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Liquid chromatography–electrospray ionisation mass spectrometry for the determination of nine selected benzodiazepines in human plasma and oral fluid

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Abstract

A new simple and rapid liquid chromatographic–mass spectrometric technique was designed for the determination of nine benzodiazepines in plasma and oral fluid. Benzodiazepines were extracted from alkalinised spiked and clinical plasma and oral fluid samples using a single step, liquid–liquid extraction procedure with diethyl ether. The chromatographic separation was performed with a Xterra® RP_{18}, 5 μm (150 × 2.1 mm i.d.) reversed-phase column using deuterated analogues of the analytes as internal standard. The recovery ranged from 70.3 to 86.9% for plasma and 63.9 to 77.2% for oral fluid. The limits of detection ranged from 0.5 to 1 ng/ml in plasma and 0.1 to 0.2 ng/ml for oral fluid. The method was validated for all the compounds, including linearity and the main precision parameters. The procedure, showed to be sensitive and specific, was applied to real plasma and oral fluid samples. The method is especially useful to analyse saliva samples from drivers undergoing roadside drug controls.

Keywords: Liquid chromatography; Benzodiazepines; Plasma; Oral fluid; Saliva; Liquid-chromatography-mass spectrometry

1. Introduction

Benzodiazepines (BZP) are a large class of psychoactive drugs, very commonly prescribed all over the world, mostly as minor tranquillizers, hypnotics and muscle relaxants [1–4]. Their clinical applications have been associated to their wide safety margin and minimal adverse side effects. From a toxicological point of view, these drugs are associated to different cases of misuse: they are often abused by illicit drug users to relieve withdrawal symptoms and, recently, the use of such substances to commit sexual abuse had spread [5]. Furthermore, they are the most documented group with regard to the influence on driving behaviour [6]. So, they are usually present in clinical and forensic toxicological cases [7–9].

A large number of analytical methods have been published so far for the determination of Benzodiazepines. Numerous gas chromatography (GC) procedures have been reported for the analysis of BZP [10–13], including NPD and ECD detectors. Generally, capillary GC coupled to mass spectrometry (MS) is the method of choice for most of the toxicological analysis. Electronic impact GC–MS provides a selective analytical procedure for the analysis of BZP in biological samples [14–17] although several authors have demonstrated that the negative-ion chemical ionisation (NCI) is a more sensitive technique [18–21]. However, this method is generally tedious to apply because BZP are very polar and thermally unstable, so they cannot be evaporated and chromatographed without decomposition, unless previous derivatisation [22]. Gas chromatography–mass spectrometry determinations have been reviewed by Maurer [23]. In recent years, the coupling of liquid chromatography (LC) to MS

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has provided a useful procedure for the analysis of organic compounds and an alternative to GC–MS in order to avoid the excessive manipulation of polar and unstable compounds. The use of HPLC–MS for clinical and forensic purposes have been reviewed by Maurer [24], Hoja [25], Marquet [26] and Van Boxclaer [27].

The excellent review done by Drummer [28] about extraction procedures and methods for the measurement of BZP in biological samples is outstanding. Finally, some references related to the determination of BZP in biological samples by HPLC–DAD [29–32] and capillary electrophoresis [33–35] must be included in order to complete a full revision of the analytical methodology.

The biological samples most commonly used for the analysis of BZP are plasma/blood and urine. The use of an alternative specimen such as saliva has been proposed [36] in the roadside places. In this context the use of saliva for the BZP detection can become a very useful tool.

For this reason we have developed a simple HPLC–MS procedure for the determination of nine BZP in human plasma and oral fluid, in order to detect small quantities. In addition, a preliminary pharmacokinetic study for two of them (midazolam and lormetazepam) was carried out in order to obtain the possible correlation between plasma and oral fluid levels.

2. Material and methods

2.1. Reagents

Midazolam, bromazepam, tetrazepam, alprazolam, lorazepam, triazolam, flunitrazepam, diazepam and lormetazepam, from Sigma Chemical (St. Louis, MO, USA). Internal standards (IS): Alprazolam-d_5, lorazepam-d_4 and diazepam-d_5, from Cerilliant (Barcelona, Spain). Formic acid (99% pure) was obtained from Merck (Barcelona, Spain). Chromasolv® grade Acetonitrile (99.98% pure) was from Riedel de Häen Sigma–Aldrich Chemie (Schnelldorf, Germany). Purified water was obtained in the laboratory using a Milli-Q water system (Le Mont-sur-Lausanne, Switzerland). A 0.1 M ammonium carbonate pH 9.30 buffer was prepared by adding a 1 M ammonium hydroxide solution to 900 ml of ammonium carbonate solution (9.6 g/l) to pH 9.3 (determined using a pH meter) and making up the solution to 1000 ml with ammonium carbonate (to oral fluid samples).

