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# The evaluation of the applicability of a high pH mobile phase in ultrahigh performance liquid chromatography tandem mass spectrometry analysis of benzodiazepines and benzodiazepine-like hypnotics in urine and blood

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ABSTRACT

A sensitive liquid chromatography tandem mass spectrometry method was developed and validated for simultaneous detection of benzodiazepines, benzodiazepine-like hypnotics and some metabolites (7-aminoflunitrazepam, alprazolam, bromazepam, brotizolam, chlordiazepoxide, chlornordiazepam, clobazam, clonazepam, clotiazepam, cloxazolam, diazepam, ethylloflazepate, flunitrazepam, flurazepam, loprazolam, lorazepam, lormetazepam, midazolam, *N*-desmethylflunitrazepam, nitrazepam, *N*-methylclonazepam (internal standard), nordiazepam, oxazepam, prazepam, temazepam, tetrazepam, triazolam, zaleplon, zolpidem, zopiclone) in urine and whole blood. Sample preparation was performed on a mixed-mode cation exchange solid phase extraction cartridge. Electrospray ionization was found to be more efficient than atmospheric pressure chemical ionization. The use of a mobile phase of high pH resulted in higher retention and higher electrospray ionization signals than the conventional low pH mobile phases. Considering the benefits of a high pH mobile phase on both chromatography and mass spectrometry, its use should be encouraged. In the final method, gradient elution with 10 mM ammonium bicarbonate (pH 9) and methanol was performed on a small particle column (Acquity C18, 1.7 μm, 2.1 mm × 50 mm). The optimized method was fully validated.

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## 1. Introduction

Benzodiazepines and benzodiazepine-like hypnotics (zaleplon, zolpidem and zopiclone) are frequently used to treat sleeping disorders, anxiety, increased muscle tone and epilepsy [1]. Since these compounds are also widely misused, efficient analytical methods are needed for detection in both clinical and forensic cases. Several methods using liquid chromatography coupled to (tandem) mass spectrometry (LC–MS(/MS)) for bio-analysis of benzodiazepines and/or benzodiazepine-related hypnotics have been described [2]. Numerous parameters can be optimized to improve the sensitivity and selectivity of a LC–MS/MS method. Modifications in sample preparation, injected sample, ionization, mobile phase, LC column can have a major impact on method performance [3]. However, time for method development is often limited. Fast and easy ways to improve a method such as switching the ionization source are preferred. The two most common used sources, electrospray (ESI) and atmospheric chemical ionization (APCI), operate through a different mechanism, which makes them suited for analysis of different types of compounds [3,4]. Unfortunately, the optimal source for a compound cannot be predicted with absolute certainty based on its structure or chemical properties, so comparison of ionization sources is necessary. No consensus has been reached for the benzodiazepine(-like) hypnotics [2]. Most studies use ESI without any comments on this choice [2]. A few authors preferred APCI over ESI simply because APCI is much less susceptible to ion suppression [5–7]. When the sensitivity of APCI and ESI was compared, APCI was selected as optimal ionization interface in two papers [8,9]. ESI obtained higher sensitivity in one publication [10]. Clearly, further research on the efficiency of APCI versus ESI for analysis of benzodiazepine(-like) hypnotics is needed. Changing the mobile phase is another easy adaptation with possible impact on method performance. The effects of the mobile phase on chromatography can be relatively well predicted. When using reversed phase chromatography, there is extended retention for uncharged compounds. This implies the use of a high pH mobile phase for analysis of compounds with a basic character such as the benzodiazepines and a low pH mobile phase when dealing with acidic compounds. Besides the pH, the selected organic solvent also influences retention. Methanol is more polar than acetonitrile, resulting in later elution times. Another advantage of methanol is its lower cost. Acetonitrile on the other hand has lower viscosity,

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resulting in lower backpressures. The effects of the mobile phase on the ionization efficiency are less predictable and understood. Since APCI will be less affected by the mobile phase composition than ESI, comparison of mobile phases is especially interesting for ESI. Low pH mobile phases (using ammonium formate/acetate and formic/acetic acid) are commonly used. The use of high pH mobile phases (using ammonium bicarbonate and ammonium hydroxide) is more rare. This can probably be explained by the simple view of ESI that states that the pH of the mobile phase determines the ionization of the analyte, making low pH mobile phases first choice for basic compounds. However, several papers report "wrong way ionization" (i.e. ionization at a pH where the compound is uncharged) and show higher efficiency for high pH mobile phases than the classical low pH mobile phases for analysis of basic compounds [11–15]. Different theories explaining this phenomenon have been described, but the exact mechanism is still unclear [16]. For the benzodiazepine(-like) hypnotics, both methanol and acetonitrile were used as organic modifiers combined with low pH buffers [2]. To the best of our knowledge, there is no study where the behavior of benzodiazepines and benzodiazepine-like hypnotics in high pH mobile phase was tested. Our target was to evaluate different mobile phases for analysis of benzodiazepine(-like) hypnotics, to compare the two ionization interfaces and to use a LC column with small particles (so-called ultrahigh performance LC (UHPLC), ultra performance LC (UPLC) or ultrafast LC (UFLC)) in order to obtain high sensitivity. The final result was an optimized LC–MS/MS method for simultaneous detection of several benzodiazepine(-like) hypnotics (7-aminoflunitrazepam, alprazolam, bromazepam, brotizolam, chlordiazepoxide, chlornordiazepam, clobazam, clonazepam, clotiazepam, cloxazolam, diazepam, ethylloflazepate, flunitrazepam, flurazepam, loprazolam, lorazepam, lormetazepam, midazolam, *N*-desmethylflunitrazepam, nitrazepam, *N*-methylclonazepam (internal standard (IS)), nordiazepam, oxazepam, prazepam, temazepam, tetrazepam, triazolam, zaleplon, zolpidem, zopiclone) in urine and whole blood.

