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Testing for zolpidem in oral fluid by liquid chromatography–tandem mass spectrometry

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Abstract

The purported enhancement of sexuality, coupled with a possible abrupt coma-inducing effect and ease of administration in spiked drinks have resulted in the use of hypnotics to facilitated a sex offence. Among these compounds, zolpidem possesses amnesic properties, is short-acting and can rapidly impair an individual. In order to document zolpidem exposure, we have developed an original analytical method in oral fluid. 500 μl of oral fluid was added to 500 μl pH 7.6 Soerensen buffer was extracted by 2 ml dichloromethane in presence of 5 ng diazepam-d_5, used as internal standard. An aliquot of the extract was injected into a 5 μm Novapak C18 column (150 mm × 2.1 mm). Reversed-phase separation was achieved in 6 min, under isocratic conditions (90% acetonitrile, 10% 2 mM ammonium formate pH 3.6) at a flow rate of 150 μl/min. Detection was achieved by tandem mass spectrometry. Molecular ions (*m/z* 308 and 290 for zolpidem and the IS, respectively) were selected in Q1 and the corresponding daughter ions (*m/z* 235 and 263 for zolpidem and *m/z* 154 and 198 for the IS) were detected in Q3 after collision with argon. Linearity was observed for zolpidem concentrations ranging from 0.2 to 100 ng/ml, and the assay was capable of detecting 0.05 ng/ml. Oral fluid was collected for 480 min after oral zolpidem administration of 10 mg to 2 subjects. In both cases, zolpidem was detectable (0.4 ng/ml) after 15 min intake. Peak zolpidem concentrations were obtained at 150 min (53.5 ng/ml) and 180 min (75.7 ng/ml), respectively. Oral fluid tested positive for zolpidem for over 8 h (9–15 ng/ml).
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1. Introduction

Insomnia, an inability to initiate or maintain sleep, affects 30–35% of people living in developed countries. In the last 30 years, benzodiazepines were the treatment of choice for short-term insomnia, but these compounds are associated with adverse effects, such as rebound insomnia, withdrawal, tolerance and dependency. Benzodiazepines act non-selectively at two central receptor sites, termed omega-1 and omega-2. The sedative action is related to omega-1 receptors, whereas omega-2 receptors are responsible for effects on memory and cognitive functioning [1].

New hypno-sedatives are now preferred over conventional benzodiazepines to treat short-term insomnia. Among them, zolpidem (Stilnox, Ambien, Ivadal) has been sold in France since 1988. This drug is an imidazopyridine derivative, with a chemical structure unrelated to benzodiazepines [2]. It is intended for once-nightly consumption at a dose of 10 mg. Despite its selective interaction with omega-1 receptors, several side-effects, including visual disturbance and hallucinations have been described. Moreover, impairment of psychomotor performance [3] and effects on recent or remote recall [4,5] can be both significant.

Zolpidem has been observed in cases of drug facilitated crimes (DFC), including robbery and sexual assault [6]. As the drug is short-acting (T_{max} = 1.7 h), it can impair an individual rapidly. Moreover, due to its amnesic properties, the victims are less able to accurately recall the circumstances under which the offence occurred.

Urine is the conventional specimen to document DFC [7] and there is little attention in the literature for the use of oral fluid. However, this specimen is recommended for workplace drug testing or for roadside testing of intoxicated drivers [8].

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The advantages of oral fluid over traditional specimens such as blood or urine are the non-invasive, and ease of collection and that sampling can be achieved under close supervision.

To evaluate the potential interest of oral fluid in the detection of zolpidem, two healthy volunteers were administered a single oral dose of 10 mg. To test for the drug, a specific and ultra-sensitive method was developed.

2. Materials and methods

2.1. Specimens

Oral fluid from 2 volunteers (male, 42 year old, 68 kg and female, 28 year old, 58 kg) was collected over 480 min by spitting at fixed time intervals in a plastic tube, without any stimulation, to obtain approximately 1 ml of sample. Subjects received a single oral dose of Stilnox (tablet at 10 mg of zolpidem tartrate) along with 100 ml water. Specimens were stored at +4 °C until analysis (achieved within 3 days).

Subjects participated in the experimental part of the study through written informed consent.

Blank oral fluid samples were obtained from laboratory personnel.

