A Simple and Rapid Method for the Identification of Zolpidem Carboxylic Acid in Urine

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Abstract

Zolpidem is a non-benzodiazepine hypnotic that has been implicated in both drug-facilitated sexual assault and drink spiking. Detection of the drug in urine is extremely difficult because of its extensive metabolism. A method is presented for the detection and quantitation of zolpidem carboxylic acid (ZCA), the major urinary metabolite of zolpidem. The metabolite was extracted from urine at pH 4.5–5.0 with chloroform/isopropanol alkylated with ethyl iodide and identified by gas chromatography–mass spectrometry in the selected ion-monitoring mode. Following a single ingestion of 10 mg zopidem, ZCA is detected for up to 72 h. The limit of detection is 2 ng/mL, with an overall recovery of 80%. Using this procedure, zolpidem was identified in two cases of alleged drug-facilitated sexual assault.

Introduction

Zolpidem (Stilnox®) is a non-benzodiazepine hypnotic commonly prescribed for sleep disorders. Although structurally unrelated to the benzodiazepines, zolpidem selectively binds to the omega-1 receptor subtype of the GABA-A receptor complex (1). Zolpidem has been mentioned as just one out of a number of drugs capable of being used in drug-facilitated sexual assault (DFSA) (2). A recent study into drink spiking by the Australian Institute of Criminology (3) found 58% of 117 victims suffered memory loss, suggesting that a drug or drugs causing amnesia may have been co-administered with a drink. Although zolpidem itself does not generally produce amnesia, Salva and Costa (4) reported that co-administration of imipramine and zolpidem did produce anterograde amnesia. It is therefore important to include zolpidem in a profile of substances suspected of being used in either drink spiking or DFSA. Zolpidem is extensively metabolized with less than 1% of the parent drug excreted (4). The major urinary metabolite (metabolite I) accounted for 51% and the minor metabolite (metabolite II) accounted for 11.5% of an administered dose (Figure 1). As the elimination half-life is approximately 2 h, there is very little chance of detecting unchanged zolpidem in a random urine taken 24–48 h after an alleged offense.

Although zolpidem has been available in parts of Europe since 1992 and in Australia since 2000, there are few reported simple procedures suitable for its detection after a single ingestion. Published methods using high-pressure liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC–MS) focus on identifying parent zolpidem rather than the major urinary metabolite. Ascalone et al. (5) studied the metabolism of zolpidem and described the four known metabolites (I–IV). The authors used HPLC to measure zolpidem and its metabolites in plasma and urine following oral administration of the drug. Urinary levels of metabolite I dropped rapidly 8 h after ingestion of 10 mg zolpidem, and it was undetectable by 30 h. Ausburger et al. (6) measured zolpidem and its metabolites in urine using the ion-pairing reagent, tetrahexylammonium hydrogen sulfate, followed by extractive alkylation with methyl iodide, and a cleanup step using SM-7 resin. GC–MS was used for identification; however, the procedure required large volumes of reagents and a cleanup step. Dona et al. (7) obtained poor sensitivity for zolpidem in urine using solid-phase extraction (SPE) and were unable to identify any metabolites using this procedure. Levine et al. (8) identified zolpidem in postmortem fluids using alkaline extraction into *n*-butyl chloride, followed by backwash into acid and re-extraction with methylene chloride. Ali-Tolppa et al. (9) included zolpidem in their GC–MS screen for benzodiazepines; however, no details were presented regarding sensitivity or recovery. Logan and Couper (10) reported a number of cases of suspected driving impairment in which zolpidem had been identified. Although the authors measured blood

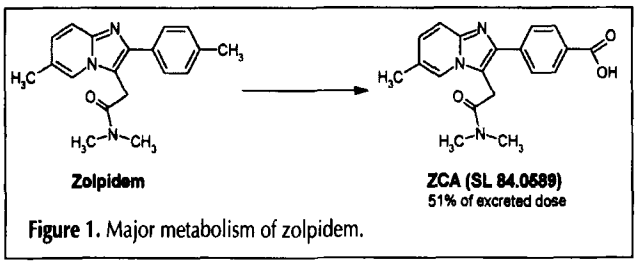

Figure 1. Major metabolism of zolpidem.

