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Development and validation of a liquid chromatography–atmospheric pressure photoionization–mass spectrometry method for the quantification of alprazolam, flunitrazepam, and their main metabolites in haemolysed blood☆

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ABSTRACT

A LC–APPI–MS method was developed and validated for the detection of alprazolam, flunitrazepam and their major metabolites in haemolysed blood. Samples were diluted with water (2:1, v:v) and extracted with a hydrophobic–lipophilic balanced copolymer. The method was fully validated according to ICH guidelines and SFSTP protocols. Deuterated internal standards of both parent drugs were used and good quantitative performance was achieved in terms of trueness and precision (repeatability and intermediate precision) since accuracy profiles were achieved within the acceptance limits (±30% for biological samples). The LC–APPI–MS method was linear over the concentration range of 1–1000 and 3–1000 ng/mL, for alprazolam and flunitrazepam, respectively. Lower limits of quantification as low as 1 ng/mL in haemolysed blood were reached and the method was successfully applied to the quantification of alprazolam, flunitrazepam and their major metabolites in real toxicological samples.

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1. Introduction

Benzodiazepines (BZD) are molecules used as psychotherapeutics that have a potent central nervous system effect. They are mainly used as tranquillizers, sedatives, anticonvulsants, and hypnotics [1,2] for the treatment of anxiety, sleep disturbances, or epilepsy [3]. They are widely consumed and their psychotropic effects often lead to behavioural disorders [4,5], dependence [6], or death by asphyxia [7]. Numerous methods for the analysis of BZD in biological matrices have already been described in the literature, mainly reporting the use of gas chromatography–mass spectrometry (GC–MS) [8–10] and liquid chromatography–mass spectrometry (LC–MS) [11–14]. Other separation techniques, such as capillary electrophoresis (CE) [15,16], micellar electrokinetic chromatography (MEKC) [17,18] and capillary electrochromatography (CEC) [17], have also been reported.

Concerning LC–MS, most applications have been conducted with electrospray ionization (ESI) [4,12,19], while atmospheric pressure chemical ionization (APCI) [20,21], fast atom bombardment (FAB) [22], matrix-assisted laser desorption ionization (MALDI) [23], and atmospheric pressure thermodesorption surface ionization (APTDSI) [24] techniques have been rarely used. To the best of our knowledge, no LC–MS methods have been published with atmospheric pressure photoionization (APPI) for BZD analysis in biological samples. However, the APPI source presents various advantages over ESI, such as a lower sensitivity to signal alterations with biological samples [25–30] and a larger linear dynamic range [31,32]. The latter is particularly well adapted to BZD analysis because of their wide therapeutic and toxicity windows [33,34]. Most studies have reported BZD analysis in urine [35–38], but other body fluids have also been investigated, such as blood [10,39,40], hair [11,41], and saliva [42]. Because direct injection of such samples in LC–MS systems present difficulties, a sample preparation is necessary. Among the available techniques, liquid–liquid extraction (LLE) [10,43,44] and solid-phase extraction (SPE) [12,14,37] are the most employed, whereas the use of solid-phase micro-extraction (SPME) [4,39] and supercritical fluid extraction (SFE) [45] have been less reported. SPE presents the advantage of a fast procedure, solvents compatibility with LC mobile phases and ease of automation over LLE.

The aim of this study was to develop and validate a method to quantify the commonly consumed BZD alprazolam and flunitrazepam, as well as their respective major metabolite, namely α-hydroxyalprazolam and 7-aminoflunitrazepam, in haemolysed blood samples, an important matrix for toxicological or forensic issues. Method selectivity was evaluated with various other BZD to avoid co-medication issues. A simple and fast SPE procedure was implemented, followed by a selective LC separation in the isocratic mode coupled to a sensitive APPI–MS detector. Quantitative performance was assessed according to the international conference on

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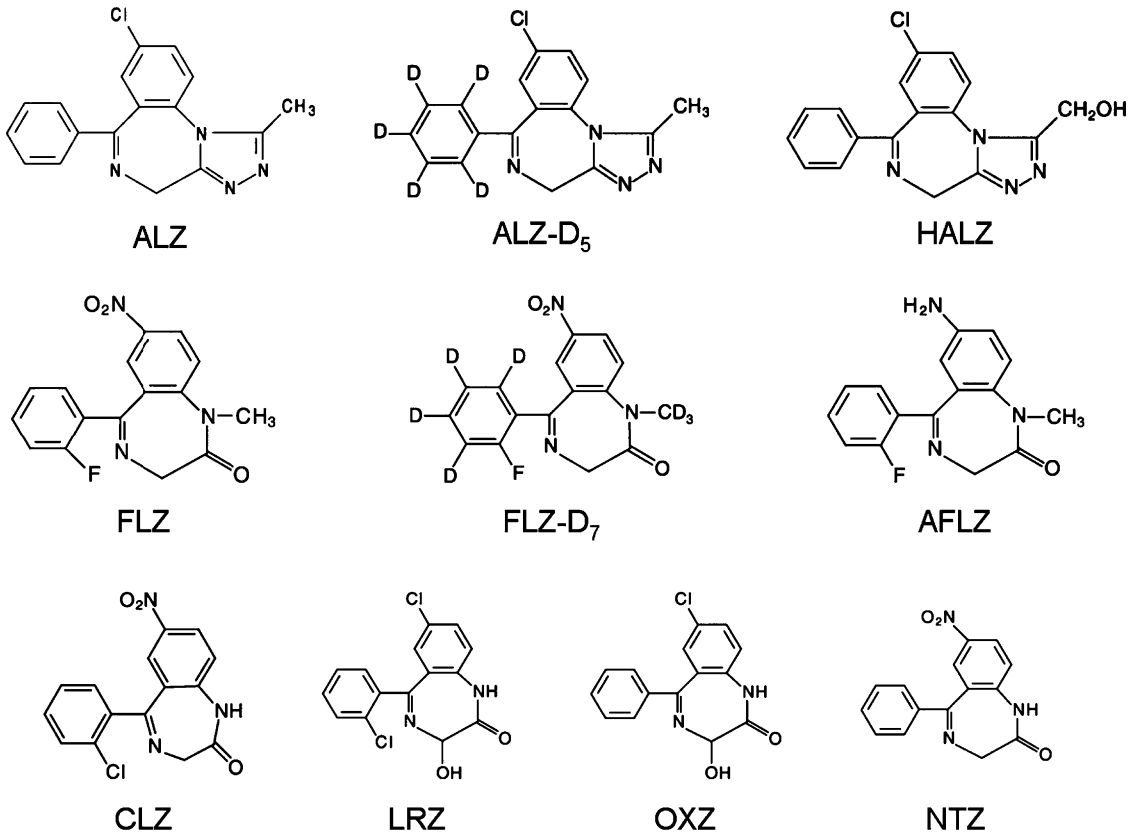

