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Journal of Chromatography B, 789 (2003) 131–138
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

# Determination of zaleplon and zolpidem by liquid chromatography–turbo-ionspray mass spectrometry: application to forensic cases

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**Abstract**

Zolpidem and zaleplon are two short-acting hypnotic agents used in Europe and in the USA. An atmospheric pressure ionisation liquid chromatography–mass spectrometry (Sciex API 150 EX) method was developed for the determination of zolpidem and zaleplon in whole blood. After single-step liquid–liquid extraction, the hypnotics were separated by gradient-elution with an ammonium formate buffer/acetonitrile eluent on an Inertsil ODS-3 column. Methaqualone was used as internal standard. The recovery was higher than 70% for both hypnotics and the internal standard. The best fit for the calibration curve was achieved, between 1 and 250 ng/ml, with $1/x$ quadratic regression. Coefficients of intra- and inter-assay variation calculated at 5, 25 and 100 ng/ml were less than 10%. The method was successfully applied to forensic cases.


*Keywords:* Intoxication; Zaleplon; Zolpidem

## 1. Introduction

Both zaleplon (Sonata®), a pyrazolopyrimidine, and zolpidem (Stilnox®, Ambien®, Ivadal®), an imidazopyridine derivative, are sedative and hypnotic agents with a chemical structure unrelated to benzodiazepines (Fig. 1) [1]. They are used in the short-term management of insomnia. Both hypnotics have several side-effects (e.g. visual disturbance, hallucinations, hypotension), including abuse potential, interactions with other CNS depressants, impairment of psychomotor performance and memory and risk of overdose. Compared with zolpidem, zaleplon produces less sedation and memory deficit and its side-effects are of shorter duration. Moreover, it was shown that zaleplon has no residual effects on driving, divided attention, or memory when taken at bedtime, 10 h before driving [2]. These two compounds are marketed in Switzerland, Germany and in the US. Zolpidem has been sold in France since 1988. The main pharmacokinetic parameters of zaleplon [1] and zolpidem [1,3] are listed in Table 1. After oral administration, zaleplon is extensively metabolised by oxidation with less than 1% of the dose excreted unchanged in urine. All zaleplon metabolites are pharmacologically inactive. Zolpidem is also converted into inactive metabolites that are mainly eliminated by renal excretion. Zaleplon interacts with other drugs by a variety of mechanisms. For instance, zaleplon potentiated the CNS-impairing effects of ethanol under various psychopharmacological settings [4]. Zolpidem intoxication can be mistaken for narcotic overdose be-

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1570-0232/03/$ – see front matter 
doi:10.1016/S1570-0232(03)00135-1

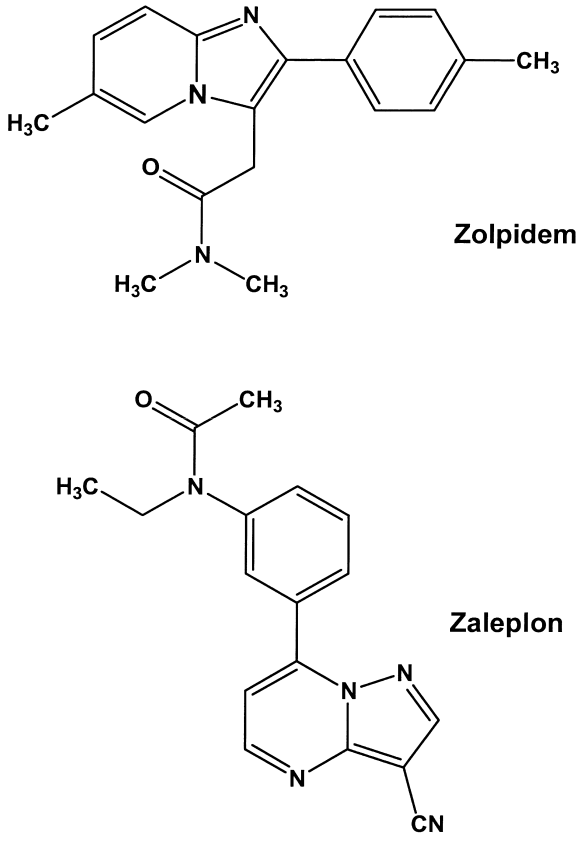


Fig. 1. Chemical structures of zolpidem, an imidazopyridine and of zaleplon, a pyrazolopyrimidine derivative.