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levels of parent zolpidem using GC–MS, they suggested that a morning urinary void would be positive for zolpidem following ingestion the previous night. Bickeboeller-Friedrich and Maurer (11) included identification of zolpidem metabolites in a systematic procedure for a number of antidepressants and related drugs in urine. However, the authors only included zolpidem metabolite II in their screening procedures, presumably because the major metabolite, a carboxylic acid, was not amenable to the authors' extraction and derivatization procedures. Villain et al. (12) described an LC–MS–MS procedure for the detection of zolpidem in the hair of alleged sexual assault victims. Using this technique, the authors were able to identify parent zolpidem up to 60 h in urine following a single 10-mg tablet of the drug. However, urine concentrations were virtually zero after 36 h. None of these procedures were deemed appropriate for the detection of low concentrations of metabolite following a single therapeutic ingestion of zolpidem. A simple procedure is presented for the MS identification of zolpidem carboxylic acid (metabolite I) in urine.

Experimental

Reagents

Zolpidem and metabolite I (SL 84.0589) were kind gifts from Sanofi-Synthelabo Australia. Mefenamic acid was obtained from Sigma Aldrich (Sydney, Australia). All solvents and chemicals were A.R. grade or better. Chloroform, dichloromethane, propan-2-ol, ethyl acetate, ethyl iodide, anhydrous potassium carbonate, sodium sulfate, and sodium acetate were purchased from Bacto Laboratories (Sydney, Australia).

High recovery 2-mL vials (Agilent 5183-2030) were purchased from Biolab (Sydney, Australia). A sodium acetate buffer (0.1M, pH 5) was used (8.2 g anhydrous sodium acetate was dissolved in 1 L deionized water and pH adjusted to 5.0 with glacial acetic acid). Ethereal diazomethane was generated from Diazald® (Sigma Aldrich) by the method of Holder et al. (13).

Instrumentation

GC–MS analysis was performed on an Agilent 6890 GC interfaced to a 5973 mass selective detector and operated in the electron impact mode. The electron multiplier was set 200 volts above the autotune value. The GC was fitted with a J&W HP-1 column (12 m × 0.2-mm i.d., 0.33-µm film thickness). The GC was operated in the splitless mode with helium as the carrier gas at 1 mL/min. The injector was operated in the pulsed pressure mode at 26 psi for 0.5 min. The injection volume was 2 µL. The injector and detector temperatures were 240°C and 280°C, respectively. The initial oven initial temperature was held at 150°C for 1 min, and then ramped at 30°C/min to 310°C with a final hold of 3 min. The MS was operated in the selected ion-monitoring mode using 269, 223, and 208 for the ethyl derivative of mefenamic acid (retention time 4.1 min) and 219, 293, and 365 for the ethyl derivative of ZCA (retention time 7.1 min).

LC–MS^n analyses were carried out on a Thermo Finnigan LCQ Deca XP Plus MS coupled to a Thermo Finnigan Surveyor LC and autosampler fitted with a Zorbax Stable Bond C_{18} column (150 mm × 3 mm, 5 µm) (Biolab). The mobile phases were 1% acetic acid (A) and methanol (B). The gradient was 95% A and 5% B linearly programmed to 10% A and 90% B over 10 min, held for 4 min, returned to 95% A and 10% B over 2 min, and held for 4 min. The injection volume was 10 µL. The MS was operated in the positive ion electrospray ionization mode. The capillary temperature was 350°C, the capillary voltage was 32 V, the ionspray voltage was 4.5 kV, the sheath and auxiliary gases were at 65 and 5 units, respectively, and the

