

Short Communication

Comparison of Zopiclone Concentrations in Oral Fluid Sampled with Intercept® Oral Specimen Collection Device and Statsure Saliva Sampler™ and Concentrations in Blood

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Abstract

A clinical study of zopiclone was performed using doses of 5 and 10 mg. Samples of oral fluid were collected using the Statsure and Intercept devices, and blood samples were collected simultaneously. Concentrations of zopiclone in samples of oral fluid and blood were determined with liquid chromatography–mass spectrometry, and concentrations in undiluted oral fluid were calculated. The concentrations of zopiclone in oral fluid were generally higher when using the Intercept compared to the Statsure device; the median oral fluid/whole blood concentration ratios were 3.8 (range 1.5–15.9) and 1.9 (range 1.2–4.6), respectively ($n = 21$). The correlation between zopiclone concentrations in oral fluid collected with the two devices was fairly poor, $r^2 = 0.35$. The results indicate that the type of sampling device may significantly affect the analytical result for zopiclone in sampled oral fluid.

Introduction

Zopiclone is the most frequently prescribed hypnotic drug in Norway. In 2008, 6.4% of the population had one or more prescriptions of zopiclone dispensed from a pharmacy with an average of 171 defined daily doses dispensed per patient (1). Zopiclone was also the most frequently found drug in a Norwegian roadside survey of drugs and driving performed in 2005–2006 (2); 1.0% of 10,816 drivers had zopiclone concentrations above 25 ng/mL in oral fluid. The Intercept oral fluid collection device was used in that study. A similar roadside survey was performed in 2008–2009; this study was a part of

the European DRUID project (3). In this study, the Statsure collection device was used. Zopiclone was also the most frequently drug detected; however, 0.7% of 9391 drivers had concentrations above 25 ng/mL in oral fluid, which is significantly lower than in the previous study ($p < 0.02$).

Drug concentrations in collected oral fluid may be affected by the sampling process and by the fluid collection device in itself (4–6). The oral fluid collection pad used in the Intercept device is made of cotton treated with a solution containing sodium chloride, citric acid, sodium benzoate, potassium sorbate, gelatine, and sodium hydroxide, according to the package insert. Some of these compounds stimulate the production of saliva and may also affect the local pH and may therefore affect the concentration of zopiclone in the sampled oral fluid. The oral fluid collection pad used in the Statsure device is made with cellulose and is not treated with any chemicals to stimulate the production of saliva (6). These differences between the sampling devices might affect the zopiclone concentration in collected oral fluid samples. In addition, the measured concentrations in oral fluid also depend on the recovery of zopiclone from the sampling device.

On this background we decided to compare the zopiclone concentrations in oral fluid collected with the two devices to see whether the use of different collection devices could explain the differences in median zopiclone concentrations observed in the two roadside surveys of drugs and driving.

Materials and Methods

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from Lab-Scan (Poch S.A., Gliwice, Poland), ammonium acetate from Merck (Darmstadt, Ger-

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many), zopiclone from European Directorate for the Quality of Medicines and Healthcare (European Pharmacopoeia, Strasbourg, France), and 7-aminoflunitrazepam-d₇ and diazepam-d₅ from Cerilliant (Round Rock, TX).

Analytical testing

Samples of oral fluid were weighed to determine the amount collected. As the specific gravity of oral fluid is 1.002–1.006 g/mL (7,8), the weight in grams is a close approximation of the volume in milliliters. The concentrations of zopiclone in samples of preserved oral fluid were analyzed by HPLC and tandem mass spectrometry (MS) after extracting with ethylacetate/heptane (4:1) (9). Five calibration standards ranging from 0.4 to 150 ng/mL in preserved oral fluid were used. The oral fluid was diluted by a factor of 3 for the Intercept device and 2 for the Stature device. 7-Aminoflunitrazepam-d₇ was used as internal standard. Quality control (QC) samples were prepared at concentrations 0.8, 27, and 78 ng/mL.

The Intercept device contains 0.8 mL buffer and the Stature device 1.0 mL. The collected oral fluid was therefore diluted with these buffer volumes after sampling. Drug concentrations in undiluted oral fluid (C_{UOF}) were calculated as follows: $C_{UOF} = C_{OFB} \times (V_{Buffer} + V_{OF}) / V_{OF}$, where C_{OFB} = drug concentration in oral fluid-buffer mixture, V_{Buffer} = volume of buffer, and V_{OF} = volume of oral fluid.