A pH 9.0 borate buffer was prepared by mixing 6.2 g of H_3BO_3 and 7.5 g of KCl with 420 ml of a solution of 0.1 M sodium hydroxide, and adding water until 1000 ml (to plasma samples).

Fresh and drug-free human plasma and oral fluid (OF). Liquid–liquid extraction: Diethylether Multisolvent, from Scharlau (Sentmenat, Spain).

Salivette® device for collecting OF, with and without citric acid stimulation (Sarstedt Nümbrecht, Germany).

2.2. Preparation of stock solutions and standards

Stock solutions of each BZP and respective IS were prepared at 1 g/l in methanol or acetonitrile and were kept at −20 °C in the dark for a maximum of 6 months. Each day, working solutions of each, containing 0.02, 0.1, 0.5 and 2.5 mg/l, for plasma and 0.002, 0.005, 0.01, 0.1 and 0.25 mg/l for saliva, were prepared by appropriate dilution with Milli-Q water. The working solutions of respective internal standards at 1 mg/l (for plasma) and 0.4 mg/l (for saliva) were prepared by appropriate dilution with methanol.

2.3. Sample preparation

To 0.5 ml of plasma were added 50 μl of a 1 mg/l IS solution, 0.5 ml of the pH 9.0 borate buffer and 8 ml of the extraction solvent (diethyl ether), in a 10-ml borosilicate tube. The tubes were shaken for 15 min then centrifuged at 3500 rpm for 10 min. The organic phase (7.2–7.8 ml) was transferred to a 10-ml borosilicate tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The dry extract was re-dissolved in 100 μl of a mixture of formic acid 0.1%–acetonitrile (90:10, v/v). The samples were transferred into autosampler vials, and 15 μl was injected into the LC–MS.

In the case of saliva samples, the process was slightly different. To 0.5 ml of saliva were added 50 μl of a 0.4 mg/l IS solution, 0.5 ml of the 0.1 M ammonic carbonate buffer, pH 9.30, and 6 ml of the extraction solvent (diethyl ether), in a 10-ml borosilicate tube. The tubes were shaken for 15 min then centrifuged at 3500 rpm for 10 min. The organic phase (5.2–5.8 ml) was transferred to a 10-ml borosilicate tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The dry extract was re-dissolved in 50 μl of a mixture of formic acid 0.1%–acetonitrile (93:7, v/v), of which 15 μl was injected into the chromatographic system.

Two sets of calibrating standards, one for plasma at 0, 1, 2, 5, 10, 25, 50, 125 and 250 ng/ml, and one for saliva at 0.2, 0.5, 1, 2, 10 and 25 ng/ml of the mixture of BZP, were prepared with each series, by spiking blank plasma and saliva samples with the appropriate working solutions.

2.4. Liquid chromatography–mass spectrometry

The HPLC system was a Waters Alliance 2795 (Waters, Watford, UK) separation Module. Chromatographic separation was performed with a Xterra® RP_{18}, 5 μm (150 × 2.1 mm i.d.) reversed-phase column (Waters, Milford, USA). The mobile phase, delivered at a flow-rate of 0.25 ml/min at room temperature, was a gradient of acetonitrile in 0.1% formic acid programmed as follows: for plasma, 10% acetonitrile during 1.5 min, increased to 72% in 14 min and decreased to 10%, i.e., original conditions, in 1 min; and for saliva, 7% acetonitrile during 2 min, increased to 72%

in 12.5 min and decreased to 7%, i.e., original conditions, in 0.5 min.

The detection was performed using a Micromass ZMD 2000 mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray ion interface. Ionisation was achieved using electrospray in the positive ionisation mode (ESI+). Nitrogen was used as nebulisation and desolvatation gas. For optimising ionisation and ion transmission conditions of each BZP and the IS, 15 μl of a 10 μg/ml solution of each compound in the mobile phase were injected without HPLC separation into the ion source. In order to obtain the highest possible intensity for quantitation and confirmation ions, fragmentation energy (cone voltage) was optimised. During this experiment, a mass range from *m*/*z* 100 to 400 was monitored. Acquisition was made in the selected ion-monitoring mode of positive ions, with a dwell time of 0.15 s. For the quantitation of each BZP the protonated molecule $[M+H]^+$ was selected as the quantifying ion and one main fragment was selected as the confirmation ion. Table 1 summarizes the conditions for the measurement of each BZP and the deuterated IS. The other main parameters settings were: drying gas temperature 300 °C, source heater temperature 115 °C, nebulisation gas flow 550 l/h, cone gas flow 100 l/h and capillary voltage 3000 V.

Data acquisition, peak integration and calculation were interfaced to a computer workstation running Mass Lynx NT 3.5 and QuanLynx 3.5 software.