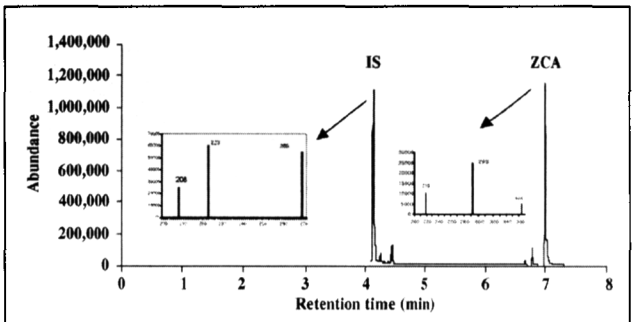

Figure 2. GC–MS of ethyl derivatives of mefenamic acid (IS) and ZCA.

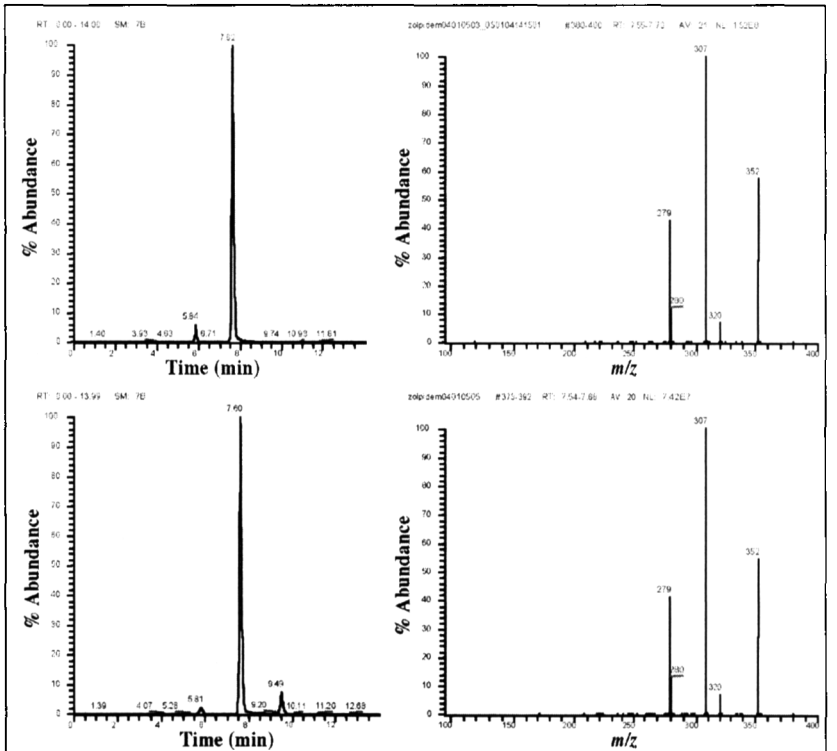

Figure 3. LC–MS–MS data for a ZCA standard (upper traces) and a urine extract (lower traces) collected 17 h after ingestion of 10 mg zolpidem.

scan range was 100–500 Da. The MS–MS precursor ions were *m/z* 338 (metabolite 1), *m/z* 352 (metabolite 1 methyl ester), and *m/z* 366 (metabolite 1 ethyl ester). The isolation peak width was 1.5 Da and the relative collision efficiency was 43%.

Preparation of standards

For the internal standard (IS), a stock solution of mefenamic acid (1 mg/mL) was prepared in methanol. This was diluted 1:100 to give a working solution of 10 µg/mL. A stock ZCA standard (1 mg/mL) was prepared in methanol. This was diluted 1:100 with methanol to give working calibrator 1 (WC1) 10 µg/mL. This was found to be stable for at least 8 weeks. WC1 was further diluted by dispensing 100 µL in 2 mL methanol to give working calibrator 2 (WC2) 0.5 ng/µL.