Concentrations of zopiclone in full blood were determined by HPLC–MS using a Waters ZQ MS with a 2695 Alliance pump (Waters, Milford, MA). Six zopiclone calibration standards with concentrations ranging from 10 to 583 ng/mL were used. QC samples were prepared at 27 and 389 ng/mL.

Calibrators and QC samples were prepared in advance in acetonitrile solutions in glass vials stored in a freezer at –20°C. After reaching room temperature, 50 µL from each solution was transferred to plastic tubes, followed by 50 µL water before 400 µL whole blood from the national blood bank services was added.

Calibrators, QC samples, and samples were all added to 50 µL of the internal standard diazepam-d₅ (2900 ng/mL) and then vortex mixed. Keeping the samples cold with an ice bath, 500 µL cold acetonitrile (approximately 4°C) was added, and each individual tube was immediately vortex mixed. Finally all the samples were shaken vigorously on a multitube vortexer for 60 s, put in a freezer at about –20°C for at least 10 min and then centrifuged for 10 min at 6000 × *g* at 4°C. The supernatant was transferred to plastic autosampler vials while keeping the samples cold and analyzed by LC–MS.

Separation was performed with a Waters Symmetry C18-column or X-terra MS-column (2.1 × 150 mm, 3.5 µm), with gradient elution at a flow rate of 0.3 mL/min. The mobile phase consisted of acetonitrile and 5 mM ammonium acetate buffer at pH 5 using a gradient from 30% to 80% acetonitrile in 8 min followed by a wash step. The pre-column volume was set to 0.95 mL. The overall cycle time was 15 min. The column temperature was held at 35°C, and the injection volume was 10 µL.

The samples were analyzed by LC–MS using positive ionization in the selected ion monitoring (SIM) mode. The capillary voltage was set to 3.0 kV, the source block temperature was

120°C, and the desolvation gas (nitrogen) was heated to 300°C and delivered at a flow rate of 500 L/h. The cone gas (nitrogen) was set to 70 L/h. System operation and data acquisition were controlled using Mass Lynx 4.1 software. Data were processed with the QuanLynx program.

The molecular ion of zopiclone (m/z 389.1) and the internal standard diazepam-d₅ (m/z 285.1) and one fragment ion of both zopiclone (m/z 245.1) and the internal standard (m/z 198.1) were recorded. Analytes were identified by comparing the retention time and ion ratio with those of the calibrators and QC samples. Based on peak heights, the response of the molecular ion of zopiclone relative to that of the internal standard was used for quantitation. A weighted (1/*x*) second-order regression line, excluding the origin, was applied to the calibration standards.

Clinical study

Sixteen healthy volunteers participated in a study of zopiclone; five of them participated two days with two different doses of zopiclone. The study was primarily designed as a double-blinded crossover study to investigate pharmacokinetics and psychomotor abilities, and a large number of samples of blood and oral fluid were taken for those purposes (results will be published separately). The time points for additional sampling of oral fluid for comparison of collection devices, and which participants to include, were decided based on practical reasons to avoid interfering with the primary study.

Doses of 5 or 10 mg zopiclone were given to 6 and 15 participants, respectively, at about 9 a.m., and blood and oral fluid samples were taken at specified time points after drug administration. The earliest sampling was performed about 1 h after administration, and the latest one about 10 h after administration. Breakfast and dinner were served about 2½–3 h and 8 h after administration of zopiclone, respectively, and oral fluid samples were collected at least 2 h after intake of food.

Collection of oral fluid and blood

The Stature Saliva Sampler (Saliva Diagnostic Systems, Framingham, MA) was used to collect a sample of oral fluid shortly before using the Intercept Oral Specimen Collection Device (OraSure Technologies, Bethlehem, PA). This sampling sequence was used in all cases because the Intercept device contains chemicals that stimulate the production of oral fluid, and the Stature device does not. In addition, samples of oral fluid were collected using the Intercept device 1–3 h before using the Stature device.