Table 1
Retention times, selected ions and cone voltages of the nine selected benzodiazepines

Compound	Retention time (min)		Selected *m*/z ratios[a]	Cone voltage (V)
	Pl	OF		
Alprazolam	11.6	12.6	**309.1**	40
			281.2	60
Bromazepam	9.8	10.8	**318.0**	30
			290.0	50
Diazepam	13.4	14.1	**285.3**	20
			257.2	60
Flunitrazepam	12.9	13.8	**314.2**	30
			268.2	60
Lorazepam	12.2	13.2	**321.0**	30
			303.1	40
Lormetazepam	13.2	14.2	**335.0**	30
			289.0	60
Midazolam	9.27	10.1	**326.1**	30
			291.3	60
Tetrazepam	11.1	12.2	**289.2**	40
			261.3	55
Triazolam	12.0	12.9	**343.1**	45
			308.1	55
Alprazolam-d_5	11.6	12.5	**314.2**	40
Diazepam-d_5	13.3	14.2	**290.3**	40
Lorazepam-d_4	12.2	13.2	**325.1**	25

[a] Quantifying ions are in bold characters.

2.5. Validation

The analytical validation was performed according the recommendations of Shah [37] and Peters [38].

Recoveries were determined in quintuplicate at two concentrations (low and high) of each BZP in plasma and saliva. For each concentration, five blank samples were fortified with the IS and the appropriate amount of each BZP, while five others only with the IS. They were extracted as previously described. The dry extracts of the fortified samples were re-dissolved in 50 μl of the reconstitution solvent, while the extracts of the blank samples were re-dissolved with 50 μl of the reconstitution solvent containing the respective nominal amounts of BZP. The latter were used as neat standards.

In addition, the recovery from the cotton-roll of Salivette® was calculated in a similar way at 1 and 25 ng/ml of each BZP in saliva. For each concentration, 10 blank saliva samples were fortified with the respective BZP and IS. Five samples of each concentration were absorbed with Salivette® devices, centrifuged and then extracted as previously described. The other five, directly extracted, were used as neat standards.

Within-day precision and accuracy were determined at three concentrations, the lower limit of quantitation (LLOQ), the upper limit of quantitation (ULOQ) and an intermediate level, by preparing and analysing on the same day six replicates for each level. Between-day precision and accuracy, as well as linearity and the lower limit of quantitation (LLOQ) were assessed by analysing each day for 6 days a set of plasma samples spiked at 1, 2, 5, 10, 20, 50, 125 and 250 ng/ml and saliva samples at 0.2, 0.5, 1, 2, 10 and 25 ng/ml, respectively. Precision, expressed as the coefficient of variation (CV) of the measured values, was expected to be less than 15% at all concentrations, except for the LLOQ, for which 20% was acceptable. In the same way, accuracy was evaluated using the mean relative error (MRE), which had to be less than 15% of theoretical values at each concentration level except for the LLOQ, for which 20% was acceptable [37,38]. Therefore, LLOQ was defined as the lowest concentration yielding between-day precision CV and MRE of less than 20% each.

The validation of a partial or increased sample volume was done by diluting 1/5 additional quality controls (QC) with a concentration five times above the ULOQ in plasma and saliva (1250 and 250 ng/ml, respectively). Five replicates of each control sample were analysed together with a calibration curve after appropriate dilution of the samples, with blank plasma or saliva, to the validated concentration range. The calculated concentrations of diluted QCs, when multiplied by their respective dilution factors must fall within the defined precision and accuracy criteria for that QC [39].

The specificity of the method was evaluated by analysing plasma and saliva samples from 10 healthy non-drug-consuming subjects.

The matrix effect on the ESI response was evaluated by using a post-column infusion system [40–42]. Mobile phase was delivered into the electrospray interface at a rate of