## 2. Materials and methods

### 2.1. Chemicals and standards

7-Aminoflunitrazepam (1 mg/ml), alprazolam (1 mg/ml), bromazepam (1 mg/ml), brotizolam (10 mg), clobazam (1 mg/ml), clonazepam (1 mg/ml), diazepam (1 mg/ml), flunitrazepam (1 mg/ml), flurazepam (1 mg/ml), loprazolam (1 mg/ml), lorazepam (1 mg/ml), lormetazepam (1 mg/ml), midazolam (1 mg/ml), *N*-desmethylflunitrazepam (1 mg/ml), nitrazepam (1 mg/ml), nordiazepam (1 mg/ml), oxazepam (1 mg/ml), prazepam (1 mg/ml), temazepam (1 mg/ml), tetrazepam (100 mg), triazolam (1 mg/ml), zaleplon (100 mg), zolpidem (1 mg/ml) and zopiclone (1 mg/ml) were purchased from LGC (Molsheim, France). Chlordiazepoxide, clorazepate and cloxazolam were ordered from various pharmaceutical companies as Librium®, Tranxene® and Akton®, respectively. Ethylloflazepate (100 mg) was purchased from Sanofi-Aventis (Huizingen, Belgium). Chlornordiazepam, clotiazepam and *N*-methylclonazepam were obtained from Roche (Brussel, Belgium). Standard solutions were prepared by diluting the stock chemicals with methanol to several concentration levels. All solutions were stored at −20 °C. Acetic acid, acetone, chloroform, ethyl acetate, methanol, monopotassium phosphate and ammonium hydroxide which are used in the sample preparation were purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli Q Water Purification System (Millipore, Brussel, Belgium). LC–MS grade acetonitrile and methanol were purchased from Biosolve (Valkenswaard, The Netherlands). All LC–MS grade mobile phase additives (formic acid, ammonium formate, acetic acid, ammonium acetate, ammonium bicarbonate and ammonium hydroxide) and β-glucuronidase (*Patella vulgata*) were purchased from Sigma–Aldrich (Bornem, Belgium). Glassware was silanized using AquaSil Siliconizing Fluid (Thermo Scientific, Breda, The Netherlands). 1.5 ml screw cap vials and 100 μl inactivated glass vial inserts were purchased from Agilent (Diegem, Belgium).

### 2.2. Instrumentation

LC–MS/MS analysis was carried out using a UFLC Shimadzu system consisting of a LC-20ADXR pump, a SIL-20ACXR autosampler, a DGU-20A3 degasser and a CTO-20A oven (Shimadzu Prominence, Antwerpen, Belgium) in combination with a 3200 QTRAP (ABSciex, Halle, Belgium) and Analyst software (version 1.5).

### 2.3. MS/MS conditions

The Turbo V ion source equipped with ESI probe used following settings: gas 1: nitrogen, 55 psi; gas 2: nitrogen, 55 psi; ion-spray voltage: 5000 V; ion-source temperature: 550 °C; curtain gas: nitrogen, 25 psi. The APCI probe operated with following parameters: gas 1: nitrogen, 40 psi; needle current: 4 μA; ion-source temperature: 475 °C; curtain gas: nitrogen, 15 psi. The mass spectrometer was operated in scheduled multiple reaction monitoring mode (sMRM) with parameters listed in Table 1. In this scan mode, MRM transitions are only monitored in a given time window around the expected retention time (RT) of the compound. Doing so reduces the number of compounds monitored at a given time and thus enhances the quality of detection.

### 2.4. LC conditions

The Acquity C18 column (1.7 μm particle size, 2.1 mm × 50 mm), fitted with a guard frit of 0.2 μm, was purchased from Waters (Zellik, Belgium). Tested mobile phases were: acetonitrile and 25 mM ammonium formate in water set at pH 3.0 with formic acid; methanol and 25 mM ammonium formate in water set at pH 3.0 with formic acid; acetonitrile and 25 mM ammonium acetate in water set at pH 4.0 with acetic acid; methanol and 25 mM ammonium acetate in water set at pH 4.0 with acetic acid; acetonitrile and 10 mM ammonium bicarbonate in water set at pH 9.0 with ammonium hydroxide and finally methanol and 10 mM ammonium bicarbonate in water set at pH 9.0 with ammonium hydroxide. The used flow rate was 0.5 ml/min. The autosampler temperature was set at 15 °C, the column oven at 40 °C. The final optimized method had following gradient conditions using 10 mM ammonium bicarbonate in water set at pH 9.0 (solvent A) and methanol (solvent B): 0–10 min: 25–90%B; 10–11 min: 90%B; 11–11.5 min: 90–25%B; 11.5–13 min: 25%B. Retention times of the benzodiazepine(-like) hypnotics using this gradient are shown in Table 1.

### 2.5. Sample preparation

Bond Elut Plexa PCX cartridges (60 mg, 3 ml) were purchased from Varian (Sint-Katelijne-Waver, Belgium). All solid phase extractions (SPE) were carried out on a Vac Elut SPS 24 (Varian, Sint-Katelijne-Waver, Belgium). The SPE cartridge was conditioned with 2 ml methanol and 2 ml 0.1 M phosphate buffer pH 6. In a silanized glass tube, 0.5 ml urine was mixed with 5 ml 0.1 M phosphate buffer pH 6 and 100 μl of an IS solution (which contains 600 ng/ml *N*-methylclonazepam). Urine samples treated with β-glucuronidase were incubated with 0.2 ml β-glucuronidase (5000 U/ml in 1.0 M acetate buffer pH 5) at 65 °C for 3 h before adding phosphate buffer and IS. When analyzing blood, 0.5 ml blood was mixed with 5 ml 0.1 M phosphate buffer pH 6 and 100 μl IS and sonicated for

**Table 1**
Analytes, RT, MRM transitions and the voltage settings declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP) and collision energy (CE).