Samples of whole blood were collected using 5-mL Vacutainer® tubes containing 20 mg sodium fluoride and 143 I.U. sodium heparin (BD Vacutainer Systems, Bellerive Industrial Estate, Plymouth, U.K.). Blood sampling was performed simultaneously with the collection of the oral fluid samples.

Oral fluid and blood samples were stored in a refrigerator at about 5°C for up to 24 h after sampling, and then either analyzed or frozen at about –20°C for later analysis.

Statistical tests

Pearson's Chi-Square test was used for comparing preva-

lences. The two-sided Wilcoxon Signed-Rank test was used for comparing paired OF/B ratios for different collection devices.

Results and Discussion

Method validation

Some validation of the analytical method for oral fluid has been published previously for a number of drugs (9), and some additional data for zopiclone are presented here. The limit of detection (LOD, defined as a peak height corresponding to the mean of negative specimens plus 3 times the standard deviation) was 0.1 ng/mL, the lower limit of quantitation (LLOQ, defined as defined as a peak height corresponding to the mean of negative specimens plus 10 times the standard deviation) was 0.3 ng/mL. The upper limit of quantitation (ULOQ) was 150 ng/mL, determined by inspection of calibration curves. The interassay precision and accuracy expressed as bias was 16 to 19% and -2 to -6% respectively ($n = 26$). Recoveries for the liquid-liquid extraction of zopiclone from the oral fluid/buffer mixtures were 60% for the StatSure buffer and 65% for the Intercept buffer. Matrix effects evaluated by the method proposed by Matuszewski et al. (10) were 165% for StatSure and 88% for Intercept when compared to neat mobile phase, and 114% and 80% when compared to extracted buffer. Relative matrix effects expressed as the coefficient of variation were 5% for StatSure and 23% for Intercept. The recoveries from the sampling devices were 74% for StatSure and 80% for Intercept devices.

For the analysis of zopiclone in blood, the interassay precisions for low and high QC samples were 12% and 5% ($n = 14$), respectively, and the accuracy expressed as bias was 1.2% and -7%. LOD and LLOQ were determined by analysing 7 different zopiclone negative whole blood specimens. A total of 11 series were analyzed, differing either in day of analysis or analyst. The LOD and LLOQ were 3 ng/mL and 7 ng/mL, respectively. The ULOQ was 1170 ng/mL.

To evaluate recovery and matrix effects, samples from six different lots of human whole blood were used. Three sets of samples were prepared. Set A consisted of extracts of the six

blank matrixes spiked before extraction, set B of extracts of the six different blank matrixes spiked after extraction with the same amount of standards as used for set A, and set C consisted of neat standard solutions. The extraction recovery (RE) was determined by measuring an extracted sample against a post-extraction spiked sample: $RE = (A/B) \times 100\%$, where A is the peak height for the extracted samples and B the peak height for post-extracted samples. The matrix effect (M) was calculated by referring the peak height of the samples spiked after extraction (B) with the height found for the neat standards (C): $M = (B/C) \times 100\%$, where B is the peak height for the post-extracted samples and C is the peak height for the neat standards.

Set A was prepared by mixing 50 μ L of a zopiclone solution in acetonitrile (two concentration levels) with 50 μ L water and 400 μ L whole blood, whereas set B was prepared by mixing 50 μ L acetonitrile, 50 μ L water, and 400 μ L whole blood. After the protein precipitation procedure 200 μ L of each extracted sample was transferred to glass tubes. 20 μ L of zopiclone solution was added to set B, and the extracts from both set A and B were evaporated to dryness under N_2 at 40°C using a Zymark Turbovap (Sotax AG, Basel, Switzerland). The residue was then dissolved in 180 μ L acetonitrile/water (50:50, v/v), and 20 μ L acetonitrile was added. To prepare the neat standards, 20 μ L zopiclone solution in acetonitrile was added to 180 μ L of the reconstitution solution.

Recovery and matrix effects were calculated at two concentration levels and found to be 76% and 128% at 29 ng/mL and 81% and 112% at 389 ng/mL, respectively. The relative matrix effect as given by the relative coefficient of variation was 15% at the low concentration level and 7% at the high level.