Fig. 1. Chemical structures of investigated BZD.

harmonization (ICH) guidelines as well as recommendations from the "société française des sciences et techniques pharmaceutiques" (SFSTP) [46–48] including the concept of total error. Various criteria, namely trueness, precision, accuracy and limit of quantification (LOQ) were used to evaluate quantitative performance. Deuterated internal standards (IS) were used to compensate for the overall method variability, including extraction and ionization variations. The fully validated method was applied to the quantification of alprazolam and flunitrazepam in toxicological cases.

2. Experimental

2.1. Chemicals

Stock solutions at 1000 μg/mL in methanol (MeOH) of flunitrazepam (FLZ), flunitrazepam-D_7 (FLZ-D_7) 100.00% isotopic purity, 7-aminoflunitrazepam (AFLZ), alprazolam (ALZ), alprazolam-D_5 (ALZ-D_5) 99.91% isotopic purity, and α-hydroxyalprazolam (HALZ) were purchased from Cerilliant (Austin, Texas, USA) and stock solutions at 1000 μg/mL in MeOH of lorazepam (LRZ), clonazepam (CLZ), nitrazepam (NTZ), and oxazepam (OXZ) were provided by Lipomed (Arlesheim, Switzerland). Chemical structures of the analytes are reported in Fig. 1. Acetonitrile (ACN) and MeOH were purchased from Panreac (Barcelona, Spain). Acetic acid 99.8%, ammonium hydroxide 25%, acetone, and toluene were obtained from Fluka. Water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA). All chemicals were of the highest purity grade commercially available and all reagents used were of HPLC grade. Human blank whole blood and real cases were obtained from the Institut Universitaire de Médecine Légale (IUML, Geneva, Switzerland). They were stored at −22 °C and defrosted at room temperature for 30 min before use. Because freezing and thawing cause hemolysis of human erythrocytes [49], they were considered as haemolysed blood samples.

2.2. Sample preparation

2.2.1. SPE

Haemolysed blood samples were centrifuged at 8000 × *g* for 10 min and 1000 μL of water was added to 500 μL of the supernatant. After vortex-mixing, the whole sample (1500 μL) was extracted by SPE on an Oasis HLB 96-well plate (10 mg sorbent) from Waters (MA, USA). Each well was conditioned with 500 μL of MeOH and equilibrated with 500 μL of water. One thousand five hundred microliters of the sample was loaded and washed with a mixture of water–MeOH (70:30, v/v). Elution was carried out with 250 μL of MeOH and the eluate was directly transferred into the injection vial.

2.2.2. Evaluation of process efficiency and matrix effect

Recoveries (*i.e.*, process efficiency) were estimated according to the methodology developed by Matuszewski et al. [50]. A first set of three standards was prepared using neat solutions of BZD in MeOH at three concentrations, namely LOQ, 50% and 100% of the studied range (Table 1). The samples were prepared by diluting appropriate

Table 1
Calibration standard (CS, $k = 3$) and quality control (QC) sample ($k = 4$) concentrations (ng/mL) used for validation of alprazolam (ALZ), flunitrazepam (FLZ) and their respective major metabolite, α-hydroxyalprazolam (HALZ) and 7-aminoflunitrazepam (AFLZ).

CS	QC	ALZ	HALZ	FLZ	AFLZ
LOQ	LOQ	1	13	3	2
	4 × LOQ	4	52	12	8
50%	50%	500	500	500	500
100%	100%	1000	1000	1000	1000

Table 2
Studied BZD with their pK_a, log D and typical blood concentrations (μg/mL).

Compound	pK_a[a]		log D[a]			Concentration in blood (μg/mL) [34]		
	Acidic	Basic	pH 2	pH 7	pH 10	Therapeutic	Toxic	Lethal
Alprazolam	–	2.3	1.9	2.5	2.5	0.005–0.05	0.1–0.4	–
α-Hydroxyalprazolam	13.1	1.2	1.6	1.7	1.7	–		
Clonazepam	11.2	1.5	2.2	2.3	2.3	0.01–0.08	0.1	–
Flunitrazepam	–	1.7	1.1	1.3	1.3	0.005–0.015	0.05	–
7-Aminoflunitrazepam	–	3.1	−0.6	0.6	0.6	–		
Lorazepam	10.8	–	2.5	2.5	2.4	0.08–0.25	0.3–0.5	–
Nitrazepam	11.4	3.2	1.0	2.2	2.2	0.03–0.1	0.2–0.3	5
Oxazepam	10.9	1.7	2.1	2.3	2.3	0.2–1.5	2	3–5

[a] pK_a and log D values were calculated using Advanced Chemistry Development software version 8.14 for Solaris (ACD/Labs, Toronto, Canada).

volumes of each BZD stock solution with MeOH (total volume of 250 μL). After mixing, the solutions were transferred into injection vials and directly injected into the LC–APPI–MS system. A second set of three standards was prepared in haemolysed blood originating from six different sources, pooled, and spiked before SPE. The samples were prepared by diluting appropriate volumes of each BZD stock solution with haemolysed blood (total volume of 500 μL). After mixing, the solutions were subjected to the above-mentioned SPE procedure. Spiked amounts were calculated to obtain the same concentrations of BZD after SPE than in samples from the first set. The mean recovery, as well as associated RSD, were determined for each BZD at each concentration by the ratio of peak areas obtained in the second set to those in the first set (Table 3).