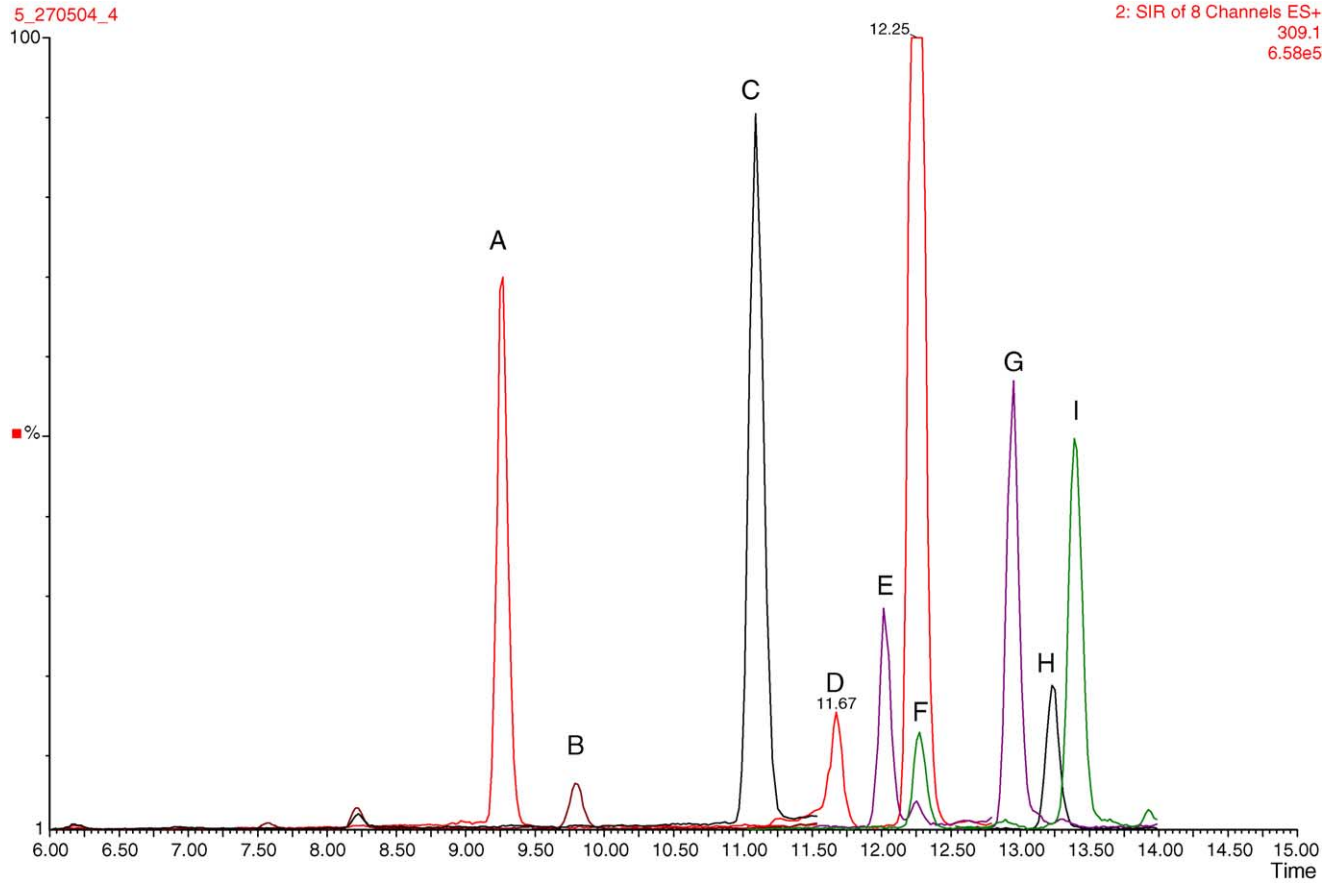

Fig. 1. LC-ES–MS ion cromatograms of a plasma sample spiked at 5 ng/ml of nine benzodiazepines.

250 μl/min while analyte was being infused, post-column, through a Valco zero dead volume tee using a Harvard Apparatus Model 11 (South Natick, MA, USA) syringe pump. Five plasma and saliva samples without BZP nor IS were extracted as previously described, reconstituted with mobile phase, and 15 μl of each plasma extract were injected onto the XTerra column. Effluent from the HPLC column combined with the infused analytes and entered the electrospray interface.

Midazolam was administered parenterally in a single 2 mg-dose to patients undergoing minor surgery. Patients were conscious during the OF sampling in all cases. Lormetazepam was administered orally at a 1 mg dose to healthy volunteers.

3. Results and discussion

Deuterated analogues are commonly used as internal standards as they are essentially identical in chemical and chromatographic properties to the respective unlabelled compounds whilst being readily distinguishable by mass spectrometry because of their mass difference. In our case alprazolam-d_5, lorazepam-d_4 and diazepam-d_5 were chosen for this purpose.

The ion chromatograms of the nine BZP are presented in Fig. 1. The retention time and the selected ions are reported in Table 1. Likewise, their respective optimised fragmentation voltages are shown in the same table as responsible to obtain the highest intensity of the base product ions. CID conditions were adjusted in order to obtain the optimum intensity for one product ion for each analyte.

No endogenous plasma and saliva components were observed at the retention times of the nine analytes nor internal standards.

The within-day precision, as well as the between-day precision and accuracy were satisfactory under all the tested concentrations (Tables 2 and 3).The linearity of the compound-to-IS peak area ratio versus the theoretical concentration was verified in plasma and saliva by using a $1/x$ weighted linear regression. The correlation coefficients were typically better than 0.99 and the curvature was tested on a set of six calibration curves (Table 4). The limits of detection (LOD), defined as the lowest tested concentration yielding a signal-to-noise ratio higher than 3, and the recoveries, calculated as described in the validation section, are shown in the same table. The LLOQs and the ULOQs, also can be seen on Table 4, and correspond to the lowest and the highest concentration level of the calibration range, respectively, for each analyte.