2.2. Chemicals and reagents

Acetonitrile and dichloromethane were HPLC grade (Merck, Darmstadt, Germany). Chemicals for the Soerensen buffer – 38.8 ml KH_2PO_4 at 9.07 g/l + 61.2 ml Na_2HPO_4 at

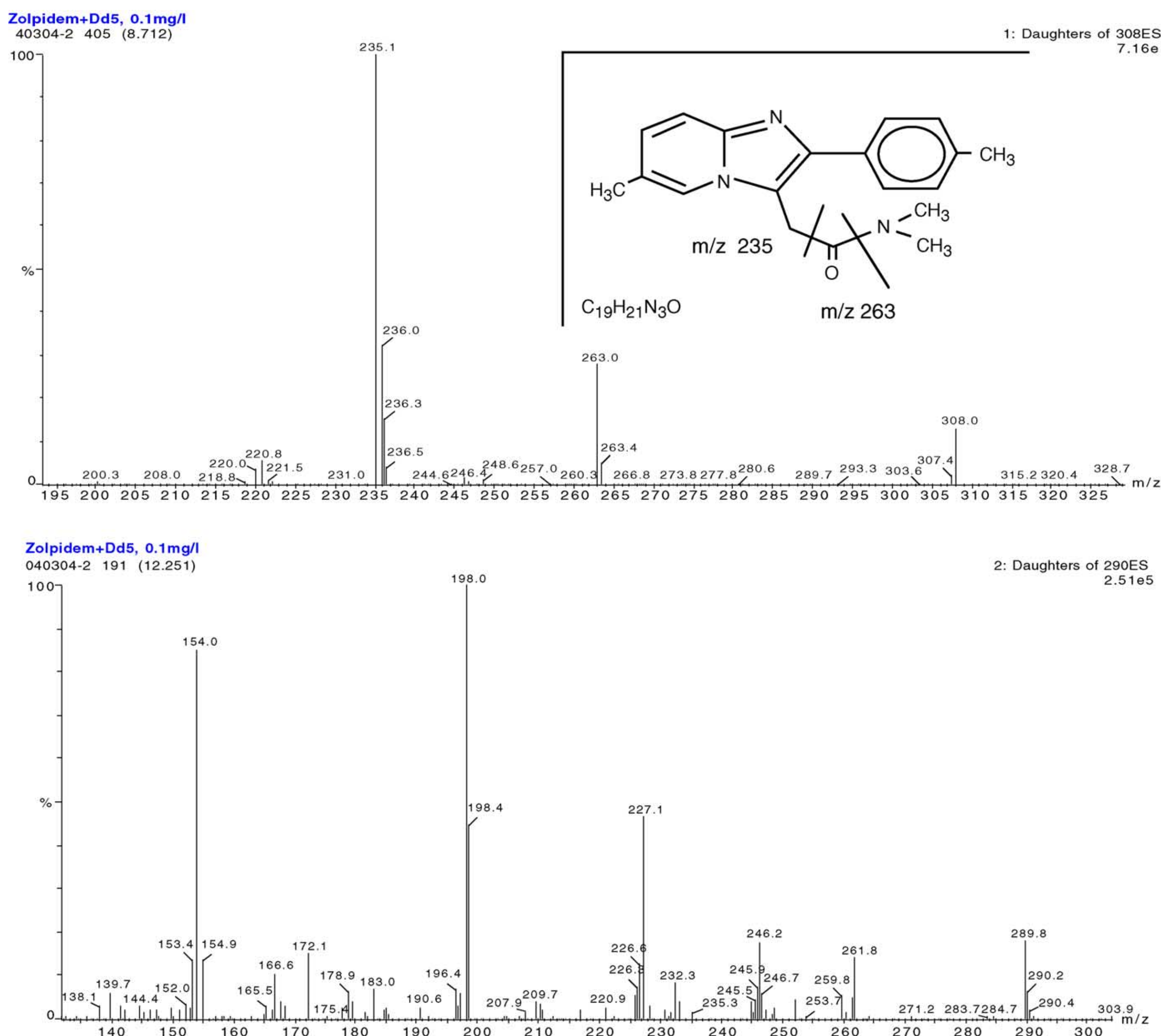

Fig. 1. Full mass spectrum of the fragmentation of the precursor ion for both zolpidem (top) and the internal standard (bottom), together with suggested fragmentation losses of zolpidem.

11.87 g/l, adjusted at pH 7.6 – and ammonium formate were purchased from Fluka (Saint-Quentin Fallavier, France). Diazepam-d_5 was purchased from Promochem (Molsheim, France).

2.3. Zolpidem extraction

Five hundred microliters of oral fluid, treated with 500 μl of Soerensen buffer were extracted by 2 ml dichloromethane, in presence of 5 ng of diazepam-d_5 used as internal standard (IS). After agitation and centrifugation (10,000 × g for 15 min), the supernatant was collected and evaporated to dryness under nitrogen flow. The residue was reconstituted by adding 50 μl of acetonitrile.