Matrix effect on LC–APPI–MS was investigated using a post-column infusion system according to Bonfiglio et al. [51]. 5 μL of mobile phase, water, and blank haemolysed blood extracted by the above-mentioned SPE procedure was injected in the LC–APPI–MS system, while a solution containing all BZD in the mobile phase at 10 ng/mL was infused post-column at a flow rate of 2 μL/min by a Harvard 11 Plus Single Syringe pump (South Natick, MA, USA). Effects associated to the elution of endogenous compounds on the analytes signal were assessed by comparing the MS response obtained with the injection of extracted blank blood to that of the mobile phase.

2.2.3. Samples used for calibration

Calibration standards (CS) were prepared through an independent method from blank haemolysed blood spiked with known concentrations of analytes and their respective deuterated IS at a fixed concentration. Three concentration levels were selected (k = 3), corresponding to low (estimated LOQ), medium (50%) and high (100%) concentrations. The investigated ranges of each BZD are summarized in Table 1. The CS were replicated twice (n = 2) on three different series (j = 3).

2.2.4. Samples used for validation

Validation standards or quality control samples (QC) were prepared through an independent method from blank haemolysed blood spiked with known concentrations of analytes and their respective deuterated IS at a fixed concentration. Concentrations of each analyte are summarized in Table 1. Four concentration levels were selected (k = 4) and QC were replicated four times (n = 4) on three different series (j = 3).

2.2.5. Application to biological samples

Two frozen blood samples (011 987 and 180 07) were provided from the IUML (Geneva, Switzerland). Sample 011 987 contained FLZ and sample 180 07 contained ALZ, both at concentrations lower than 100 ng/mL. They were defrosted under agitation at room temperature for 30 min. Both samples were centrifuged at 8000 × g for 10 min. One thousand microliters of an aqueous solution of FLZ-D_7 at 180 μg/mL was added to 500 μL of the supernatant of sample 011 987. One thousand microliters of an aqueous solution of ALZ-D_5 at 110 μg/mL was added to 500 μL of the supernatant of sample 180 07. Each sample was then extracted by SPE as described in Section 2.2.1. Based on the available volumes, sample 011 987 was extracted twice (N = 2) and sample 180 07 three times (N = 3).

2.3. Instrumentation

All experiments were performed on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a binary pump. Five microliters of the sample was injected on an XBridge Shield 100 mm × 2.1 mm, 3.5 μm analytical column from Waters (MA, USA). The mobile phase, acetate buffer 20 mM pH 5-ACN (67:33, v/v), was delivered in the isocratic mode at 200 μL/min. The LC system was coupled to an Agilent Series 1100 MSD single quadrupole equipped with an orthogonal APPI source (PhotoMate). Nitrogen was used as both the nebulizing (5 L/min) and drying gas (250 °C). The vaporizer temperature was set at 250 °C, nebulizer pressure at 45 psig and capillary voltage at +2000 V. Post-column infusion of acetone as a dopant was achieved at 20 μL/min by a Harvard 11 Plus Single Syringe pump (South Natick, MA, USA). Detection of protonated FLZ, AFLZ, ALZ, HALZ, LRZ, CLZ, NTZ, and OXZ was conducted in the selected ion monitoring (SIM) mode at m/z 314, 284, 309, 325, 321, 316, 282, and 287, respectively, with a dwell time of 71 ms. The Chemstation A.10.03 software (Agilent Technologies) was used for instrument control, data acquisition and data handling.

3. Results and discussion

The aim of this study was to develop and validate a LC–APPI–MS method for the analysis of two broadly used BZD in haemolysed blood, namely alprazolam (ALZ) and flunitrazepam (FLZ), as well as their major metabolites, α-hydroxyalprazolam (HALZ) and 7-aminoflunitrazepam (AFLZ). Metabolites quantification was carried out to establish a BZD intake even in the case of an undetectable amount of the substrate. Lorazepam (LRZ), clonazepam (CLZ), nitrazepam (NTZ), and oxazepam (OXZ) were included in the evaluation of the method selectivity to avoid quantification problems due to potential co-medication.

The set of compounds includes weak bases (ALZ, FLZ, and AFLZ, pK_a < 3.1), a weak acid (LRZ, pK_a > 10.8) and ampholytes with both weak functions (CLZ, NTZ, OXZ and HALZ) (Table 2). Since BZD possess broad therapeutic and toxicity windows (*e.g.*, between 5 and 400 ng/mL for ALZ [34]), the analytical procedure should therefore enable quantification over a wide concentration range.

3.1. Method development

3.1.1. SPE

A hydrophilic–lipophilic sorbent (Oasis HLB) was selected. In order to determine the most suitable operating conditions, experi-

ments were first performed on neat standard solutions spiked with all compounds and then transferred to haemolysed blood samples.

Analyte retention on the sorbent during the loading step was compared in acidic and neutral media (pH 2.5 and 7). As expected, the Oasis HLB was not able to fully retain analytes under acidic conditions, due to the lower hydrophobicity of BZD (Table 2) than at neutral pH. However, the latter allowed compound retention and was therefore selected. The washing step was then studied with mixtures of water/MeOH between 0% and 100% of MeOH. The optimal amount of MeOH was 30% for minimal compound loss at this stage. Finally, increasing volumes of MeOH (100 μL, 250 μL and 500 μL) for elution were evaluated and 250 μL was found to be optimal for a complete elution from a 10 mg sorbent cartridge.