Table 2
Within-day precision and accuracy for the determination of nine benzodiazepines in plasma (Pl) and oral fluid (OF) samples

Compound		Precision (R.S.D., %)			Mean relative error[a] (%)		
		Low level	Medium level	High level	Low level[b]	Medium level[c]	High level[d]
Alprazolam	Pl	7.7	4.2	2.7	4.0	4.7	4.8
	OF	6.9	2.1	3.3	7.7	1.3	2.4
Bromazepam	Pl	11.7	13.0	7.2	1.2	6.8	1.8
	OF	11.3	10.8	9.8	9.3	1.6	2.1
Diazepam	Pl	9.5	2.0	3.7	6.0	1.5	0.8
	OF	8.5	2.4	0.9	0.8	4.9	4.6
Flunitrazepam	Pl	6.3	2.9	6.9	3.5	3.0	0.7
	OF	5.5	6.5	1.1	0.1	3.0	1.8
Lorazepam	Pl	8.9	3.5	3.6	5.0	6.6	3.3
	OF	8.4	3.8	2.3	1.1	1.4	4.0
Lormetazepam	Pl	7.7	8.6	1.2	1.5	8.5	3.6
	OF	9.2	7.4	2.2	0.8	5.2	0.7
Midazolam	Pl	2.3	3.4	8.9	0.8	8.2	5.8
	OF	13.4	3.8	5.2	3.3	5.7	6.6
Tetrazepam	Pl	5.3	6.7	7.9	4.3	7.3	6.8
	OF	6.4	3.9	2.3	7.5	5.2	0.9
Triazolam	Pl	5.6	8.2	6.7	0.2	1.7	4.7
	OF	9.7	8.1	2.8	5.8	5.7	2.7

[a] Mean relative error = |Mean measured value − theoretical value| × 100/theoretical value.
[b] Low level, the LLOQ of each coumpoud.
[c] Medium level, 50 ng/ml in plasma and 2 ng/ml in OF samples.
[d] High level, the ULOQ of each compound.

Table 3
Between-day precision and accuracy for the determination of 9 benzodiazepines in plasma (Pl) and oral fluid samples (OF)

Compound		Precision (R.S.D., %)			Mean relative error[a] (%)		
		Low level[b]	Medium level[c]	High level[d]	Low level[b]	Medium level[c]	High level[d]
Alprazolam	Pl	5.7	4.2	2.1	13.3	3.4	0.6
	OF	5.1	3.9	1.7	1.1	1.2	0.4
Bromazepam	Pl	8.3	9.9	2.6	1.2	1.1	2.4
	OF	12	4.7	2.5	7.7	5.9	0.1
Diazepam	Pl	12.6	4.6	1.3	2.6	1.8	1.9
	OF	9.1	2.2	0.9	10.0	6.4	2.5
Flunitrazepam	Pl	14.7	4.1	2.0	1.4	4.4	3.5
	OF	8.2	8.4	1.4	7.9	0.4	1.2
Lorazepam	Pl	13.8	4.6	1.3	5.4	2.3	2.3
	OF	6.6	4.3	1.3	8.6	5.2	2.1
Lormetazepam	Pl	12.8	2.4	3.4	1.3	3.0	1.1
	OF	8.1	7.0	1.8	5.0	4.0	0.9
Midazolam	Pl	13.7	3.3	2.8	5.0	6.9	4.0
	OF	12.5	5.4	2.9	3.3	3.2	0.1
Tetrazepam	Pl	14.8	5.8	2.0	5.8	8.3	3.8
	OF	6.2	3.5	1.4	14.3	6.4	2.8
Triazolam	Pl	11.7	4.5	1.0	1.6	3.2	4.5
	OF	13.3	4.6	2.5	6.4	4.2	0.7

[a]Mean relative error = |Mean measured value − theoretical value| × 100/theoretical value. [b]Low level, corresponding with the LLOQ of each compound. [c]Medium level, corresponding with 50 ng/ml in plasma and 2 ng/ml in OF samples. [d]High level, corresponding with the ULOQ of each compound.

Table 4
Calibration data, limits of detection, and recoveries for the examined compounds