cause they present similar symptoms [5]. Many acute non-lethal intoxications [6,7] and a few with fatal outcome [7–12] have been reported for zolpidem. A total of 344 cases of intentional acute overdose have been reviewed retrospectively. It is noteworthy that half of the patients ingested other substances (psychotropic drugs and alcohol) concomitantly [7]. Very high levels (blood: 7.9 μg/ml) were measured in a fatality involving zolpidem [13]. Although the blood concentration was very high suggesting drug overdose, the cause of death was drowning. This suggests that, unless other CNS depressive agents are taken with zolpidem, death attributable to zolpidem overdosage alone is an unlikely occurrence [7,13]. A suicide case involving zolpidem overdose and hypothermia has also been reported [14]. As far as we know, data concerning zaleplon are very rare. At present, immunoassays dedicated to the detection of zaleplon or zolpidem in biofluids are not yet commercially available, therefore their detection relies only on chromatographic procedures. Several analytical methods, using HPLC with fluorescence [14–18] or diode-array detection [19] or GC [9–11,13,20], have been already described for the determination of zolpidem. One reports the use of thermospray liquid chromatography tandem mass spectrometry for the profiling of zolpidem metabolites [21].

Table 1
Main pharmacokinetic parameters of zolpidem (Stilnox®) and zaleplon (Sonata®)

| Parameter | Zolpidem | Zaleplon |
|---|---|---|
| Defined daily dose (DDD) (mg) | 10 | 10 |
| $C_{max}$ (ng/ml) | 140 | 29 |
| $T_{max}$ (h) | 1.7 | 1.0 |
| $T_{1/2}$ (h) | 2.4 | 1.2 |
| $F$ (%) | 67 | 31 |
| $V$ (l/kg) | 0.5 | 1.4 |

Several LC–MS techniques with atmospheric pressure ionisation (API) have been reported for hypnotics belonging to the benzodiazepines class [22]. Here we present a new API LC–MS method able to quantify in one single analysis zaleplon and zolpidem, two non-benzodiazepine hypnotics, in the low therapeutic range. The usefulness of this method is illustrated with several forensic cases involving zaleplon or zolpidem use.

## 2. Experimental

### 2.1. Materials and solvents

Drug standards of zolpidem and methaqualone were purchased from Tocris (Anawa Trading, Zürich, Switzerland) and Cooper (Fribourg, Switzerland), respectively. Zaleplon was a gift from Wyeth (Zug, Switzerland). A 10 mg/10 ml stock solution of each compound in methanol was prepared and stored at −20 °C. Working standards were prepared by dilution in methanol (10 and 1 μg/ml) using a Hamilton syringe (Hamilton, Bonaduz, Switzerland). Acetonitrile (HPLC grade, quality gradient) was obtained from Riedel-de Haën (Fluka, Buchs, Switzerland). Ammonium hydroxide solution 25%, formic acid solution 50%, ethyl acetate (analytical

reagent grade) and ammonium formate (Biochemika grade) were purchased from Fluka. Dichloromethane (Suprasolv grade), *n*-hexane and ammonium chloride (analytical reagent grade) were purchased from VWR Merck Eurolab (Dietikon, Switzerland). Deionized water was purified by a Milli-Q system (Millipore). The ammonia buffer was prepared as follows: ammonium chloride was added to 1 l of distilled water up to saturation, and the pH was then adjusted to 9.5 with a 25% ammonium hydroxide solution. The buffer was stored at room temperature.

### 2.2. Biological specimens

The blank blood samples were obtained from the local hospital blood bank. Blood samples from intoxicated people were taken during autopsy (peripheral blood), at hospital or from drivers suspected of driving under the influence of drugs. They were collected in 5.5-ml S-Monovettes containing 1 mg fluoride/ml and 1.2 mg EDTA/ml blood as preservatives (Sarstedt, Sevelen, Switzerland).