This SPE procedure was implemented on haemolysed blood samples spiked with all analytes. Potential interference of endogenous compounds during SPE and LC–MS analysis could thus be emphasized. Haemolysed blood was first centrifuged and the supernatant diluted with two volumes of water as a sample pretreatment before SPE. As already observed elsewhere [26], dilution prior to online SPE removed matrix effects encountered with APPI for some analytes, whereas ESI was subjected to such effects in all cases. In this study, as an off-line SPE was operated, qualitative information on matrix effects resulting from a simple dilution of the sample prior to SPE was retrieved from a post-column infusion setup (2.2.2) [51]. As no interferences were observed (data not shown), dilution was selected for its ease and to limit co-precipitation risks. Quantitative results on matrix effects were obtained through the application of the method described by Matuszewski et al. [50]. Recoveries on elution were calculated at low and high concentration levels (estimated LOQ and 100%, respectively, see Table 1) by comparing results obtained with neat standards not extracted on SPE (2.2.2). Mean recoveries were between 83% and 119% for all compounds and RSD values were lower than 10% (Table 3), confirming no matrix effects. The simple dilution of the sample prior to SPE was therefore considered as adapted to off-line SPE combined with APPI.

Table 3
SPE elution step: mean recovery of haemolysed blood samples spiked with the studied BZD at two concentration levels.

	Mean recovery (%)		RSD (%)	
	at LOQ	at 100%	at LOQ	at 100%
Alprazolam	102.9	116.0	3.2	9.6
α-Hydroxyalprazolam	95.1	106.7	6.3	6.6
Flunitrazepam	96.8	106.2	2.4	3.0
7-Aminoflunitrazepam	107.7	119.2	6.0	6.0
Lorazepam	99.9	113.8	6.2	7.6
Clonazepam	86.4	96.0	3.3	2.7
Nitrazepam	84.4	96.4	5.5	3.3
Oxazepam	87.2	92.4	6.5	1.1

3.1.2. LC–APPI–MS

Since there were two pairs of isobaric compounds (*e.g.*, FLZ/ALZ-D_5, *m*/*z* 314 and LRZ/FLZ-D_7, *m*/*z* 321), a chromatographic separation with sufficient resolution was necessary. Three columns were compared (Waters XBridge 100 mm × 2.1 mm, 3.5 μm, Waters XBridge Shield 100 mm × 2.1 mm, 3.5 μm and Thermo Hypersil Gold 100 mm × 2.1 mm, 5 μm) and mobile phases at various pH and compositions were tested. The optimal chromatographic conditions for BZD separation were found using HPLC modeling software (Osiris 4.1.1.2, Datalys, Grenoble, France) according to a procedure described in the literature [52]. The XBridge Shield presented the best compromise between resolution of both isobaric couples and analysis time, with a mobile phase made of acetate buffer 20 mM pH 5-ACN (67:33, v/v). The mobile phase was delivered at 200 μL/min to fulfill APPI requirements regarding the maximal affordable flow rate [53]. Under these chromatographic conditions, complete separation (Rs > 1.5) of isobaric compounds was achieved, with a total analysis time of 10 min (Fig. 2). Isotopic abundance of $^{35}Cl/^{37}Cl$ occurred in most investigated BZD and accounted for small peaks on relatively close (in term of *m*/*z*) extracted chromatograms. For instance, ALZ-D_5 (*m*/*z* 314) presents a small *m*/*z* difference with CLZ (*m*/*z* 316). Therefore, a small peak corresponding to ALZ-D_5 was found on CLZ chromatogram around 5.5 min.

Since HLB elution required pure MeOH, chromatographic performance might be affected by the direct injection of the organic fraction [54]. Hence, evaporation and reconstitution in water was considered. The influence of the injection solvent on the chromatographic separation was evaluated by the injection of standard solutions diluted in increasing proportions of MeOH. Pure MeOH

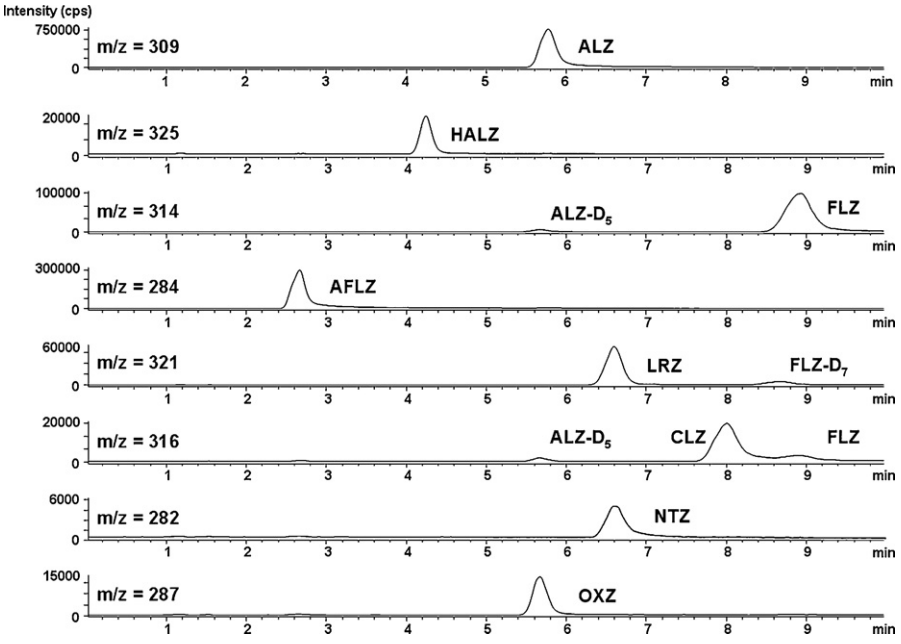

Fig. 2. Chromatograms obtained by the injection of studied BZD diluted in MeOH at 250 ng/mL.