Compound	Biological sample	Calibration range (ng/ml)	Linearity ($n = 6$)	R^2	S.D. (on slope)	LOD[a] (ng/ml)	Recovery (%) ($n = 5$)	
							Low[b]	High[c]
Alprazolam	Pl	1–400	$y = 0.004x + 0.0015$	0.999	0.0002	0.5	77.5	76.7
	OF	0.5–50	$y = 0.0212x + 0.0039$	0.999	0.0041	0.2	70.6	67.6
Bromazepam	Pl	5–250	$y = 0.003x + 0.0024$	0.996	0.0008	1	85.9	84.0
	OF	0.5–25	$y = 0.0066x + 0.0014$	0.998	0.0022	0.2	70.3	70.5
Diazepam	Pl	1–250	$y = 0.0053x + 0.0026$	0.999	0.0003	0.5	81.6	89.0
	OF	0.2–50	$y = 0.0161x + 0.0028$	0.999	0.0012	0.1	77.2	67.2
Flunitrazepam	Pl	1–400	$y = 0.0061x + 0.0011$	0.998	0.0003	0.5	82.3	91.0
	OF	0.2–50	$y = 0.0202x + 0.001$	0.999	0.0069	0.1	76.1	72.1
Lorazepam	Pl	1–250	$y = 0.0032x + 0.0018$	0.999	0.0001	0.5	71.9	78.0
	OF	0.5–25	$y = 0.0295x + 0.0036$	0.998	0.0029	0.2	72.7	72.3
Lormetazepam	Pl	1–400	$y = 0.0018x + 0.0006$	0.998	0.0001	0.5	77.7	82.4
	OF	0.2–50	$y = 0.006x + 0.0007$	0.999	0.0011	0.1	70.5	71.0
Midazolam	Pl	1–250	$y = 0.005x + 0.0017$	0.996	0.0013	0.5	70.3	71.6
	OF	0.2–25	$y = 0.0143x + 0.0021$	0.998	0.0029	0.1	68.3	69.9
Tetrazepam	Pl	1–250	$y = 0.0074x + 0.001$	0.996	0.001	0.5	86.9	96.8
	OF	0.2–50	$y = 0.0276x + 0.0024$	0.998	0.0014	0.1	72.1	69.3
Triazolam	Pl	2–250	$y = 0.0149x - 0.0004$	0.994	0.0007	0.5	74.5	69.9
	OF	0.2–50	$y = 0.0118x + 0.001$	0.998	0.0011	0.1	63.9	64.9

[a] Defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1.
[b] Low levels, 5 ng/ml in plasma and 2 ng/ml in OF samples.
[c] High levels, 125 ng/ml in plasma and 25 ng/ml in OF samples.

Table 5
Evaluation of partial sample dilution in plasma and oral fluid samples, and recoveries of benzodiazepines from the cotton roll of the Salivette® devices

Compound		Dilution 1/5 ($n = 6$)		Recovery from Salivette® (%) ($n = 5$)	
		R.S.D. (%)	Mean relative error[a]	1 ng/ml	25 ng/ml
Alprazolam	Pl	1.6	0.2		
	OF	2.6	3.4	101.2	82.7
Bromazepam	Pl	12.8	2.3		
	OF	9.2	1.5	91.6	90.0
Diazepam	Pl	2.4	0.7		
	OF	2.2	3.8	68.0	67.7
Flunitrazepam	Pl	3.1	0.8		
	OF	1.6	0.6	88.8	78.7
Lorazepam	Pl	2.5	1.4		
	OF	1.6	1.8	65.8	70.3
Lormetazepam	Pl	2.9	1.4		
	OF	1.5	7.2	81.8	73.5
Midazolam	Pl	9.7	3.9		
	OF	10.0	2.7	52.2	55.0
Tetrazepam	Pl	5.4	7.3		
	OF	3.7	3.6	55.0	53.7
Triazolam	Pl	5.9	3.7		
	OF	5.8	13.2	89.8	87.4

[a] Mean relative error = |Mean measured value − theoretical value| × 100/theoretical value.