### 2.3. Instrumentation

The LC–MS system consisted of two high-pressure Perkin-Elmer Series 200 micro pumps, a Series 200 autosampler and a Lee-Visco-Jet micro-mixer with 10-μl internal volume connected to an Applied Biosystems MDS Sciex API 150EX single quadrupole system (Applera Europe, Rotkreuz, Switzerland). For atmospheric pressure ionisation, a turboionspray interface was used. The modules were controlled by a MacIntosh computer running OS 8.1 and data collection was performed using MassChrom 1.4 software. Quantitative results were processed with TurboQuan 1.0 software. The calibration curves were obtained by weighted ($1/x$) least-squares quadratic regression analysis.

### 2.4. Extraction procedure

The internal standard (100 μl methaqualone in 1 μg/ml methanol) and the zolpidem and zaleplon standards were added to 10-ml Pyrex SVL tubes (GlasKeller, Basel, Switzerland) and taken to dryness under $N_2$. Then 1 ml whole blood and 1 ml saturated ammonia buffer, pH 9.5 were added and mixed for 1 min with a vortex mixer. After addition of 5 ml of a mixture of dichloromethane: hexane:ethyl acetate (5:4:1; v/v/v) with a digital dispenser (Calibrex 520, Socorex, ReactoLab, Servion, Switzerland), the hypnotics were extracted for 30 min on a horizontal shaker at 200 backward and forward motions/min (Edmund Bühler, GlasKeller, Basel). After centrifugation (30 min, 2000 rpm), the upper phase was collected, taken to dryness under $N_2$ and the dried extract was reconstituted into 100 μl of the HPLC starting eluent prior to LC–MS analysis. Blood specimens were appropriately diluted (generally two or ten times) in order to yield results inside the quantitation range.

### 2.5. Chromatography

A Chrompack Inertsil ODS-3 column (150 mm × 2.0 mm I.D., particle size 3 μm) and guard columns packed with the same material were purchased from Stehelin (Basel, Switzerland). Zolpidem and zaleplon were separated at room temperature (23 °C) by gradient-elution using a flow-rate of 200 μl/min and an acetonitrile/1 m*M* ammonium formate buffer eluent. The pH was adjusted to 4.0 with formic acid. For the first 2 min, an isocratic elution was performed with 10% acetonitrile. From then on, a gradient was started and the amount of acetonitrile increased linearly to 60% in 15 min. This concentration was maintained for 3 min. Subsequently, the system was returned to its initial conditions in 1 min and equilibrated for 10 min before injection of the next sample.

### 2.6. Mass spectrometric conditions

The mass spectrometer was used in the positive mode. Nitrogen was used as nebulizing, heater and curtain gas. The gas flows were set to ~1.2 (nebulizer gas setting: 12), 7 and 1.0 l/min (curtain gas setting: 10), respectively. The turbo probe temperature was 475 °C and the ionspray voltage was set at 5000 V. The voltages of the orifice and of the focusing ring were optimised for each ion separately by flow injection at a flow-rate of 200 μl/min. The composition of the eluent was 50% acetonitrile and 50% formate ammonium buffer. The following ions corresponding to the protonated molecules were monitored in the SIM mode for quantification: 308.21 (zolpidem), 306.21 (zaleplon) and 251.11

(methaqualone). The dwell-time was set at 200 ms. The following orifice and focusing ring voltages were applied: zolpidem: 40 and 175 V, zaleplon: 35 and 175 V, methaqualone: 20 and 150 V, respectively. Calibration of the mass analyser was performed by infusion of a PPG (polypropylene glycol) standard mixture using a Harvard syringe pump at a flow-rate of 0.6 ml/h.

### *2.7. Validation*

The evaluation of linearity was carried out with blood. Blank matrix was spiked with the internal standard (100 ng methaqualone/ml) and with zolpidem and zaleplon at eight different levels, yielding calibration points at 1, 2, 5, 10, 25, 50, 100 and 250 ng/ml. The recoveries were determined by spiking a blood-blank matrix with 100 ng/ml of zaleplon, zolpidem and methaqualone before and after sample preparation ($n=4$). Matrix suppression was studied by extracting a blank matrix and spiking the extract with both hypnotics and the internal standard at a concentration of 100 ng/ml in the organic layer ($n=4$).

These samples were compared to reference samples that were prepared by spiking the same substances into pure extraction solvent. The suppression effect was then assessed by calculating the area ratio between the peak areas obtained in the presence and in the absence of co-extracted potential interferences. In order to determine the limit of detection, samples containing very low concentrations of zolpidem and zaleplon (0.5 and 0.1 ng/ml) were prepared and analysed between two blank probes. The selectivity of the method was studied by including in each batch one blank sample containing no internal standard and one specimen spiked with methaqualone only. Furthermore, samples known to contain a single hypnotic were monitored and checked for the absence of the other one.