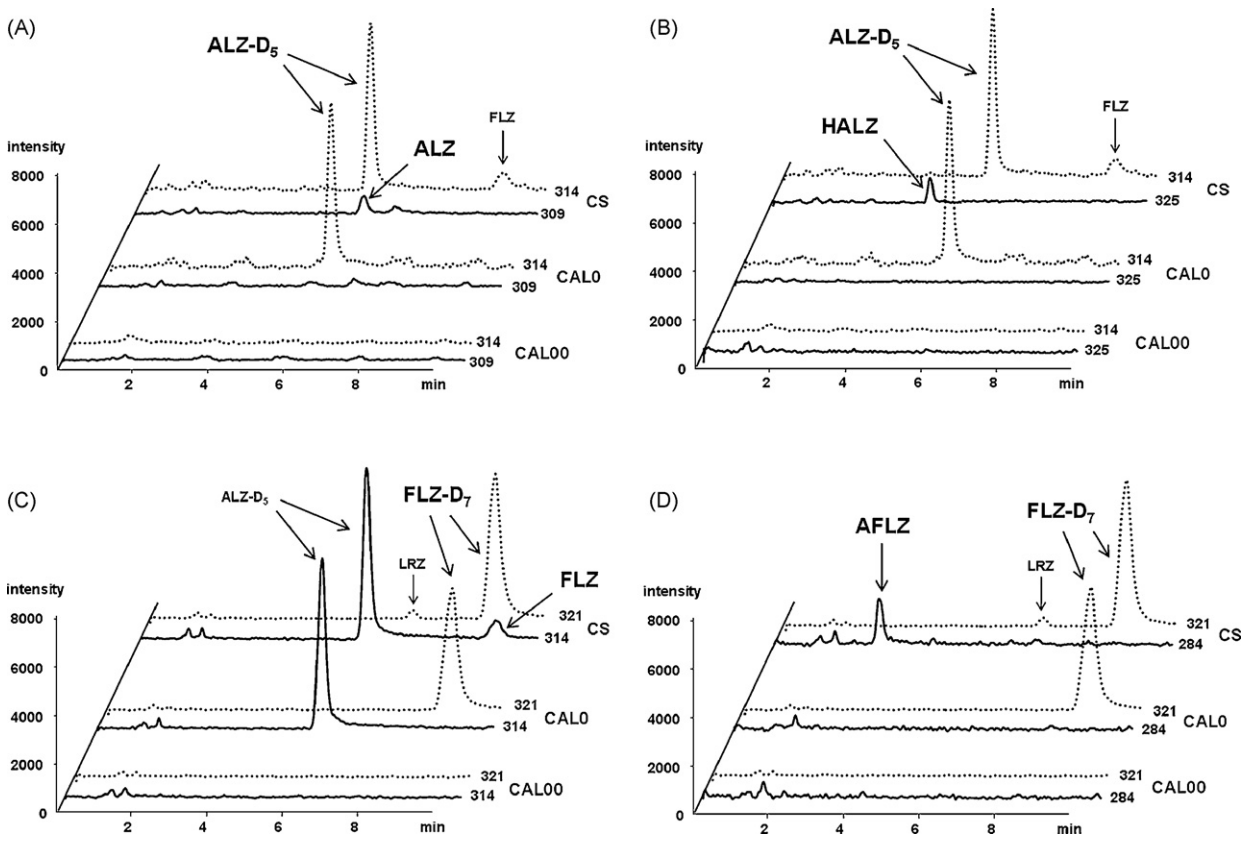

Fig. 3. (A) Typical chromatograms of ALZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with ALZ-D_5 (CAL 0) at 220 ng/mL and a calibration standard (CS) containing ALZ at 1 ng/mL, (B) typical chromatograms of HALZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with ALZ-D_5 (CAL 0) at 220 ng/mL and a calibration standard (CS) containing HALZ at 13 ng/mL, (C) typical chromatograms of FLZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with FLZ-D_7 (CAL 0) at 360 ng/mL and a calibration standard (CS) containing FLZ at 3 ng/mL, and (D) typical chromatograms of AFLZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with FLZ-D_7 (CAL 0) at 360 ng/mL and a calibration standard (CS) containing AFLZ at 2 ng/mL. XIC of ALZ (m/z = 309), HALZ (m/z = 325), ALZ-D_5 (m/z = 314), FLZ (m/z = 314), AFLZ (m/z = 284), and FLZ-D_7 (m/z = 321).

did not alter the resolution, probably due to the low injected volume (5 μL) and to the retention factors between 2 and 8.

Source parameters were optimized with standard solutions and the best compromise for all compounds was selected. It has to be noted that the selected capillary voltage in the APPI was quite high (+2 kV) compared to standard settings. However, no ionization was observed by switching off the lamp, indicating that ionization under these conditions was exclusively due to photoionization and not ESI-like mechanisms. BZD photoionization was evaluated with and without dopant, since the latter could significantly improve the ionization process [55–58]. The two most common dopants, toluene and acetone [56], were investigated. Both were added at 10% of the mobile phase flow rate (*i.e.*, 20 μL/min) since ionization efficiency reaches a maximum at this proportion [55]. This was done through post-column infusion with a tee placed between the column and the detector, to prevent from chromatographic and/or solubility issues compared to direct addition in the mobile phase. The use of toluene was deleterious compared to the no dopant situation, while acetone significantly improved signals (factor 1.5 on ALZ, HALZ, FLZ and AFLZ, n = 6) and was therefore selected (data not shown). It has to be noted that the use of acetone as a dopant in APPI was generally found in the literature to be less efficient than toluene regarding proton transfer. The latter was also proved to promote charge transfer, a mechanism unachievable with acetone [57,59]. However, in the case presented here, pseudo-molecular ions were mainly observed over molecular ions in all cases when acetone was used rather than toluene, as already observed in a previous study [26].

Finally, sensitivity was compared in ESI and APPI with standard solutions of all used benzodiazepines and was found better in APPI by a factor 3 with ALZ and a factor 2 with FLZ. The use of APCI could be a good alternative to APPI for the ionization of BZD with chromatography performed at high flow rates (>1 mL/min) due to its ionization mechanism [55].