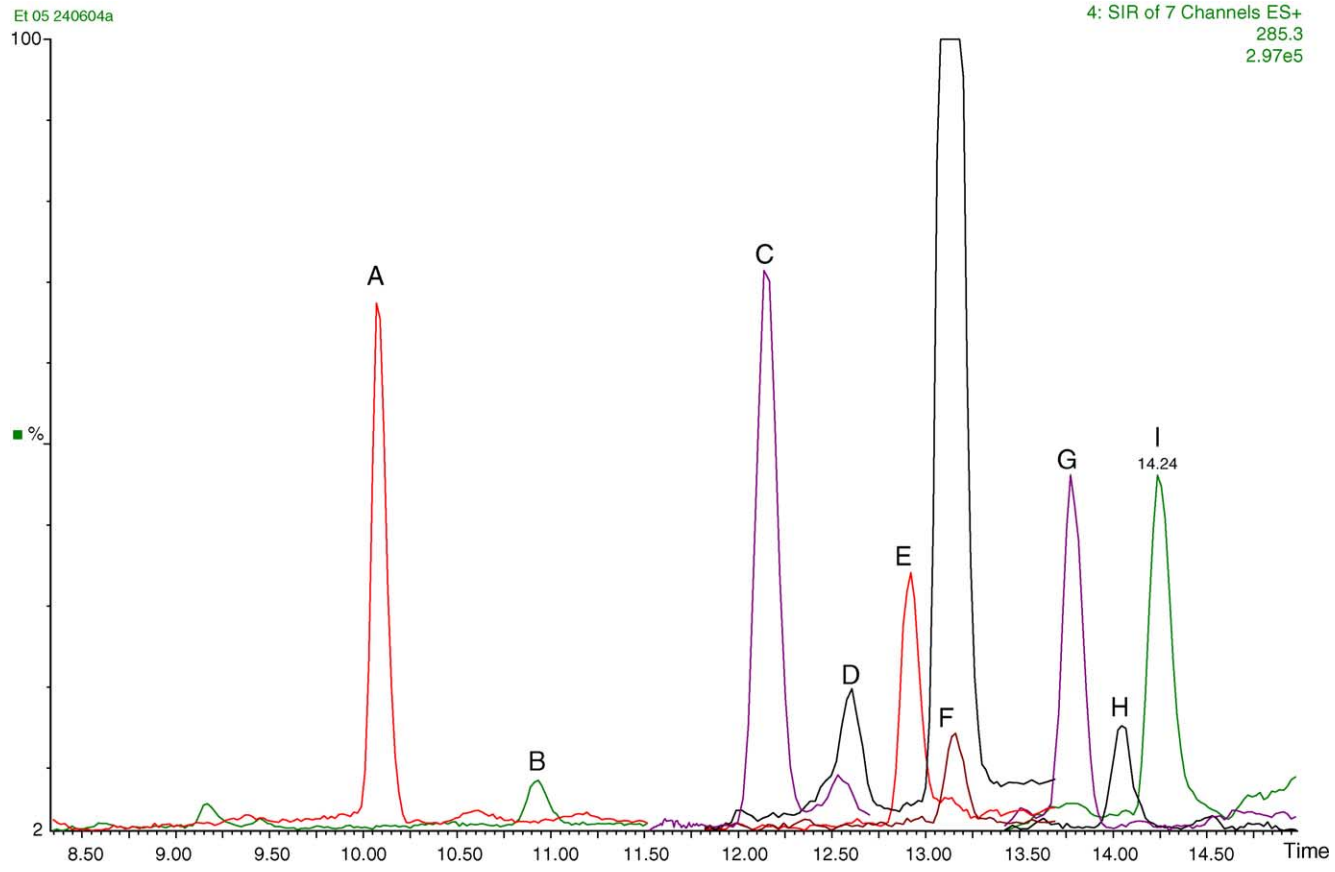

Fig. 2. LC-ES–MS ion cromatograms of an OF sample spiked at 0.5 ng/ml of nine benzodiazepines.

As an alternative to the extension of the calibration curve for the measurement of concentrations, which are above the highest calibration standard, the sample can be re-analysed after dilution with blank matrix [40–42]. The validation of this procedure showed the following results: the 1/5 dilution of a plasma concentration of 1250 ng/ml and a saliva concentration of 250 ng/ml for each BZP yielded results within the defined precision and accuracy criteria (Table 5). The same table also shows the recoveries of the nine BZP from the Salivette device. In our study the recovery from the cotton

Table 6
Results of the kinetic study done in patients undergoing lormetazepam treatment

Case number	Extraction time	Concentration found (ng/ml)		
		Plasma	Oral fluid (Salivette without stimulation)	Oral fluid (Salivette with stimulation)
1	−5	N.A.	0.00	0.00
	30	N.A.	3.39	2.01
	60	4.56	1.48	0.72
	90	5.50	0.45	0.30
	120	2.58	<LOQ	<LOQ
	150	3.92	<LOQ	<LOD
	180	3.73	<LOD	<LOD
	300	N.A.	N.A.	N.A.
	360	2.58	N.A.	N.A.
2	−5		0.00	0.00
	30		0.77	0.45
	60		0.49	0.27
	90		0.35	0.24
	120		<LOQ	<LOQ
	180		<LOQ	<LOD
	240		<LOD	<LOD
	420		<LOD	<LOD

roll of Salivette® was very variable, and ranged from only 52.2% for midazolam and 101.2% for alprazolam, respectively. This means that more than 50% of the drug present in saliva can remain in the cotton, which represents another difficulty in the interpretation of saliva results: It is very risky, as we showed, to establish a relation between plasma/saliva concentrations for selected BZP, and in any case specific studies for each new drug should be done. In any case the proposed analytical procedure is sensitive enough as to detect very low saliva concentrations, which permit to diagnose recent consumption of the nine BZP studied. Figs. 1 and 2 show the ion chromatograms of a plasma sample spiked at 5 ng/ml and a saliva sample spiked at 0.5 ng/ml with BZP, respectively.

No suppressive effect was detected in the region of interest after the evaluation of the ion suppression effect by the post-column infusion system.

Finally, Tables 6 and 7 show the results of the kinetic study done in patients undergoing midazolam or lormetazepam treatment. Fig. 3 represents the ion chromatogram of a saliva sample from a patient undergoing lormetazepam treatment. In summary, we have developed a simple and rapid method for the BZP quantitation in plasma and oral fluid by using LC–MS. The procedure is sensitive and specific and involves a very simple liquid–liquid extraction method. It has been validated and applied to real samples. The method is especially useful to analyse saliva samples from drivers undergoing road side drug controls.