Comparison of zopiclone concentrations in samples of oral fluid and blood

A total of 21 parallel samples of oral fluid using StatSure and Intercept devices were obtained combined with simultaneously taken blood samples. The analytical results are presented in Figure 1. The results indicated a significant difference in OF/B ratios for zopiclone using the two sampling devices ($p = 0.001$). The mean and median OF/B ratios for the Intercept device were 4.7 and 3.8, respectively, whereas the mean and median OF/B ratios for the StatSure device were 2.3 and 1.9. Wide variations

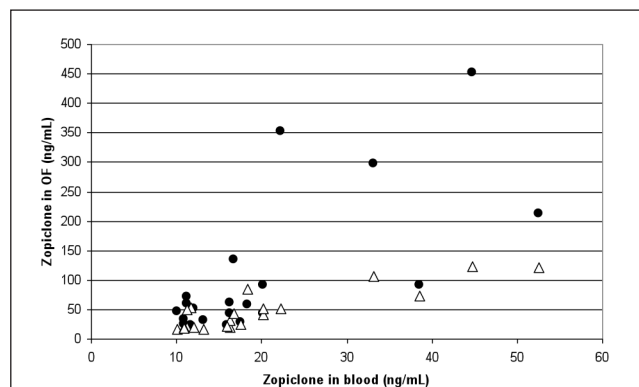


Figure 1. Concentrations of zopiclone in oral fluid (un-diluted) collected with Intercept (●) and StatSure (Δ) sampling devices in relation to zopiclone concentrations in blood.

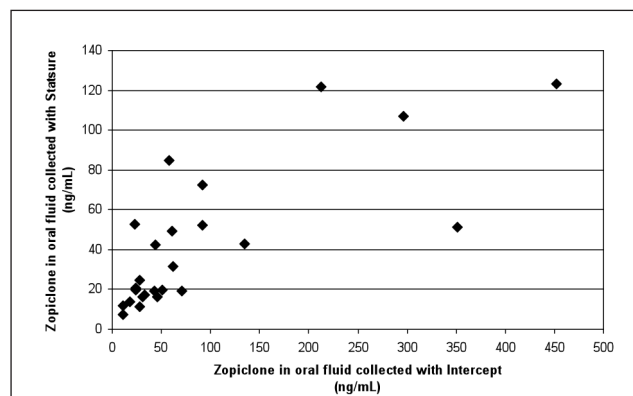


Figure 2. Concentrations of zopiclone in oral fluid (un-diluted) collected with the StatSure sampling device compared to the Intercept device.

of OF/B ratios were obtained; the ranges were 1.5–15.9 for Intercept and 1.2–4.6 for StatSure. The OF/B ratios seemed not to be normally distributed, and the “apparent” relative standard deviations were 46% and 75% for the StatSure and Intercept devices, respectively.

Paired samples (from the same subjects) taken with the Intercept device 1–3 h before using the StatSure device showed a mean and median OF/B ratio of 5.5 and 3.8 ($n = 21$), respectively, indicating that taking an oral fluid sample with the StatSure device shortly before using the Intercept device did not significantly affect the Intercept OF/B ratios ($p = 0.86$).

The zopiclone concentration ratio between oral fluid and plasma has previously been found to be 2.3; however, the method for sampling oral fluid was not described (11). Using a plasma/blood ratio of 1.0 (12), the concentration ratio between oral fluid and blood (OF/B ratio) would also be 2.3; this is similar to the mean OF/B ratio we observed for the StatSure device. In a recent study using the Intercept device, the mean OF/B ratio for zopiclone in four subjects was 3.7 (13), which is lower than the mean OF/B ratio we observed in this study. However, the median values were similar.

The concentrations of zopiclone in undiluted oral fluid are presented in Figure 2. The correlation between drug concentrations using those two sampling kits was fairly poor ($r^2 = 0.35$).

The results of this study indicate that the two sampling devices for oral fluid produced quite different analytical results for zopiclone. The differences in the two sampling devices may explain the observed differences in zopiclone concentrations in the two Norwegian roadside surveys of drugs and driving. The Intercept sampling pad contains saliva-stimulating agents that also affect the local pH of oral fluid in the mouth, and it may therefore also affect the equilibrium for zopiclone between blood and oral fluid. This may, at least in part, explain differences in OF/B ratios for the two devices. Studies of the recovery of zopiclone from the two collection devices did not reveal any marked differences (results not shown).

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