| | RT (min) | Q1 mass | DP (V) | EP (V) | CEP (V) | Q3 mass | CE (V) | Q3 mass | CE (V) |
|---|---|---|---|---|---|---|---|---|---|
| 7-Aminoflunitrazepam | 2.0 | 284.2 | 58 | 10.0 | 30 | 135.1 | 39 | 227.1 | 36 |
| Alprazolam | 4.9 | 309.2 | 66 | 7.5 | 30 | 205.2 | 53 | 281.2 | 30 |
| Bromazepam | 3.7 | 316.1/318.1 | 53 | 7.5 | 32 | 182.1 | 44 | 182.1 | 44 |
| Brotizolam | 5.2 | 393.1 | 56 | 11.0 | 43 | 210.1 | 57 | 314.1 | 32 |
| Chlordiazepoxide | 5.4 | 300.2 | 53 | 5.5 | 30 | 227.0 | 34 | 282.0 | 33 |
| Chlornodiazepam | 5.4 | 305.1 | 63 | 6.5 | 32 | 165.1 | 40 | 206.1 | 47 |
| Clobazam | 4.6 | 301.2 | 50 | 8.0 | 30 | 224.2 | 45 | 259.2 | 30 |
| Clonazepam | 4.2 | 316.1 | 61 | 7.5 | 31 | 214.0 | 54 | 270.0 | 34 |
| Clotiazepam | 6.4 | 319.1 | 45 | 9.0 | 35 | 154.1 | 41 | 291.0 | 30 |
| Cloxazolam | 6.5 | 349.1 | 68 | 9.0 | 35 | 177.1 | 28 | 305.0 | 35 |
| Diazepam | 5.8 | 285.2 | 63 | 8.5 | 30 | 154.1 | 37 | 193.2 | 44 |
| Ethylloflazepate | 6.2 | 361.1 | 55 | 5.0 | 29 | 259.1 | 46 | 287.0 | 29 |
| Flunitrazepam | 4.2 | 314.2 | 40 | 7.5 | 31 | 239.1 | 40 | 268.1 | 25 |
| Flurazepam | 7.0 | 388.2 | 43 | 7.5 | 43 | 315.0 | 31 | 317.0 | 27 |
| Loprazolam | 5.4 | 465.2 | 68 | 9.5 | 43 | 252.1 | 57 | 408.0 | 33 |
| Lorazepam | 4.9 | 321.1 | 50 | 5.0 | 30 | 229.1 | 41 | 274.9 | 35 |
| Lormetazepam | 5.3 | 335.1/337.1 | 50 | 10.0 | 32 | 289.1 | 30 | 291.1 | 30 |
| Midazolam | 5.9 | 326.2 | 71 | 7.0 | 35 | 249.1 | 47 | 291.1 | 36 |
| *N*-desmethylflunitrazepam | 3.7 | 300.1 | 58 | 6.5 | 31 | 198.1 | 53 | 254.1 | 34 |
| Nitrazepam | 4.0 | 282.2 | 65 | 9.0 | 29 | 180.2 | 51 | 236.1 | 34 |
| *N*-methylclonazepam (IS) | 4.7 | 330.2 | 60 | 10.0 | 37 | 255.0 | 45 | 284.0 | 34 |
| Nordiazepam | 5.6 | 271.1 | 58 | 4.5 | 25 | 140.1 | 39 | 165.1 | 40 |
| Oxazepam | 4.8 | 287.2 | 45 | 7.5 | 31 | 241.0 | 30 | 269.0 | 25 |
| Prazepam | 7.1 | 325.2 | 60 | 10.0 | 37 | 140.1 | 48 | 271.0 | 32 |
| Temazepam | 5.1 | 301.2/303.2 | 46 | 6.5 | 30 | 255.0 | 29 | 257.0 | 32 |
| Tetrazepam | 6.9 | 289.2 | 63 | 7.5 | 29 | 197.2 | 43 | 225.2 | 41 |
| Triazolam | 4.9 | 343.1 | 76 | 7.5 | 33 | 239.1 | 59 | 308.1 | 38 |
| Zaleplon | 3.4 | 306.2 | 53 | 9.5 | 31 | 236.1 | 36 | 264.1 | 30 |
| Zolpidem | 4.9 | 308.2 | 56 | 5.5 | 32 | 235.2 | 47 | 236.2 | 38 |
| Zopiclone | 3.9 | 389.2 | 33 | 4.0 | 56 | 217.1 | 43 | 245.1 | 25 |

The collision cell exit potential (CXP) was set at 4 V. The collision gas (CAD) was set at medium. Because of the sMRM scan mode, each MRM transition was only detected 45 s before the expected RT and 45 s after. Underlined transitions were most abundant and used for quantification.

15 min to fragment red blood cells. The mixtures were vortexed and centrifuged. The supernatant was loaded on the SPE cartridge. The loaded cartridge was washed with 1 ml water, 1 ml 0.01 M acetic acid, and finally, after the column was dried for 4 min, 50 μl methanol. The SPE column was then dried for another minute. The analytes were eluted with 3× 1 ml acetone–chloroform (1:1) and 3× 1 ml 2% ammoniated ethyl acetate. The eluates were evaporated to dryness at room temperature. The residues were reconstituted in 100 μl methanol–water (25:75) and 5 μl was injected into the LC–MS/MS system.

### 2.6. Method validation

The method was validated according to internationally accepted recommendations [4]. For extended information about the used method validation, the reader is referred to our previous validation studies [11,15]. Urine and whole blood were spiked to obtain seven concentration levels: 2 ng/ml (limit of quantification (LOQ), 5 ng/ml for loprazolam), 10 ng/ml (lowest), 25 ng/ml (LOW), 100 ng/ml (medium (MED)), 250 ng/ml (HIGH), 500 ng/ml (highest) and 5000 ng/ml (above calibration range (ACR)). Selectivity was evaluated with blank samples from different sources ($n=10$ each for both urine and blood with 5 post-mortem samples, no IS was added during sample preparation) and zero samples ($n=2$ including one post-mortem sample, IS was added during sample preparation). Matrix effects (ME) and recovery (RE) were determined at two concentration levels: 25 ng/ml and 500 ng/ml urine or blood. At both levels a standard, pre-extraction spiked samples ($n=5$ with 3 post-mortem samples) and post-extraction spiked samples ($n=5$ with 3 post-mortem samples) were analyzed. For each compound, ME and RE were calculated with following equations:

$$\text{ME\%} = \left(\frac{B}{A}\right) \times 100$$

$$\text{RE\%} = \left(\frac{C}{B}\right) \times 100$$

where $A$ is the peak area from pure standard, $B$ from post-extraction spiked samples and $C$ from pre-extraction spiked samples. ME were acceptable if between 75% and 125% with a coefficient of variation (CV% = standard deviation/mean × 100) lower than 15% or 20% (if the concentration is near the limit of detection) [7]. In a similar way, CV% of the RE was used to evaluate the acceptance of the efficiency of extraction [7]. To create calibration curves, blank urine and blood samples ($n=6$ at each concentration level) were spiked to obtain calibration standards at LOQ, lowest, LOW, MED, HIGH and highest calibration level. The peak area ratio of analyte and IS was plotted versus analyte concentration. The regression line was calculated using a weighted least-squares linear regression model. Analysis of residual plots revealed the inverse of the squared concentration as appropriate weight factor. Daily calibration curves using the same concentrations ($n=1$ at each concentration level) were prepared for all following experiments. The limit of quantification was defined as the lowest point of the calibration curve and fulfilled the criteria for LOQ based on precision and accuracy data. In previous studies, we determined the limit of detection (LOD) using a specific calibration curve in the range of the LOD [11,15]. However, the resulting LODs had signal-to-noise (S/N) values often much higher than 3, which is commonly used as definition of LOD in the LC–MS/MS literature [11,15]. Therefore, in this study the S/N ratio of the qualifier transition at the LOQ was measured and used to calculate a concentration at which the S/N would be 3:1. The calculated LODs were evaluated by analysis of blank urine and blood enriched with decreasing amounts of the analytes situated around the calculated LOD (200 pg/ml, 100 pg/ml, 50 pg/ml and 20 pg/ml). The concentration level at which the qualifier compound could be detected with a S/N ratio of at least 3 was selected as LOD. To evaluate precision and accuracy, quality control

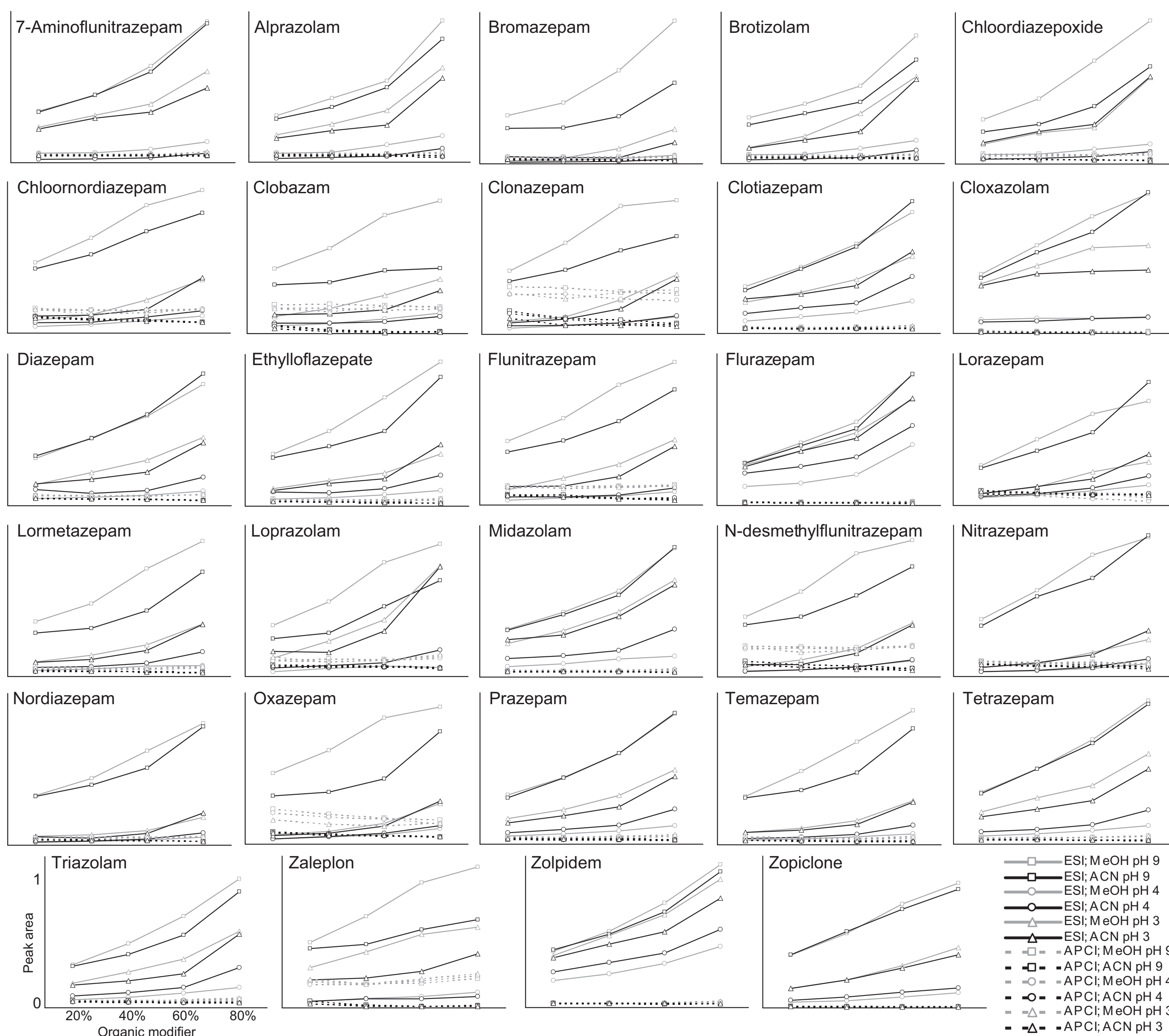


**Fig. 1.** Comparison of different combinations of mobile phase and ionization interface. Flow injection analysis of stock solutions containing 100 ng/ml of the compound was performed for each combination of ESI or APCI and the different mobile phases at 20%, 40%, 60% and 80% organic modifier (measuring points are indicated by the symbols). The percentage organic modifier (acetonitrile (ACN) or methanol (MeOH)) is plotted on the *x*-axis, the measured peak area normalized to the maximum value of the method with maximum response on the *y*-axis. pH 9 indicates 10 mM ammonium bicarbonate in water set at pH 9.0 with ammonium hydroxide, pH 4 indicates 25 mM ammonium acetate in water set at pH 4.0 with acetic acid, pH 3 indicates 25 mM ammonium formate in water set at pH 3.0 with formic acid.