Table 7
Results of the kinetic study done in patients undergoing midazolam treatment

Case number	Concentration found (ng/ml)	Sample extraction time (min)		
		10	30	50
1	Plasma	197.87	54.29	43.36
	OF	13.98[a]	2.56[a]	1.04
2	Plasma	282.9	140.6	51.8
	OF	*N.A.*	*N.A.*	N.A.

Case number	Concentration found (ng/ml)	20	40	60
3	Plasma	1101.74	156.7	28.19
	OF	1.98[a]	0.57	0.57
4	Plasma	50.64	25.48	30.2
	OF	0.79[a]	1.42[a]	0.22[a]
5	Plasma	308.87	26.29	15.04
	OF	1.73[a]	2.16[a]	N.A.

N.A., no sample available.
[a] Not enough volume of sample (<500 μl).

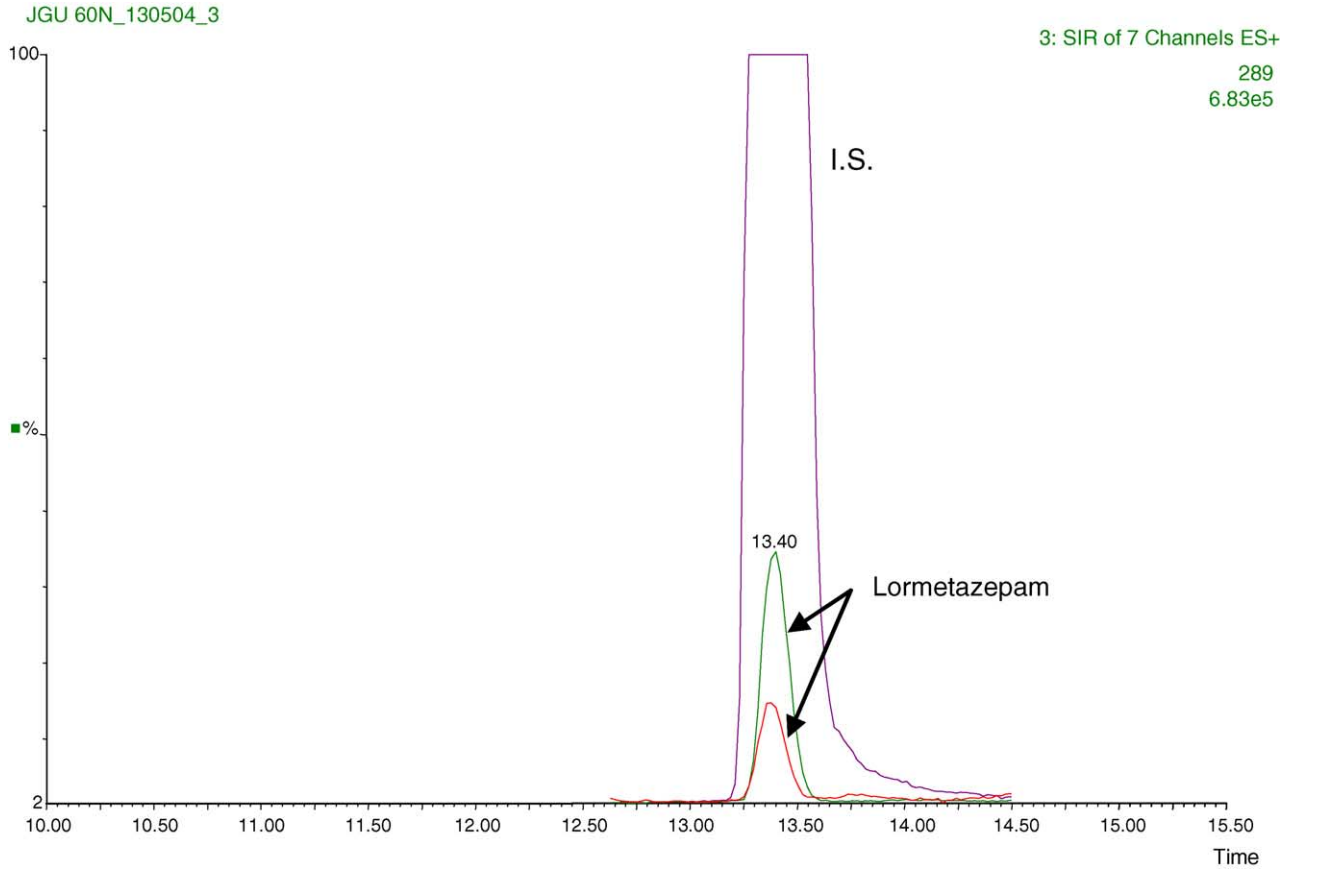

Fig. 3. Selected ion chromatograms of OF sample collected at 60 min after administration of single oral dose of 1 mg lormetazepam with Salivette® device. The concentration measured was: 1.48 ng/ml.

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