3.2. Quantitative performance

In order to compensate for the overall method variability, including extraction and ionization variations, deuterated IS of ALZ and FLZ were employed. Quantitative determinations of ALZ, HALZ, FLZ and AFLZ are presented.

3.2.1. Selectivity

Because endogenous compounds might still be present after solid-phase extraction and induce ionization alterations, potential matrix effects were evaluated, as mentioned in Section 3.1.1. Since no interference was observed, method selectivity was further investigated by comparing chromatograms obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with IS (CAL 0, ALZ-D_5 and FLZ-D_7 at 220 and 360 ng/mL, respectively), and a CS at the estimated LOQ. As illustrated in Fig. 3, no interferences were observed at retention times corresponding to analytes of interest and IS, although six independent sources of haemolysed blood were tested. It has to be noted that the peak appearing at 8 min in Fig. 3A and B corresponded to FLZ, while the peak appearing at 6 min in Fig. 3C corresponded to ALZ-D_5. Both

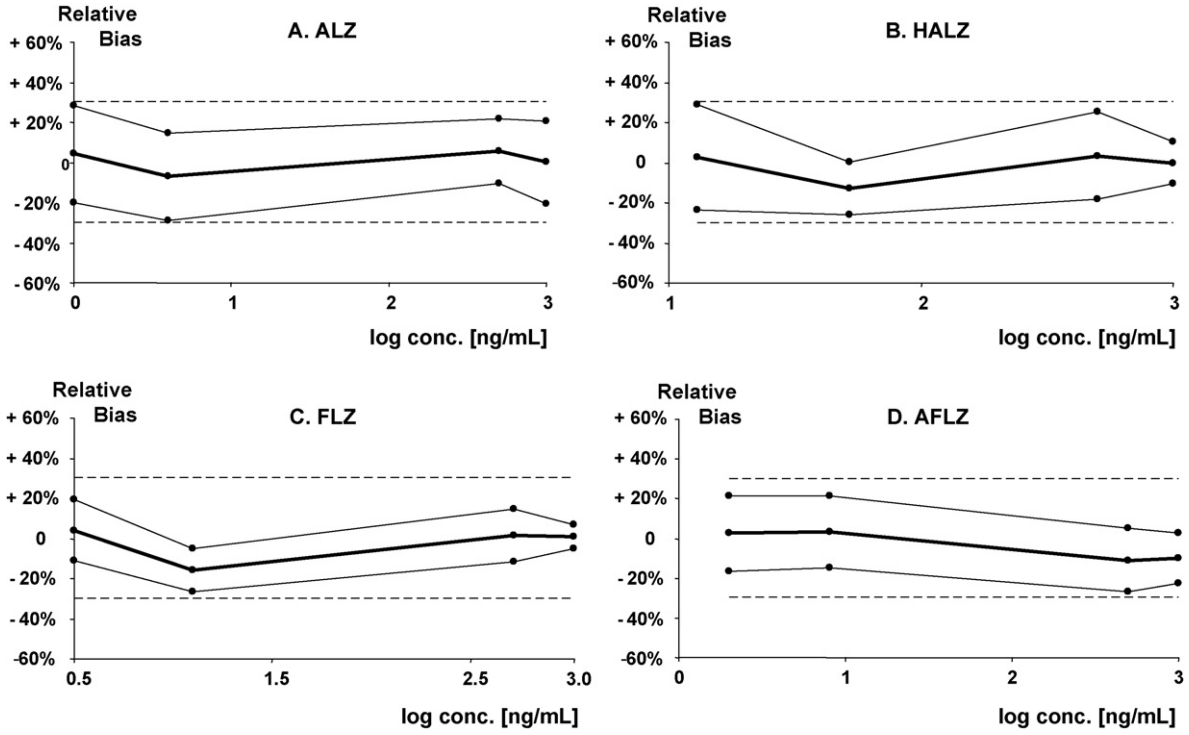

Fig. 4. Accuracy profiles expressed as confidence intervals for (A) ALZ, (B) HALZ, (C) FLZ, and (D) AFLZ.

compounds are isobaric (m/z 314) and could not be resolved with a single quadrupole mass spectrometer. Fig. 3C and D also revealed another peak at 7 min, attributable to LRZ that presents the same m/z value (321) to that of FLZ-D_7. A selective LC separation was thus mandatory prior to MS detection with a single quadrupole analyser to prevent from this issue.

3.2.2. Validation

Quantitative performance was evaluated according to SFSTP validation guidelines on three separate series ($j = 3$). According to SFSTP 2003 recommendations [47], validation protocol V5 was followed. The latter recommends three concentration levels ($k = 3$) with two repetitions ($n = 2$) for CS. To improve accuracy profiles, four concentration levels ($k = 4$) with four repetitions ($n = 4$) for QC, both prepared in the biological matrix, were selected. Trueness and precision were estimated for each concentration level after the selection of the most suitable response function. Trueness corresponds to the difference between the true value and the mean recalculated concentration of QC (experimental value). Trueness was expressed in terms of relative bias (%) for each level of concentration and for every compound. Precision corresponds to the dispersion level among a series of measurements from multiple samplings. In this study, precision was estimated with variances of repeatability (s_r^2) and intermediate precision (s_R^2), calculated on the estimated concentrations as described in [60] and finally expressed as RSD. Accuracy corresponds to the total error including systematic (trueness) and random (precision) errors. For the construction of accuracy profiles used to evaluate the total error of the method, SFSTP 1997 [46] recommendations were followed. Confidence intervals were calculated for each compound with fixed degrees of freedom ($df = j \cdot (n - 1)$) at a unilateral level of risk $\alpha = 10\%$. These confidence intervals gave the lower and upper confidence limits associated to the experimental value.

