention times and correct transition ratios. Of the compounds tested, the methadone metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) was the only identified interferent. At EDDP concentrations >10,000 μg/L, zolpidem-d<sub>6</sub> suppression was apparent; however, the analyte remained unaffected yielding zolpidem quantitation differences >20% from target at low QC concentration. ZCA was not affected by any tested interferent. Finally, 60 additional de-identified pain compliance specimens (containing various licit and illicit analytes) were extracted and analyzed for possible endogenous and exogenous interferences. Of these, one had interference with zolpidem (but not the metabolite); this is believed to be related to EDDP concentrations >10,000 μg/L in this specimen. Analyte stability in urine (n = 5) was evaluated under three conditions: freeze–thaw, autosampler and processed sample stability. For freeze–thaw stability, concentration changes ranged from −2.8 to 7.6% for zolpidem and −1.2 to 10.6% for ZCA. For autosampler stability, concentration changes ranged from −4.7 to 3.9% for zolpidem and −4.4 to 0.4% for ZCA. For processed sample stability, concentration changes ranged from −5.8 to −2.1% for zolpidem and −2.5 to −0.1% for ZCA.

Results and Discussion

With over 43 million prescriptions filled per year in the USA alone, zolpidem is the most prescribed sleep aid and is widely available (3). However, to date, no study has examined use of zolpidem metabolites for compliance determinations. Zolpidem is extensively metabolized and exhibits short detection windows in urine, potentially yielding false-negative results in clinical and forensic settings. Thus, detection and quantification of both parent and metabolite in a rapid, high-throughput assay is a significant advancement for this compound and here we report the impact of metabolite inclusion on compliance determinations.

Samples

A total of 3,142 directed analysis urine samples were obtained from chronic pain patients over 5 months. Results were de-identified and examined for zolpidem and ZCA prevalence.

Prevalence of metabolite in urine

Of 3,142 specimens, zolpidem was detected greater than LOQ in 720 (22.9%), whereas ZCA was detected in 1,579 (50.3%) (Table II). Two specimens (0.06%) contained zolpidem greater than ULOQ and 45 specimens (1.43%) contained ZCA greater than ULOQ. Normalized median (range) zolpidem and ZCA concentrations were 28.3 (4.08–805) μg/L and 2.038 (4.53–23,000) μg/L, respectively (Figure 3). Only five specimens (0.16%) contained zolpidem alone (median concentration 488 μg/L). As ZCA was observed without parent zolpidem in 864 samples, addition of this metabolite increased detection rates by 27.5% in this group.

We previously reported (18, 19) that inclusion of metabolites for compliance determinations in chronic pain patients is crucial for maximizing accuracy. In the present report, zolpidem positive determinations were increased from 22.9 to 50.3%, representing 864 additional positive identifications of zolpidem use in 5 months when compared with analyzing for zolpidem alone at the same LOQ. Others have noted (13, 14) that zolpidem detection windows can be increased with inclusion of ZCA in routine analysis. While the present method improved detection rates specifically related to inclusion of the metabolite, this study cannot provide information regarding improvement in zolpidem intake detection windows. Further controlled administration studies would be beneficial in more precisely defining these windows under acute and chronic dosing regimens. Administration studies with immediate- and controlled-release zolpidem may also provide additional insight into ZCA production and excretion rates, although the similar elimination half-lives between the products [mean 2.6 and 2.8 h, respectively (4, 11)] suggest minor impact on detection periods or concentrations.

Monitoring for zolpidem use or misuse may be prudent for pain practitioners during compliance assessment. According to data from the National Health and Nutrition Examination Survey, more than half of zolpidem users in the USA are using

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<td>Pain Compliance Cohort Descriptive Statistics for Samples within the Linear Range (4–1,000 μg/L for Zolpidem and 4–10,000 μg/L for ZCA)</td>
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<td>Zolpidem</td>
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All concentrations are microgram per liter and normalized to urine specific gravity.
other sedating medications, of which opioids and benzodiazepines are most common (20). Zolpidem may cause adverse drug reactions including daytime drowsiness, dizziness, hallucinations, agitation or bizarre behavior, sleepwalking and ‘sleep driving’ (4). The high incidence of polypharmacy and significance of adverse effects related to zolpidem may pose increased risk for pain patients. Emergency department visits related to adverse effects of zolpidem have increased 220% from 2005 to 2010; more than half of visits involved concomitant use of other medications, particularly opioids and benzodiazepines. Women and elderly patients (>65 years old) may be at increased risk and have been involved in a disproportionate number of emergency department visits related to zolpidem intake (21). Sex-related pharmacokinetic differences with lower clearance observed in women (22) may be a factor; consequently, in January 2013, the US Food and Drug Administration required manufacturers to lower recommended dosages of zolpidem for females (23).

In addition to legitimate widespread use as a sedative–hypnotic, zolpidem also has been implicated in both DFSA (7, 13) and driving under the influence of drugs (DUID) (24, 25) forensic casework. Although urinary zolpidem and ZCA concentrations cannot be used for impairment determination, detection of these compounds (principally the metabolite) can provide evidence for zolpidem intake, be it known or unknown. This is particularly important in DFSA casework, where victims may not immediately present for evaluation. Based on these findings, it is recommended to include zolpidem metabolite to minimize false-negative potential in these scenarios.

Although we identified numerous specimens containing ZCA with no zolpidem parent, only five samples in the present study contained detectable zolpidem with no corresponding ZCA. Upon further review, two of these samples were from a single individual with samples taken on two separate occasions. This individual was also positive for illicit drugs and had the highest concentrations of zolpidem in the entire cohort. The three remaining samples were investigated further, and we determined that these samples had no metabolites present for all other compounds detected. While substantial CYP enzyme inhibition could yield observed patterns, sample adulteration also should be considered as a possible explanation. Corrected zolpidem concentrations of 13.5–1,700 μg/L (4.1–1,440 μg/L uncorrected) indicate that some technical knowledge (or luck) would be required for adulteration as only 0.2–72 μg of parent zolpidem (0.004–1.4% of a typical 5 mg dose) would need to be added to a 50 mL urine sample to produce observed concentrations. Nevertheless, adulteration remains a plausible hypothesis and while metabolite testing identified 99% of zolpidem use in the present study, inclusion of both analytes is recommended during routine analysis for maximum accuracy in compliance determinations.

### Conclusions

Inclusion of ZCA in zolpidem urine assays is useful for accurate determinations in compliance or DFSA/DUID scenarios. Improvement in detection of zolpidem intake is critical as non-compliance can lead to censure in monitoring scenarios; the present study provided a 27.5% increase in detection rates for a chronic pain patient population at the same LOQ. This improvement in detection may also be useful in DFSA/DUID casework where determinations based on zolpidem alone may yield poor detection of ingestion. The present method is sensitive, specific and can detect and quantify both parent zolpidem and its primary urinary metabolite ZCA. The rapid extraction and analysis are beneficial and should be easily adopted in both forensic and clinical laboratories. This new method provides novel insight into zolpidem urinary excretion and will improve our ability to detect zolpidem intake in clinical and forensic drug testing casework.

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