Procedure

Urine aliquots (2 mL) for 3 calibrators, blank, and patient samples were dispensed into 10-mL screw capped tubes. The IS (20 µL) was added and vortex mixed. ZCA working calibrator WC2 (20, 50, and 100 µL) were added to calibration tubes to give concentrations of 5, 10, and 20 µg/L, respectively. To each tube was added 1 mL sodium acetate buffer and 7 mL chloroform/isopropanol. The tubes were capped and rolled for 30 min, after which they were centrifuged at 2000 rpm for 3 min. The aqueous phase was aspirated and the remaining organic phase briefly vortex mixed with a small amount of anhydrous sodium sulfate. The organic phase was then evaporated to dryness at 60°C under nitrogen.

Ethylation

Extracts were alkylated according to the method of Shaw and Kunerth (14). To each extract were added 200 µL acetone, 50 mg potassium carbonate, and 20 µL ethyl iodide. The tubes were capped and let stand at 60°C for 30 min. The acetone phases were transferred to 2-mL high recovery autosampler vials, gently evaporated to dryness, and the extracts reconstituted in 100 µL ethyl acetate. Two microliters were injected into the MS. A typical GC–MS trace is shown in Figure 2.

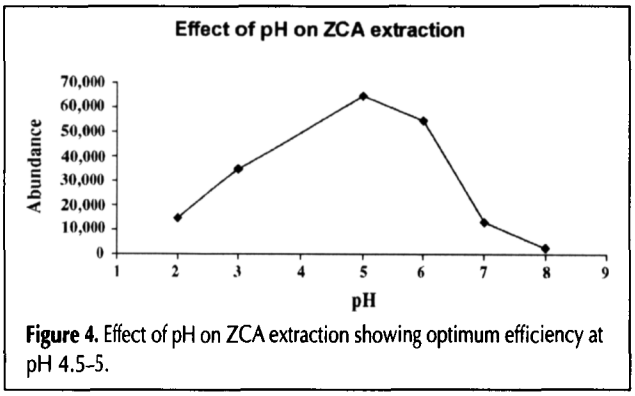

Figure 4. Effect of pH on ZCA extraction showing optimum efficiency at pH 4.5–5.

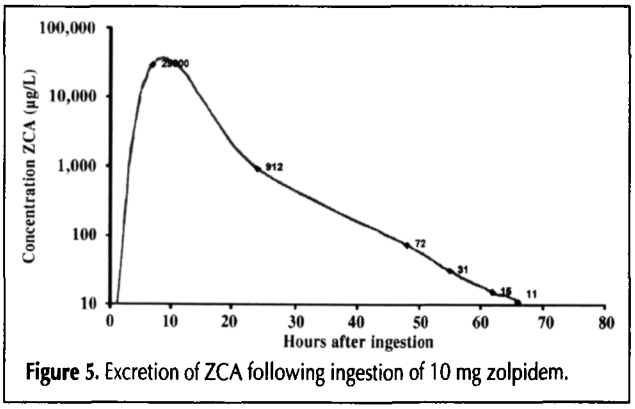

Figure 5. Excretion of ZCA following ingestion of 10 mg zolpidem.

LC–MS studies

The presence of ZCA in urine was also confirmed by LC–MS–MS analysis of the methylated and ethylated derivatives. The LC–MS–MS data for the methylated derivative is shown in Figure 3. The mass spectrum of the methylated derivative has a protonated molecular ion at *m/z* 352 and shows product ions at *m/z* 320 ($MH^+ - CH_3OH$), *m/z* 307 ($MH^+ - CH_3NHCH_3$), and *m/z* 279 ($MH^+ - CH_3NHCH_3 - CO$). The MS of the ethylated derivative showed corresponding ions at *m/z* 366 (MH^+), *m/z* 321, and *m/z* 293.

Optimization of method

pH. The optimum pH for extraction was determined by extracting spiked urines at various pHs and comparing the relative abundances. The results are shown in Figure 4.

Extraction solvent. The optimum extraction solvent was determined to be chloroform/isopropanol (5:1). This gave a higher recovery than either chloroform/isopropanol (9:1) or chloroform alone. Dichloromethane was later found to give similar results to chloroform.