samples (at LOQ, LOW, MED, HIGH and ACR concentrations, $n = 2$ at each concentration level) were extracted as unknown samples and analyzed on each of 8 days. Accuracy, expressed as bias, and precision, expressed as repeatability (within-day) and time-different intermediate precision (combination of within- and between-day effects) were calculated using following equations:

$$\text{Bias\%} = \left[\frac{X-\mu}{\mu}\right] \times 100$$

$$\text{Repeatability\%} = \left(\frac{MS_w^{0.5}}{X}\right) \times 100$$

$$\text{Intermediate precision\%} = \left[\frac{((MS_B + (n-1)\cdot MS_w)/n)^{0.5}}{X}\right] \times 100$$

where $X$ is the mean calculated concentration, $\mu$ is the nominal concentration, $MS_w$ is the mean square within days calculated by one-way ANOVA, $MS_B$ is the mean square between days calculated by one-way ANOVA and n is the number of observations each day. The acceptance limits are <15% for precision and within 15% of the nominal value for bias, except at LOQ where <20% for precision and within 20% of the nominal value for bias are acceptable. The stability of processed samples (at LOW and HIGH concentrations, $n = 8$ at each concentration level, all 8 were pooled in one vial) was tested by injection of the samples every 3 h over a total time of 9 h, followed by regression analysis in which the peak area of the analytes at each concentration was plotted versus injection time. A significantly negative slope ($p < 0.05$) would indicate instability. For determination of freeze/thaw and bench top stability, processed samples (at LOW and HIGH concentration level, $n = 6$ at each concentration level) were frozen at −20 °C for 21 h and kept

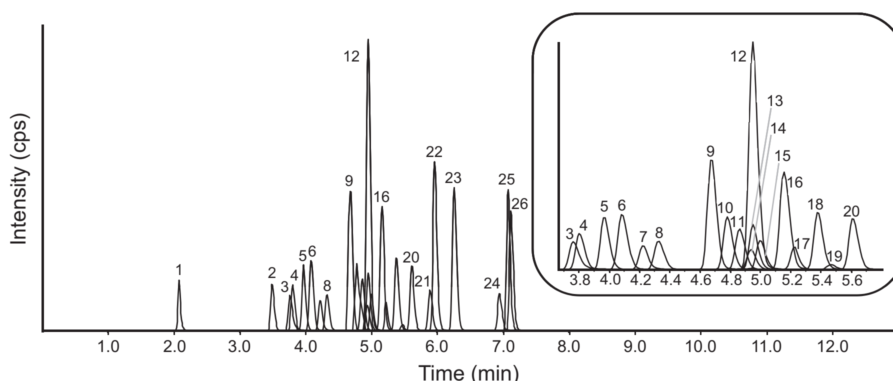


**Fig. 2.** Chromatogram of an extracted urine sample spiked at the low concentration level. In order of elution: (1) 7-aminoflunitrazepam, (2) zaleplon, (3) bromazepam, (4) *N*-desmethylflunitrazepam, (5) zopiclone, (6) nitrazepam, (7) clonazepam, (8) flunitrazepam, (9) clobazam, (10) IS, (11) oxazepam, (12) zolpidem, (13) alprazolam, (14) lorazepam, (15) triazolam, (16) temazepam, (17) brotizolam, (18) lormetazepam, (19) loprazolam, (20) nordiazepam, (21) diazepam, (22) midazolam, (23) ethylloflazepate, (24) tetrazepam, (25) flurazepam, (26) prazepam. A detailed chromatogram between 3.7 min and 5.8 min is shown in the box. Because of the difficult availability of standards for chlordiazepoxide, chlornordiazepam, clotiazepam and cloxazolam, these compounds could only be qualitatively identified and are not shown in this chromatogram. Their retention times can be found in table.

at room temperature for 3 h. The concentrations of the analytes were calculated before (control sample) and after three of these cycles (stability sample). Stability was assumed when the ratio of the means (stability samples versus control samples) was within 90–110% and the 95% confidence interval of the stability sample mean was within 80–120% of the control mean. To determine carryover, 5 μl methanol was injected as blank after every sample and blank matrix extracts were injected after the highest calibrator. As proof of applicability, some real-life forensic samples were analyzed.

## 3. Results and discussion

### 3.1. Method development

For each compound, two most abundant MRM transitions were selected for analysis (Table 1). For clorazepate, no $[M-H^+]$ ion was found, but a molecule with *m/z* 271 is present, as previously described [17]. Clorazepate seems to transform into nordiazepam during ionization, which should be kept in mind when interpreting nordiazepam concentrations. Next, six mobile phases and two ionization interfaces were investigated to determine which combination resulted in highest ionization efficiency (Fig. 1). The two ionization sources reacted differently to the increasing portion organic modifier. As expected, more organic modifier results in more ionization by ESI [18]. For APCI, there was no signal increase with increasing organic portion: the signal was stable or there was even a slight decrease. The aqueous part of the mobile phase has more impact on the ionization efficiency of ESI than the organic part: the classical low pH mobile phases appeared less efficient than the high pH mobile phases, while there was no or little difference between methanol and acetonitrile. APCI was in general less influenced by the composition of the mobile phase. The only significant difference was better ionization by methanolic mobile phases for some compounds. Low or high pH had no influence on the APCI signal. For all compounds, ESI combined with methanol and an ammonium bicarbonate buffer at pH 9 was optimal for high sensitivity. Moreover, benzodiazepines have higher retention on a reversed phase column when using this mobile phase. Our findings together with the increasing number of papers demonstrating the benefits of high pH mobile phases and the development of more and more not purely silica based columns compatible with this high pH all encourage the use of high pH mobile phases in LC–MS/MS analyses [11–15,19,20]. We tried to minimize matrix effects, the major disadvantage of LC–MS/MS, by avoiding co-elution of disturbing compounds in three ways: efficient sample preparation, efficient separation using the small particles of the UFLC columns and finally increased separation time. A universal mixed-mode SPE procedure was adapted from the literature and slightly modified [21]. We decided to work with the non-silica based equivalent of the Bond Elut Certify columns, because of better pH stability [11]. We tested 1 ml of wash solvent instead of the original volumes. The results were satisfying for the acid wash, but the increase of 50 μl methanol wash to 1 ml resulted in severe decrease in recovery since several compounds eluted from the SPE cartridge. Elution was done in steps of 1 ml with a total volume of 6 ml for optimal elution of all compounds. Two different elution steps (acetone–chloroform (step 1) and 2% ammoniated ethyl acetate (step 2)) were used. Both steps are necessary for complete elution, since some compounds elute only in step 1 (clobazam and zaleplon), others only in step 2 (7-aminoflunitrazepam, alprazolam, bromazepam, chlordiazepoxide, clotiazepam, cloxazolam, flurazepam, loprazolam, midazolam, nordiazepam, tetrazepam, zolpidem, zopiclone) while the other compounds are eluted by both solvents. The evaporated extracts were reconstituted in 100 μl methanol:water (25:75). The type and percentage of organic solvent and the pH of the injection solution can influence the peak performance of the analytes [4]. Therefore an injection solution similar to the mobile phase should be chosen. We used 25% methanol (as at the start of the gradient used) combined with water. To imitate the exact mobile phase composition, ammoniumbicarbonate at pH 9 should be used instead of water. However since no abnormal peak shape was seen, no buffer was added to the injection solution. A LC column with small particle size was used in order to gain high sensitivity. To increase the efficiency of separation and thus decrease matrix effects, a UFLC column was combined with a gradient run over 10 min with a total run time of 13 min (Fig. 2). No hydrolysis step was used during preparation of urine samples because the sensitivity of our method was high enough for adequate detection of the parent drug and some phase 1 metabolites. Moreover instability of some compounds during hydrolysis has been described [22,23]. The effect of omitting hydrolysis was tested on real-life forensic urine samples during method validation.