## 3. Results and discussion

A chromatographic profile of all ions (TIC) and extracted ion chromatograms (XIC) of zolpidem ($m/z=308.2$), zaleplon ($m/z=306.2$) and of the internal standard (methaqualone, $m/z=251.1$) at a concentration of 100 ng/ml are shown in Fig. 2. All peaks are symmetric and well resolved. No interference of other molecules could be detected. Matrix suppression was found to be insignificant for all three substances and remained lower than 10% at the tested concentration (100 ng/ml). In a first experiment, the mean recoveries were found in the range of 70–76% at 100 ng/ml ($n=4$). In a second experiment carried out with new unscratched tubes, a mean recovery higher than 90% could be determined for all three substances. Thus, binding of the molecules to the glass can be postulated and this mechanism is very likely enhanced when the glass walls are scratched exposing more active groups. At 0.1 ng/ml, the signal-to-noise ratio was ~5 for both hypnotics indicating that their detection limits (LOD) are ~0.1 ng/ml. Typical calibration curves are shown in Fig. 3; they were constructed on eight points ranging from 1 to 250 ng/ml whole blood. The best curve fitting was achieved with a $1/x$ weighted quadratic regression in the range 1–250 ng/ml. The correlation coefficient $r^2$ was always higher than 0.999. Furthermore, low values were not underestimated and the $y$ intercept was close to zero. The technique of non-linear calibration has been applied before with success [23].

The method accuracy ranged from 91.4 to 100%. Precision data were recorded on day 1 and at different days. The within-day (WD) and between-day precisions (BD) were determined at 5, 25 and 100 ng/ml ($n=5$) and found to be satisfactory and less than 10%. The main statistical parameters dealing with the validation of the LC–MS method are displayed in Table 2.

For the limit of quantification (LOQ), the lowest point of the calibration curve, 1 ng/ml for both compounds, was adopted. At this concentration, an accuracy of 89.4 and 86.5% was determined for zolpidem and zaleplon, respectively. The precision remained lower than 20% for both compounds (16.6% for zolpidem and 5.9% for zaleplon, five determinations).

## 4. Case reports

The method is routinely used for the determination of zaleplon and zolpidem in forensic cases. Results