2.4. LC–MS/MS procedure

A 10 μl aliquot of the extract was injected onto the column (5 μm Novapak C18, 150 mm × 2.1 mm i.d., 3.5 μm) protected by a 0.5 μm frit. Each 6 min chromatographic run was carried out with an isocratic binary mobile phase of 90% acetonitrile – 10% 2 mM NH_4COOH pH 3.6, at a flow rate of 150 μl/min. The HPLC system was a Waters Alliance 2695.

Table 1
MRM transitions and conditions for the measurement of zolpidem and the internal standard

Compound	Parent ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Zolpidem	308.2	235.3	70	35
		263.2	70	26
Diazepam-d_5	290.1	154.1	60	30
		198.2	60	30

Detection was carried out by a Micromass Quattro Micro tandem mass spectrometer equipped with an ion spray atmospheric pressure (electrospray) interface. Nitrogen (purity grade 99.95%) was employed as nebulizer gas and curtain gas. The instrument was operated in the positive ionization mode. The following conditions were found to be optimal for the analysis of zolpidem and the IS: capillary voltage, 1.0 kV; source block temperature, 120 °C; and desolvatation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 550 l/h.

In order to establish appropriate multiple reaction monitoring (MRM) conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation (CID) of both species was

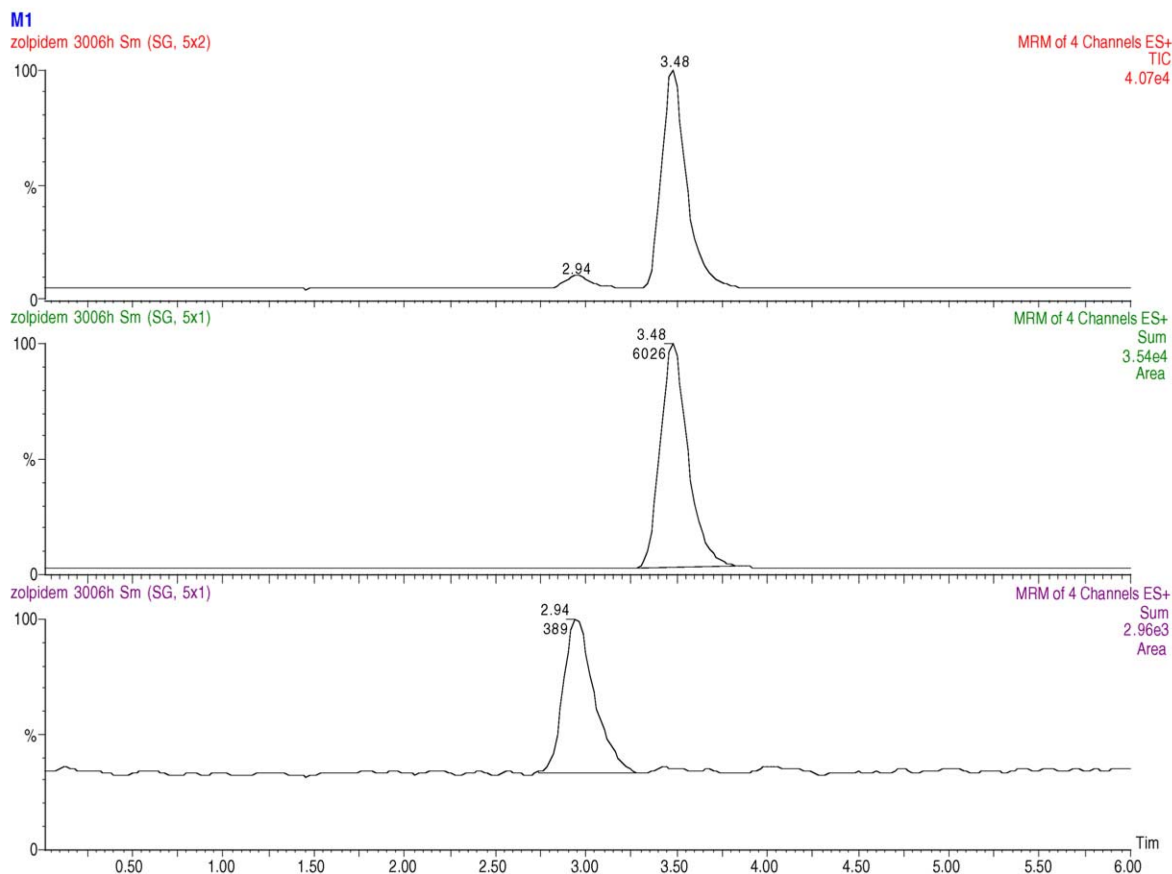

Fig. 2. Chromatogram obtained after extraction by the established procedure of an oral fluid specimen. Zolpidem was quantified at the concentration of 0.4 ng/ml. Top: MRM chromatogram, mid: sum of the 2 daughter ions of diazepam-d_5, and bottom: sum of the 2 daughter ions of zolpidem.