### 3.2. Method validation

No interferences were found in blank samples and zero samples. This proves the high selectivity of the method, provided by

**Table 2**
Matrix effects and recovery of the LC–MS/MS method for analysis of urine and blood.

| | Low concentration urine | | High concentration urine | | Low concentration blood | | High concentration blood | |
|---|---|---|---|---|---|---|---|---|
| | ME% (CV%) | RE% (CV%) | ME% (CV%) | RE% (CV%) | ME% (CV%) | RE% (CV%) | ME% (CV%) | RE% (CV%) |
| 7-Aminoflunitrazepam | 72.8 (5.0) | 28.2$^{*}$ (13.5) | 79.1 (6.9) | 56.3$^{*}$ (9.6) | 78.8 (7.9) | 29.3$^{*}$ (14.1) | 71.1 (2.0) | 61.6$^{*}$ (14.5) |
| Alprazolam | 88.8 (2.7) | 49.2$^{*}$ (13.3) | 89.5 (5.0) | 76.0$^{*,**}$ (11.0) | 91.8 (14.8) | 68.3$^{*}$ (15.5) | 91.2 (5.35) | 99.4$^{*,**}$ (14.8) |
| Bromazepam | 83.7 (13.4) | 81.2 (6.9) | 91.7 (8.8) | 88.9 (6.4) | 88.1 (11.1) | 77.8 (12.3) | 86.4 (5.6) | 85.4 (15.1) |
| Brotizolam | 85.8 (2.3) | 83.7 (6.4) | 90.7 (7.7) | 83.1 (7.5) | 89.9 (13.5) | 79.0 (13.1) | 89.5 (4.8) | 78.1 (14.2) |
| Clobazam | 75.3 (15.0) | 90.1 (9.1) | 83.3 (8.9) | 89.9 (4.1) | 91.9 (8.7) | 75.0 (11.6) | 87.1 (3.3) | 80.9 (14.6) |
| Clonazepam | 74.7 (7.0) | 78.2$^{**}$ (10.2) | 81.1 (9.7) | 81.2 (5.2) | 80.2 (14.7) | 56.9$^{**}$ (15.4) | 80.8 (9.8) | 70.3 (14.7) |
| Diazepam | 97.2 (3.6) | 83.8 (8.0) | 95.7 (10.6) | 85.2 (8.9) | 87.5 (5.3) | 72.7 (15.2) | 96.5 (2.1) | 72.8 (14.3) |
| Ethylloflazepate | 88.5 (3.2) | 85.7$^{**}$ (7.7) | 92.0 (5.9) | 80.3 (6.5) | 83.0 (9.3) | 66.9$^{**}$ (11.0) | 88.7 (4.1) | 73.2 (15.9) |
| Flunitrazepam | 75.6 (6.8) | 80.3 (8.3) | 81.9 (8.5) | 80.8 (4.0) | 71.8 (13.9) | 65.8 (13.5) | 77.2 (14.1) | 78.7 (11.0) |
| Flurazepam | 100.6 (6.8) | 84.6 (15.0) | 96.2 (2.3) | 84.3 (6.1) | 110.4 (13.3) | 75.8 (11.8) | 94.4 (1.7) | 78.7 (14.1) |
| Loprazolam | 81.2 (4.0) | 53.2 (14.3) | 84.4 (12.9) | 62.8 (10.7) | 83.7 (14.9) | 61.1 (10.4) | 86.8 (3.3) | 56.0 (12.7) |
| Lorazepam | 87.7 (8.0) | 89.1 (6.4) | 88.7 (5.1) | 96.7 (13.7) | 88.0 (9.5) | 82.3 (9.1) | 89.5 (2.5) | 97.5 (9.5) |
| Lormetazepam | 78.8 (5.7) | 84.4 (6.1) | 85.5 (11.0) | 91.5 (7.3) | 76.3 (6.7) | 77.8 (13.3) | 89.9 (4.7) | 78.9 (14.9) |
| Midazolam | 96.5 (5.9) | 55.4 (12.8) | 94.3 (4.8) | 64.1 (5.9) | 89.7 (9.0) | 54.8 (10.5) | 92.4 (2.8) | 58.8 (15.6) |
| *N*-desmethylflunitrazepam | 76.5 (13.5) | 85.0 (8.8) | 86.2 (9.9) | 88.1 (4.4) | 82.3 (12.0) | 70.4 (12.1) | 83.8 (4.4) | 81.7 (13.0) |
| Nitrazepam | 80.0 (6.6) | 65.0 (9.7) | 84.0 (7.3) | 80.5 (2.9) | 82.7 (13.1) | 65.2 (15.2) | 81.3 (6.2) | 77.7 (12.8) |
| *N*-methylclonazepam (IS) | 74.9 (5.3) | 78.5 (10.9) | 74.2 (8.8) | 82.0$^{**}$ (7.3) | 76.5 (7.0) | 63.8 (14.4) | 74.3 (9.2) | 63.4$^{**}$ (14.0) |
| Nordiazepam | 90.7 (10.7) | 78.9$^{**}$ (9.5) | 88.4 (6.4) | 84.8 (4.4) | 83.9 (5.8) | 58.0$^{*,**}$ (13.9) | 85.0 (2.7) | 75.5$^{*}$ (11.3) |
| Oxazepam | 78.9 (10.8) | 78.9 (10.3) | 84.5 (10.8) | 87.0 (6.8) | 77.6 (11.7) | 71.8 (14.4) | 81.3 (6.6) | 78.2 (11.4) |
| Prazepam | 94.4 (3.2) | 78.7$^{**}$ (12.5) | 94.1 (3.4) | 73.1 (15.1) | 85.2 (14.8) | 53.9$^{**}$ (15.9) | 93.5 (2.4) | 63.1 (13.9) |
| Temazepam | 84.3 (2.8) | 89.6$^{**}$ (9.4) | 90.1 (7.7) | 89.6 (9.2) | 86.8 (15.4) | 71.0$^{**}$ (14.6) | 87.8 (4.7) | 79.8 (12.3) |
| Tetrazepam | 98.7 (3.6) | 51.7$^{*}$ (14.4) | 92.4 (3.5) | 71.3$^{*}$ (14.2) | 92.2 (9.0) | 56.0 (14.1) | 91.9 (3.6) | 57.6 (15.0) |
| Triazolam | 75.4 (5.8) | 81.3 (13.4) | 82.5 (9.0) | 91.8 (9.7) | 82.2 (12.7) | 74.2 (12.7) | 85.2 (2.6) | 81.8 (15.5) |
| Zaleplon | 81.5 (7.9) | 85.1 (8.6) | 82.3 (9.4) | 91.7 (3.9) | 84.5 (5.2) | 81.7 (15.8) | 85.2 (10.5) | 80.8 (14.7) |
| Zolpidem | 97.2 (13.4) | 4.1 (24.0) | 97.6 (2.5) | 2.2 (50.6) | 102.3 (8.1) | 14.3 (52.2) | 96.1 (1.6) | 9.9 (40.8) |
| Zopiclone | 102.1 (11.4) | 42.7 (55.0) | 90.3 (8.7) | 44.0 (16.2) | 104.7 (13.2) | 31.9 (75.5) | 90.3 (6.8) | 45.6 (15.4) |