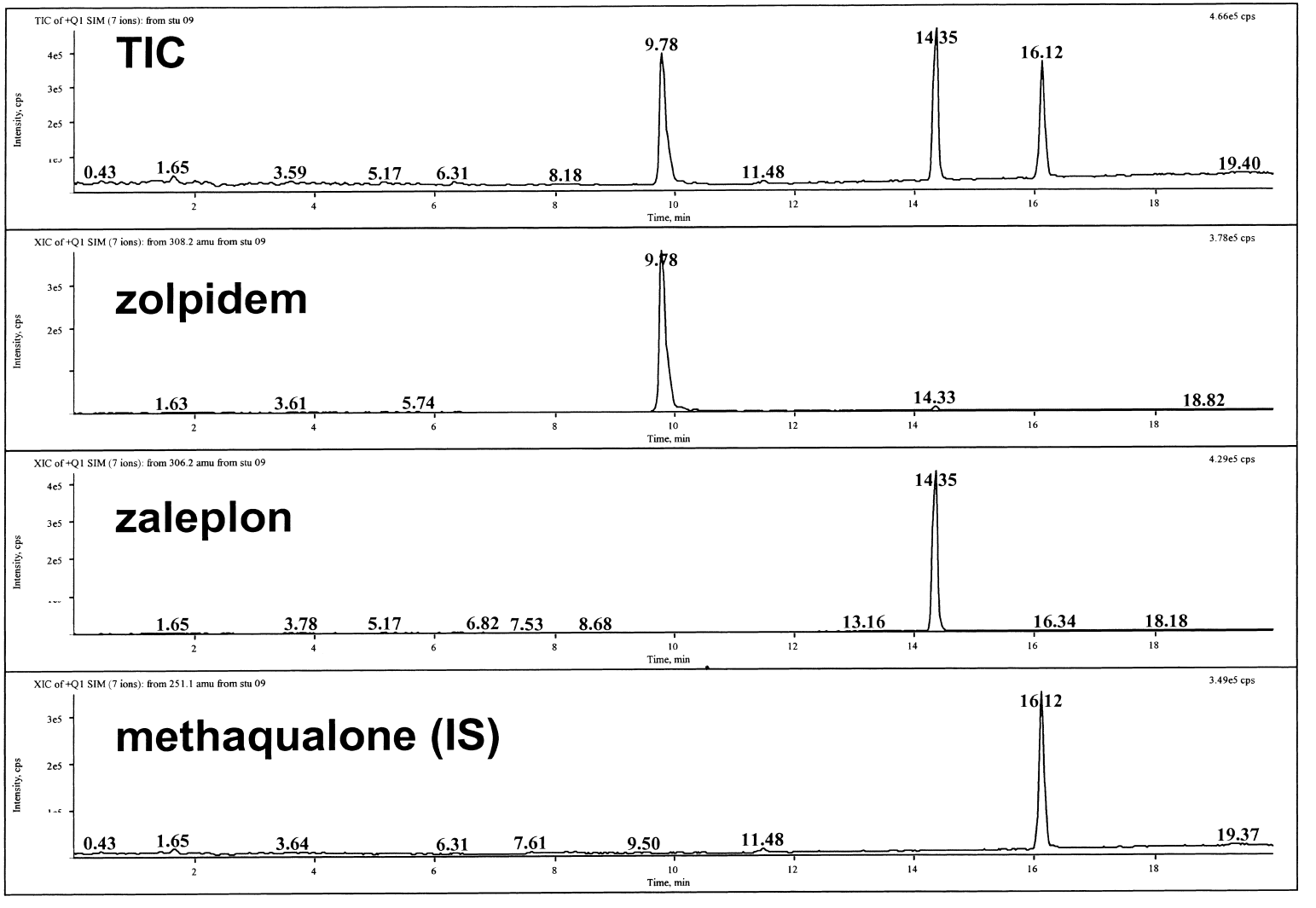


Fig. 2. LC–MS profiles of zolpidem, zaleplon, methaqualone (I.S.) and total ion chromatogram (TIC) of a blood extract containing 100 ng/ml of each molecule.