3.2.2.1. Regression model selection. Since the calibration curve affects validation results, various regression models were compared. Ordinary least squares (OLS), OLS forced through zero, external standard at the highest CS level, OLS after logarithmic transformation of both concentration (x) and response (y), OLS after square root transformation of both concentration (x) and response (y) and weighted least squares (WLS) with two weighting factors ($1/x$ and $1/x^2$) were tested for each compound. QC concentrations were back calculated *via* slope and intercept from each tested response function to determine the mean bias and the confidence limits (lower and upper). The acceptance limit for accuracy profiles was fixed at ±30%, in accordance with the most recent regulatory recommendations [61]. These profiles were used as a decision tool to estimate the method ability to quantify samples with an accepted risk ($\alpha = 10\%$). Fig. 4 shows selected accuracy profiles for ALZ, HALZ, FLZ and AFLZ. The developed method was considered accurate over the investigated concentration ranges for every compound since the lower and upper confidence limits did not exceed the acceptance limits (±30%) (Tables 4A–D). The most appropriate regression model covering the whole concentration range was then selected. The most suitable calibration model was the WLS with $1/x^2$ as a weighting factor for ALZ, HALZ and FLZ, whereas for AFLZ the best model was the linear regression after logarithmic transformation.

3.2.2.2. Linearity. A linear method gives results directly proportional to the concentration of the analyte over the investigated range. For each compound, a linear regression model was applied to the recalculated QC concentrations *vs.* experimental concentrations. The slope, intercept and coefficient of determination were calculated for each model. In all cases, slopes and intercepts were between 0.900–1.010 and −0.42–4.86, respectively. The R^2 values were higher than 0.9992, indicating that the developed method was linear for the tested compounds.

3.2.2.3. Limit of quantification (LOQ). The lower limit of quantification (LLOQ) defines the lowest amount of analyte that can be measured in the matrix under the experimental conditions with a defined accuracy. Since the lowest concentration levels for each

Table 4A
Validation results for ALZ ($j = 3$; $k = 4$; $n = 4$).

Validation criterion	Alprazolam
Trueness	
Relative bias (%)	
1 (ng/mL)	4.2
4 (ng/mL)	−7.1
500 (ng/mL)	5.6
1000 (ng/mL)	0.1
Precision	
Repeatability/intermediate precision (RSD, %)	
1 (ng/mL)	9.4/13.2
4 (ng/mL)	4.8/12.0
500 (ng/mL)	8.3/8.8
1000 (ng/mL)	4.5/11.2
Accuracy	
Lower/upper limits of the total error (%)	
1 (ng/mL)	−20.1/28.5
4 (ng/mL)	−29.2/14.9
500 (ng/mL)	−10.6/21.8
1000 (ng/mL)	−20.5/20.7
Linearity	
Range (ng/mL)	[1;1000]
Slope	1.006
Intercept	4.8596
R^2	0.9992
LLOQ (ng/mL)	1

Table 4C
Validation results for FLZ ($j = 3$; $k = 4$; $n = 4$).

Validation criterion	Flunitrazepam
Trueness	
Relative bias (%)	
3 (ng/mL)	4.1
12 (ng/mL)	−15.7
500 (ng/mL)	1.4
1000 (ng/mL)	0.8
Precision	
Repeatability/intermediate precision (RSD, %)	
3 (ng/mL)	5.8/8.2
12 (ng/mL)	4.4/5.9
500 (ng/mL)	7.1/7.1
1000 (ng/mL)	3.3/3.3
Accuracy	
Lower/upper limits of the total error (%)	
3 (ng/mL)	−11.0/19.2
12 (ng/mL)	−26.5/−4.9
500 (ng/mL)	−11.6/14.4
1000 (ng/mL)	−5.2/6.8
Linearity	
Range (ng/mL)	[3;1000]
Slope	1.010
Intercept	−0.3241
R^2	1.0000
LLOQ (ng/mL)	3

compound were included in the acceptance limits, they were considered as the LLOQ (1, 13, 3 and 2 ng/mL for ALZ, HALZ, FLZ and AFLZ, respectively). In comparison with ESI and APCI, the LOQ achieved for ALZ and FLZ in APPI with our single quadrupole MS was analogous to those obtained in the literature in ESI with triple quadrupole MS or ion trap MS (in the 0.5–5.0 ng/mL range) [11,62–66] and in APCI with single quadrupole MS or ion trap MS (in the 1.0–5.0 ng/mL range) [20,44,67,68].

3.2.2.4. Trueness and precision. Trueness was acceptable in each case (threshold of ±15%) as the relative bias was lower than 12.8% except for FLZ at 12 ng/mL (−15.7%) (Tables 4A–D). However, this higher value was compensated by good precision, keeping the total error, expressed as the confidence interval, under ±30%. Regarding precision, RSD values were satisfactory, since they were all between 3.3% and 10.1% for repeatability, and between 3.3% and 14.3% for intermediate precision. The best results were obtained with FLZ (3.3/7.1% and 3.3/8.2% for repeatability and intermediate precision, respectively).

3.2.3. Application to biological samples

The applicability of the developed method was illustrated with the analysis of two real haemolysed blood samples (180 07 and 011 987). A preliminary LC–MS screening was first performed by

Table 4B
Validation results for HALZ ($j = 3$; $k = 4$; $n = 4$).

Validation criterion	α-Hydroxyalprazolam
Trueness	
Relative bias (%)	
13 (ng/mL)	2.9
52 (ng/mL)	−12.8
500 (ng/mL)	3.6
1000 (ng/mL)	−0.2
Precision	
Repeatability/intermediate precision (RSD, %)	
13 (ng/mL)	10.1/14.3
52 (ng/mL)	3.9/7.1
500 (ng/mL)	10.0/11.8
1000 (ng/mL)	4.1/5.8
Accuracy	
Lower/upper limits of the total error (%)	
13 (ng/mL)	−23.3/29.0
52 (ng/mL)	−25.7/0.2
500 (ng/mL)	−18.1/25.2
1000 (ng/mL)	−10.7/10.4
Linearity	
Range (ng/mL)	[13;1000]
Slope	1.005
Intercept	0.585
R^2	1.000
LLOQ (ng/mL)	13

Table 4D
Validation results for AFLZ ($j = 3$; $k = 4$; $n = 4$).