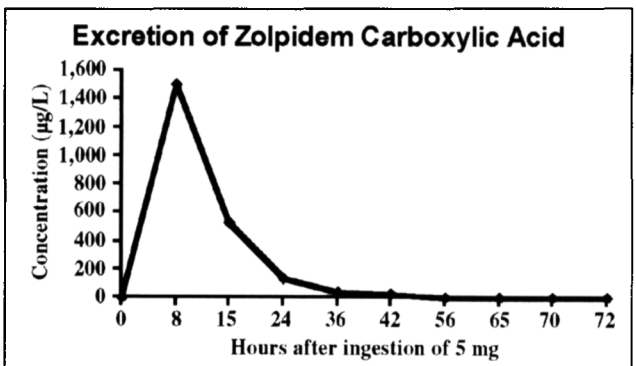

Figure 6. Excretion of ZCA following ingestion of 5 mg zolpidem.

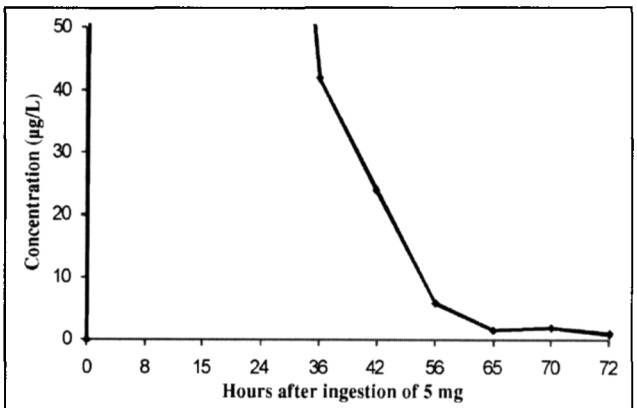

Figure 7. Excretion of ZCA 30–72 h following ingestion of 5 mg zolpidem.

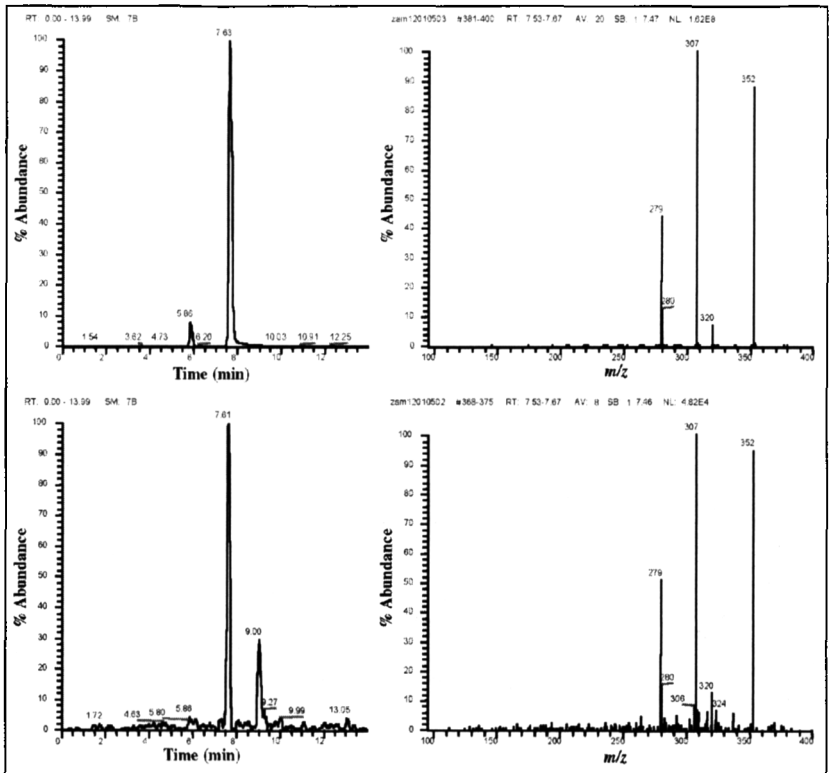

Figure 8. LC–MS–MS traces from a ZCA standard (upper traces) and an extract of a 3-year-old urine from an alleged DFSA case (lower trace).