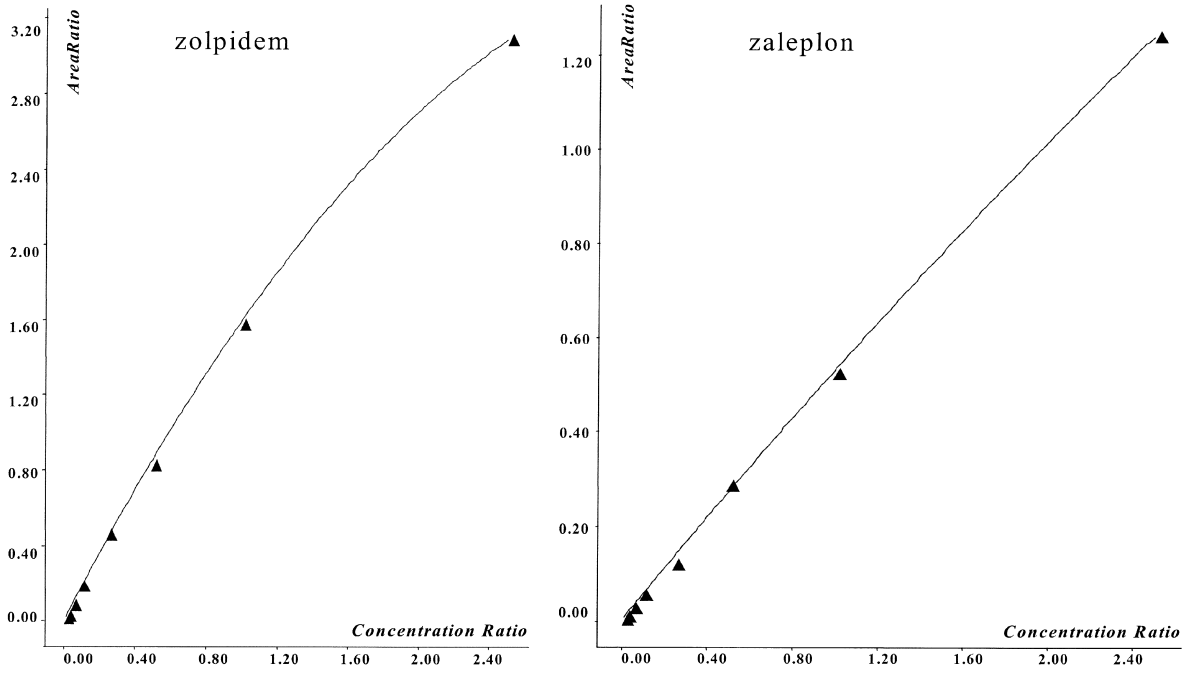


Fig. 3. Calibration curves of zolpidem and zaleplon from 1 to 250 ng/ml whole blood. Equations for zolpidem: $y = -0.23810*x^2 + 1.83078*x + 0.00508$; and for zaleplon: $y = -0.02253*x^2 + 0.55476*x + 0.00162$.

concerning a suicide attempt with zolpidem and two car accidents involving zaleplon ingestion, one with a fatal issue, are presented in Table 3.

### 4.1. Case 1

A murderess attempted to commit suicide by ingesting a dozen of tablets of Stilnox®. Blood and urine samples were taken ~6 h later. A comprehensive drug screening carried out by immunoassays and GC–MS revealed the presence of zolpidem only. A toxic blood level of 2.5 mg/l and a urine concentration of 1.9 mg/l were measured by LC–MS.

### 4.2. Case 2

A poly-drug user died as a result of a stolen-car accident. Ethanol (1.6 g/kg) and zaleplon were detected in blood. A therapeutic level of 37.4 ng/ml peripheral blood was determined by LC–MS suggesting that the driver was under the combined influence of alcohol and zaleplon. Zaleplon is known

Table 2
Accuracy (deviation from the target value), within-day (WD) precision and between-day (BD) precision

| Drug | Concentration (ng/ml) | Accuracy (%) | WD precision, RSD (%) ($n=5$) | BD precision, RSD (%) ($n=5$) |
|---|---|---|---|---|
| Zolpidem | 5 | 96.0 | 4.2 | 5.5 |
| | 25 | 95.2 | 9.9 | 4.8 |
| | 100 | 91.4 | 9.9 | 7.6 |
| Zaleplon | 5 | 100.0 | 8.0 | 6.7 |
| | 25 | 98.8 | 2.4 | 4.5 |
| | 100 | 98.0 | 2.4 | 2.0 |

RSD, relative standard deviation.

Table 3
Case reports for zaleplon and zolpidem

| Case | Circumstances | Zolpidem (ng/ml) | Zaleplon (ng/ml) | Conclusions |
|---|---|---|---|---|
| 1 | Suicide attempt by a murderess, ingestion of 12 tablets of Stilnox® | 2500 | – | Acute intoxication with zolpidem |
| 2 | Death of a poly-drug user in a stolen-car accident | – | 37.4 | Driving and car accident under the combined influence of alcohol (1.7 g/kg) and zaleplon |
| 3 | Car accident, ingestion of six tablets of Sonata® 10 mg | – | 40.3 | Driving and car accident under the influence of zaleplon |

Blood levels were determined by LC–MS.

to interact with alcohol and to potentiate its CNS depressant effects [4].

### *4.3. Case 3*

In the last case, a driver reported to have ingested about six tablets of Sonata® each containing 10 mg zaleplon before having a car crash. Zaleplon only was detected in urine following a drug screening performed by immunoassays and GC–MS on urine and blood specimens taken ~2 h later. A therapeutic concentration of zaleplon (40.3 ng/ml) was determined in the blood. The urine level was 35.4 ng/ml. Taking into account an elimination half-life of 1 h, the blood level at the time of accident could have been four times higher, a concentration which is much higher than therapeutic $C_{max}$ levels reported for zaleplon (28.9±13.9 ng/ml for a 10-mg oral dose). This calculation suggests that the driver was under the influence of zaleplon at the time of the car accident with dramatically hampered driving capacity.

## 5. Conclusions

A new and validated method for the determination of zolpidem and zaleplon in whole blood has been developed and successfully applied to forensic cases. Furthermore, turbo-ionspray LC–MS proved to be sensitive, specific and suitable for routine analysis of forensic cases.

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