Low concentration represents 25 ng/ml urine or blood, high concentration stands for 500 ng/ml. *N*-methylclonazepam was always used at 600 ng/ml. ME and RE were calculated using absolute peak areas.

* Significant difference in ME or RE between low and high concentrations in that matrix.

** Significant difference between blood and urine at that concentration level (one-way ANOVA, Bonferroni test, $p < 0.05$).

the combination of retention time, two transitions and their relative abundances as identification criteria. Because of the difficult availability of standards for chlordiazepoxide, chlornordiazepam, clotiazepam and cloxazolam, these compounds could only be qualitatively identified and were not included in the further method validation. ME and RE values were calculated with absolute peak areas. The ME values were acceptable, ranging from 71.1% to 110.4% with CV% lower than 15.4% (Table 2). When evaluating the RE, unacceptable high variation was seen for zolpidem and zopiclone, in both urine and whole blood (in this matrix, only at the low concentration for zopiclone) (Table 2). Both compounds were removed from the quantitative method since the used IS was unable to compensate for such high variability. Deuterated zolpidem and zopiclone as IS could offer a solution for this problem. For the other compounds, RE calculated with the peak area was between 28.2% and 99.4% with CV% lower than 15.9% (Table 2). In seven situations, there was a significant difference in RE between urine and blood, in six cases efficiency of extraction was better in urine (Table 2). When there was a difference in RE between the two concentration levels (in six situations), efficiency of extraction was better at the high concentration level (Table 2). For 7-aminoflunitrazepam and alprazolam, the difference in RE between the low and high concentration level was too high to be compensated by the IS, therefore the calibration range was limited to the medium concentration level for these compounds. The linearity, LODs and LOQs are listed in Table 3 together with the known therapeutic concentrations [24–26]. Plasma concentrations are given as the plasma to whole blood ratio is unknown for most of the benzodiazepines and benzodiazepine-like hypnotics. All precision and accuracy values were acceptable: precision values ranged from 2.5% to 15.6% and from 2.4% to 20.9% at LOQ level; accuracy values ranged from −15.4% to 15.2% and from −15.7% to 19.8% at LOQ level. Samples containing concentrations outside the calibration range (ACR) could be successful quantified by dilution with blank matrix prior to extraction. No instability or carryover was observed. The method was successfully used for the analysis of several real-life forensic samples. We analyzed 10 cases with benzodiazepine(-like) hypnotics present in blood at subtherapeutic or therapeutic concentrations (except for one case with toxic levels of alprazolam). For the corresponding urine samples, sample preparation with and without treatment with β-glucuronidase was compared. In the samples treated with β-glucuronidase we found alprazolam (detected in 1 sample), bromazepam (in 2 samples), clonazepam (in 2 samples), diazepam (in 4 samples), lorazepam (in 5 samples), lormetazepam (in 4 samples), midazolam (in 1 sample), nordiazepam (in 5 samples), oxazepam (in 5 samples), temazepam (in 4 samples) and zolpidem (in 1 sample). When analyzing the enzyme-untreated samples, lorazepam was not found in two samples and oxazepam in one sample. However, in all three samples a parent molecule was detected (lormetazepam for lorazepam; nordiazepam for oxazepam) making the detection of lorazepam and oxazepam not required for correct interpretation. Moreover, for forensic interpretation blood concentrations are more valuable than urine concentrations. Blood is a useful specimen to establish recent ingestion of drugs and found concentrations can be used to establish a possible dose that was taken [1]. In urine, concentrations of drugs and their metabolites are generally higher than in blood, but the presence of a drug in urine cannot be interpreted as being pharmacologically active [1].