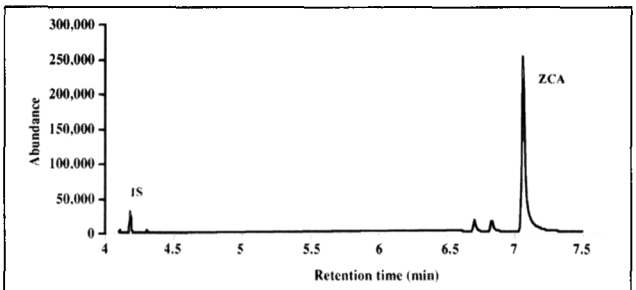

Figure 9. High concentration of ZCA found in the urine of victim of an alleged DFSA (not quantitated).

Excretion studies. Following the ingestion of a single 10-mg tablet of zolpidem (Stilnox), urine samples were collected at various times up to 72 h. Urines were initially subjected to our routine laboratory procedures for parent zolpidem by extraction at pH 9 with dichloromethane/isopropanol (9:1), followed by GC–MS operated in full scan mode. Separate aliquots of urines were then extracted for ZCA as described previously.

Results

Routine extraction procedures failed to detect any unchanged zolpidem, even in the early morning sample (8 h) following ingestion (2200 h the previous night). By contrast, ZCA was identified up to 72 h after ingestion. The excretion of ZCA is shown in Figure 5.

In a second study, one half-tablet of zolpidem (5 mg) was taken, and urine samples were collected at various times up to 72 h and extracted for ZCA as described previously. The results are shown in Figures 6 and 7.

DFSA case 1

A number of previously analyzed urine samples from unsolved alleged DFSA cases (15) that had been stored at –20°C were analyzed for ZCA. Using this procedure, one three-year-old sample was found to contain traces of ZCA. The presence of the metabolite was confirmed by re-extracting an aliquot of urine, derivatizing ZCA with diazomethane, and analyzing the substance by LC–MS. The results are shown in Figure 8.

DFSA case 2

In a more recent case, a female made coffee for a male companion and herself. Before drinking the coffee, she went to the bathroom. A short time after drinking the coffee, she felt dizzy, nauseous, and "jelly-legged". She remembered being assaulted by the male companion but was unable to move her limbs. A high concentration of ZCA was found in the urine. This is shown in Figure 9. In this particular case, parent zolpidem was also identified by the routine screening procedures as described previously.

Discussion

Although zolpidem has been available as a hypnotic for many years, there has been a paucity of information on its abuse potential or its implication in DFSA. Because of the drug's extensive metabolism, zolpidem may not always be identified in routine laboratory screening procedures. As the major urinary metabolite is a carboxylic acid, one must utilize an acidic extraction and derivatization procedure for its identification. Although this method was developed using ethylation, methyl iodide can be substituted as it works in a similar manner with the major ions being 14 Da less. Although the original method development utilized chloroform as an extraction solvent, dichloromethane has since been substituted throughout our routine laboratory procedures. As expected, there was no noticeable difference in recovery or cleanliness of extracts on GC–MS. As pure ZCA is not yet commercially available, once analytical procedures have been established, the method may be used as a qualitative screen by using a positive urine control collected 8–12 h following the ingestion of 10 mg zolpidem. The metabolite can be found in urine for 2–3 days following the ingestion of a single therapeutic dose of zolpidem. An Australian study into drink spiking (3) found that in the majority of cases of DFSA, anterograde amnesia occurred. Importantly, the alleged victim in case 2 did not experience any amnesia,

thus lending support that zolpidem, when administered alone, may not be implicated in such cases.

Conclusions

A procedure has been developed for the identification and quantitation of the major urinary metabolite of zolpidem. It has been found to be suitable for the detection of zolpidem in cases of spiked drinking and DFSA. The method is rapid, simple, and can be performed by laboratory technicians.

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