performed. Collision gas (argon) pressure was maintained at 3.0×10^{-3} mbar and the collision energy (eV) was adjusted to optimize the signal for the two most abundant product ions. (Table 1).

Molecular ions (*m/z* 308 and 290 for zolpidem and the IS, respectively) were selected in Q1 and the corresponding daughter ions (*m/z* 235 and 263 for zolpidem and *m/z* 154 and 198 for the IS) were detected in Q3.

2.5. Method validation

A standard calibration curve was obtained by preparing standards containing 0.2, 0.5, 1, 5, 10, 50 and 100 ng/ml of spiked zolpidem. Within-batch and between-batch precisions were determined using fresh oral fluid, spiked with zolpidem at 0.2 and 10 and 40 ng/ml, respectively.

The detection limit (LOD) was evaluated by decreasing concentrations of zolpidem until a response equivalent to three times the background noise was observed.

Recovery was established at 10 ng/ml, by comparing the representative peak area of zolpidem extracted from negative control oral fluid spiked at the final concentration of 10 ng/ml with the peak area of a methanolic standard at the same concentration.

3. Results and discussion

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous materials present in oral fluid. There was no interference detectable in blank oral fluid.

A full mass spectrum of the fragmentation of the precursor ion for both zolpidem and the internal standard, together with suggested fragmentation losses of zolpidem which result in the product ions used for analysis are given in Fig. 1.

Figs. 2 and 3 are typical chromatograms obtained from authentic oral fluid specimens, with concentrations of zolpidem of 0.4 and 41 ng/ml, respectively.

Retention times of zolpidem and the deuterated internal standard were 2.9 and 3.5 min, respectively. Diazepam-d_5 was choosen as IS as there is no commercially deuterated zolpidem available on the market and because the method