**Table 3**
Linearity, LODs and LOQs of the final LC–MS/MS method for analysis of urine and blood.

| | Linear range (ng/ml) | Therapeutic range plasma (ng/ml) | 95% CI slope urine | 95% CI intercept urine | $R^2$ urine | 95% CI slope blood | 95% CI intercept blood | $R^2$ blood | LOD urine (pg/ml) | LOD blood (pg/ml) | LOQ (ng/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 7-Aminoflunitrazepam | 2–100 | 0.8–2 | 0.012–0.014 | 0.024 to 0.033 | 0.9930 | 0.015–0.018 | −0.071 to 0.064 | 0.9925 | 20 | 20 | 2.0 |
| Alprazolam | 2–100 | 5–20 | 0.026–0.028 | 0.012 to 0.018 | 0.9976 | 0.045–0.052 | 0.015 to 0.045 | 0.9948 | 100 | 50 | 2.0 |
| Bromazepam | 2–500 | 80–200 | 0.026–0.028 | −0.011 to 0.001 | 0.9946 | 0.028–0.032 | −0.008 to 0.009 | 0.9937 | 50 | 50 | 2.0 |
| Brotizolam | 2–500 | 1–20 | 0.016–0.017 | −0.001 to 0.006 | 0.9946 | 0.016–0.018 | −0.001 to 0.007 | 0.9925 | 50 | 50 | 2.0 |
| Clobazam | 2–500 | 100–600 | 0.074–0.083 | 0.033 to 0.072 | 0.9900 | 0.084–0.092 | 0.033 to 0.071 | 0.9919 | 100 | 100 | 2.0 |
| Clonazepam | 2–500 | 10–80 | 0.017–0.019 | 0.005 to 0.012 | 0.9947 | 0.018–0.019 | 0.011 to 0.016 | 0.9978 | 50 | 50 | 2.0 |
| Diazepam | 2–500 | 125–2000 | 0.034–0.036 | −0.004 to 0.005 | 0.9969 | 0.031–0.035 | −0.013 to 0.008 | 0.9960 | 50 | 100 | 2.0 |
| Ethylloflazepate | 2–500 | 30–50 | 0.085–0.094 | −0.011 to 0.027 | 0.9915 | 0.082–0.090 | −0.047 to −0.013 | 0.9927 | 50 | 100 | 2.0 |
| Flunitrazepam | 2–500 | 5–15 | 0.025–0.027 | 0.001–0.009 | 0.9954 | 0.025–0.026 | 0.003 to 0.012 | 0.9953 | 50 | 50 | 2.0 |
| Flurazepam | 2–500 | 5–10 | 0.067–0.073 | −0.140 to −0.113 | 0.9936 | 0.067–0.076 | −0.142 to −0.103 | 0.9941 | 50 | 50 | 2.0 |
| Loprazolam | 5–500 | 5–10 | 0.002–0.003 | −0.012 to 0.005 | 0.9941 | 0.0020–0.0023 | −0.012 to 0.007 | 0.9924 | 200 | 200 | 5.0 |
| Lorazepam | 2–500 | 20–250 | 0.012–0.013 | 0.007 to 0.012 | 0.9939 | 0.010–0.011 | 0.014 to 0.020 | 0.9908 | 50 | 50 | 2.0 |
| Lormetazepam | 2–500 | 5–25 | 0.045–0.049 | −0.005 to 0.012 | 0.9940 | 0.047–0.051 | −0.003 to 0.014 | 0.9944 | 50 | 50 | 2.0 |
| Midazolam | 2–500 | 40–100 | 0.039–0.046 | −0.005 to 0.026 | 0.9933 | 0.038–0.042 | −0.024 to 0.008 | 0.9935 | 50 | 50 | 2.0 |
| *N*-desmethylflunitrazepam | 2–500 | 10–150 | 0.023–0.026 | 0.001 to 0.011 | 0.9944 | 0.025–0.027 | 0.0002 to 0.009 | 0.9952 | 50 | 50 | 2.0 |
| Nitrazepam | 2–500 | 30–100 | 0.037–0.040 | 0.011 to 0.024 | 0.9954 | 0.038–0.041 | 0.013 to 0.026 | 0.9953 | 50 | 50 | 2.0 |
| Nordiazepam | 2–500 | 20–800 | 0.047–0.050 | −0.014 to 0.003 | 0.9952 | 0.042–0.047 | −0.019 to 0.003 | 0.9942 | 50 | 50 | 2.0 |
| Oxazepam | 2–500 | 200–1500 | 0.029–0.031 | 0.002 to 0.013 | 0.9952 | 0.027–0.030 | −0.004 to 0.009 | 0.9929 | 200 | 200 | 2.0 |
| Prazepam | 2–500 | 200–700 | 0.064–0.071 | −0.037 to 0.005 | 0.9911 | 0.049–0.054 | −0.037 to −0.013 | 0.9909 | 50 | 50 | 2.0 |
| Temazepam | 2–500 | 20–1000 | 0.077–0.085 | 0.019 to 0.052 | 0.9933 | 0.076–0.084 | 0.018 to 0.050 | 0.9927 | 50 | 50 | 2.0 |
| Tetrazepam | 2–500 | 50–600 | 0.026–0.028 | −0.150 to 0.096 | 0.9914 | 0.020–0.022 | −0.005 to 0.003 | 0.9930 | 50 | 50 | 2.0 |
| Triazolam | 2–500 | 2–20 | 0.025–0.027 | −0.009 to 0.001 | 0.9947 | 0.026–0.029 | −0.008 to 0.004 | 0.9923 | 50 | 50 | 2.0 |
| Zaleplon | 2–500 | 1–100 | 0.026–0.028 | 0.003 to 0.013 | 0.9949 | 0.030–0.032 | 0.010 to 0.021 | 0.9950 | 20 | 20 | 2.0 |

The linear range, known therapeutic concentration range, the 95% confidence interval (95%CI) around the slope and intercept of the calibration curve, $R^2$ and LOD and LOQ are shown. If no matrix is mentioned in the heading, values are equal for urine and blood.

## 4. Conclusions

We presented a highly sensitive UFLC–MS/MS method for the analysis of several benzodiazepines, benzodiazepine-like hypnotics and their metabolites in urine and whole blood. ESI resulted in higher ionization than APCI, as did a high pH mobile phase compared to a low pH mobile phase. To the best of our knowledge, this is the first method using such a high pH mobile phase for the analysis of benzodiazepine(-like) hypnotics, resulting in high sensitivity. Considering the benefits of a high pH mobile phase on both chromatography and mass spectrometry, its use should be encouraged. The method was fully validated. Because lack of analytical standards, chlordiazepoxide, chlornordiazepam, clotiazepam and cloxazolam were only be qualitatively detected. For zolpidem and zopiclone certain validation parameters were not in the acceptable range, excluding these compounds from the quantitative method. Use of deuterated standards for these compounds should solve the problem. The method is successfully used for the analysis of several real-life forensic samples.

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