Validation criterion	7-Aminoflunitrazepam
Trueness	
Relative bias (%)	
2 (ng/mL)	2.7
8 (ng/mL)	3.2
500 (ng/mL)	−11.0
1000 (ng/mL)	−9.9
Precision	
Repeatability/intermediate precision (RSD, %)	
2 (ng/mL)	10.0/10.4
8 (ng/mL)	8.1/9.8
500 (ng/mL)	7.4/8.7
1000 (ng/mL)	4.9/6.8
Accuracy	
Lower/upper limits of the total error (%)	
2 (ng/mL)	−16.4/21.7
8 (ng/mL)	−14.7/21.1
500 (ng/mL)	−26.8/4.9
1000 (ng/mL)	−22.3/2.6
Linearity	
Range (ng/mL)	[2;1000]
Slope	0.900
Intercept	0.4183
R^2	1.0000
LLOQ (ng/mL)	2

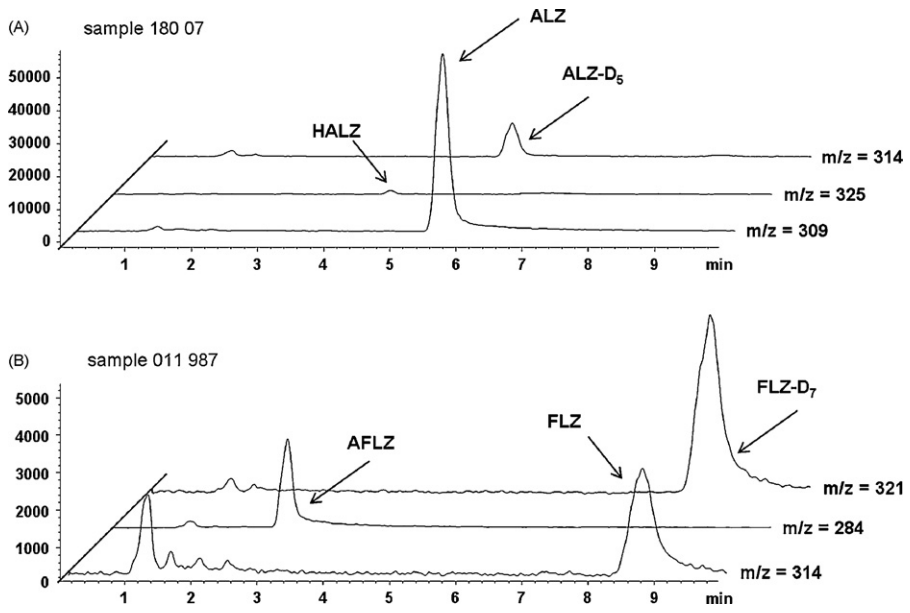

Fig. 5. Chromatograms obtained from the injection of toxicological samples. (A) haemolysed blood sample 180 07 spiked with ALZ-D_5 at 220 ng/mL and (B) haemolysed blood sample 011 987 spiked with FLZ-D_7 at 360 ng/mL.

the Institut Universitaire de Médecine Légale which showed that sample 180 07 contained ALZ and sample 011 987 contained AFLZ. The method was applied to both samples and quantification of each compound was based on a calibration curve obtained on the same day. CS with the same concentrations as those used during the validation process ($k = 3$) were injected twice ($n = 2$) and the same data treatment was used (WLS with $1/x^2$ as weighting factor for ALZ, HALZ and FLZ and OLS after logarithmic transformation for AFLZ). The confidence interval was calculated with Formula 1.

$$\bar{x} = t_{\mathrm{df},\alpha}\sqrt{\frac{s_r^2}{N} + s_g^2} \quad (1)$$

where $\bar{x}$ is the mean result and N is the number of analyses. The $t_{\mathrm{df},\alpha}$ (Student constant depending on the degrees of freedom (df) and on the level of significance α), s_r^2 and s_g^2 (intra and inter-series variances) values were determined during validation *via* the regular ANOVA-based variance decomposition. Since an important part of the overall variability was attributed to repeatability, samples were analysed twice (sample 011 987) and thrice (sample 180 07) to decrease the confidence interval. In routine analysis, this can be carried out in a single measurement according to the obtained validation results. Chromatograms of both samples are given in Fig. 5.

A concentration of 7.6 ± 0.5 ng/mL of FLZ was found in sample 011 987, but the sample also contained 27.5 ± 9.6 ng/mL of AFLZ, indicating that the majority of the FLZ was metabolized (see Table 2). Concerning sample 180 07, it contained 64.6 ± 18.2 ng/mL of ALZ and 24.7 ± 3.5 ng/mL of HALZ, a concentration within the toxicity window considering plasma values [34]. However, no data were found in the literature to evaluate these values in haemolysed blood samples.

4. Conclusion

A method for determining alprazolam, flunitrazepam and their major metabolites in haemolysed blood was developed. The sample preparation was achieved by a fast and easy SPE without evaporation or reconstitution. A selective and sensitive LC–APPI–MS analysis was carried out in less than 10 min. The use of the APPI source was found to be an excellent alternative to ESI since it lead to similar sensitivities without matrix effects. A validation strategy based on accuracy profiles was applied on alprazolam, flunitrazepam and their major metabolites to demonstrate the methods ability to quantify these compounds in haemolysed blood over a wide concentration range. Selectivity, trueness, precision and the lower limit of quantification were calculated by applying an appropriate regression model and good performance was achieved (accuracy was included in the acceptance limit of ±30%). The developed technique was finally applied to rapidly quantify alprazolam, flunitrazepam and their metabolites in two toxicological samples.

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