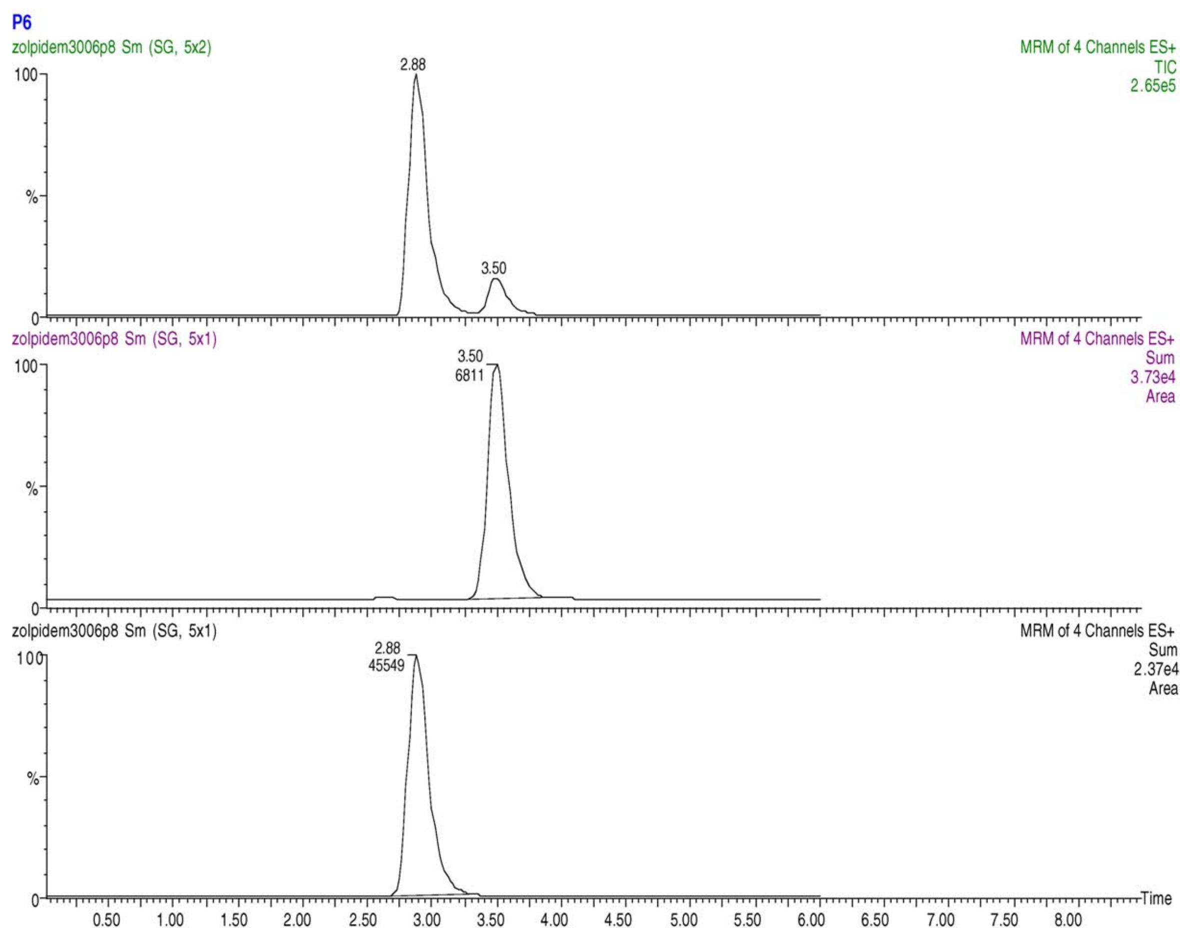

Fig. 3. Chromatogram obtained after extraction by the established procedure of an oral fluid specimen. Zolpidem was quantified at the concentration of 41 ng/ml. Top: MRM chromatogram, mid: sum of the 2 daughter ions of diazepam-d_5, and bottom: sum of the 2 daughter ions of zolpidem.

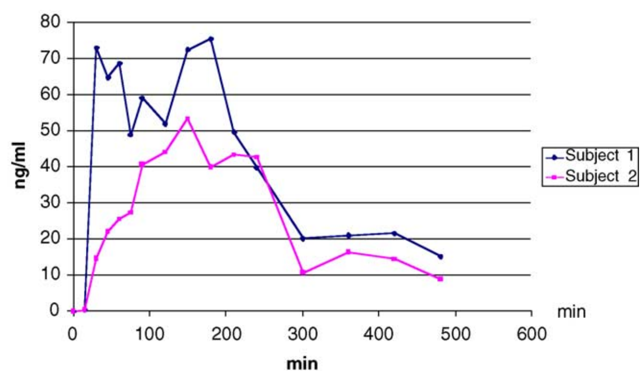

Fig. 4. Excretion pattern in oral fluid of zolpidem after oral administration of 10 mg to 2 subjects.

is part of a general screening for benzodiazepines and hypnotics.

The calibration curve corresponds to the linear regression between the peak-area ratio of zolpidem to IS and the final concentration of the drug in spiked oral fluid. Responses for zolpidem were linear in the range 0.2–100 ng/ml, with a correlation coefficient of 0.997. The within-batch precisions were 15.1 and 8.8%, as determined by analyzing 8 replicates of specimens spiked with zolpidem concentration at 0.2 and 10 ng/ml, respectively. The between-batch precision was 15.3%, as determined by analyzing 8 replicates of specimens spiked with zolpidem concentration at 40 ng/ml.

The limit of quantitation was the first point of the calibration curve, that is 0.2 ng/ml, and the limit of detection was 0.05 ng/ml. Recovery of zolpidem at 10 ng/ml was estimated at 96.5%.

After oral administration of 10 mg zolpidem to 2 subjects, 16 specimens of oral fluid were collected over 480 min. The excretion pattern of zolpidem in oral fluid is given Fig. 4. In both cases, zolpidem was detectable (0.4 ng/ml) after 15 min intake. Peak zolpidem concentrations were obtained after 150 min (53.5 ng/ml) and 180 min (75.7 ng/ml), respectively. Oral fluid still tested positive for zolpidem at 8 h (9–15 ng/ml).

In comparison with data from blood pharmacokinetics obtained in the literature [4], it appears that the oral fluid/blood ratio is lower than 1. These results suggest that oral fluid will not enhance the window of detection of zolpidem in comparison with blood, and therefore there is no need to collect it in drug-facilitated crimes, in addition to the 3 mandatory specimens (blood, urine, hair).

4. Conclusion

This sensitive, specific and reproducible method developed is suitable for the detection and quantification of zolpidem in human oral fluid. It is easy to use and is particularly rapid, as a valid result can be obtained within 1 h. This technology may find useful applications, in forensic cases, but the definition of legally defensible cut-off values would require more data.

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