A fast liquid chromatography-tandem mass spectrometry method for determining benzodiazepines and analogues in urine. Validation and application to real cases of forensic interest

Abstract

A fast liquid chromatographic/tandem mass spectrometric method was developed for the simultaneous determination in human urine of seventeen benzodiazepines, four relevant metabolites together plus zolpidem and zopiclone. The sample preparation, optimized to take into account the matrix effect, was based on enzymatic hydrolysis and liquid–liquid extraction. The separation of the twenty-three analytes was achieved in less than eight minutes.

The whole methodology was fully validated according to UNI EN ISO/IEC 17025:2005 rules and 2006 SOFT/AAFS guidelines. Selectivity, linearity range, identification (LOD) and quantitation (LOQ) limits, precision, accuracy and recovery were evaluated. For all the species the signal/concentration linearity was satisfactory in the 50–1000 ng/mL concentration range. The limits of detection ranged from 0.5 to 30 ng/mL and LOQs from 1.7 to 100.0 ng/mL. Precisions were in the ranges 5.0–11.8%, 1.5–11.0% and 1.1–4.4% for low (100 ng/mL), medium (300 ng/mL) and high (1000 ng/mL) concentration, respectively. The accuracy, expressed as bias% was within ±25% for all the analytes. The recovery values, evaluated at 300 ng/mL concentration, ranged from 56.2% to 98.8%. The present method for the determination of several benzodiazepines, zolpidem and zopiclone in human urine proved to be simple, fast, specific and sensitive. The quantification by LC–MS/MS was successfully applied to 329 forensic cases among driving re-licensing, car accidents and alleged sexual violence cases.

Keywords

- Benzodiazepines;
- Validation;
- Urine;
- Case report;
- Fast chromatography

1. Introduction

Owing to their target effect on the central nervous system (CNS), benzodiazepines are the most prescribed drugs worldwide as tranquilizers, hypnotics, anesthetics, anticonvulsants or muscle relaxants, to treat sleeplessness, depression, anxiety and epilepsy. The side-effects of these drugs are similar and mainly consist in dizziness, prolonged sleep, as well as reduced ability to concentrate, that can easily lead, for instance, to driving impairment. Whenever taken in combination with other CNS-depressant such as alcohol, benzodiazepines may cause severe respiratory depression [1]. Even if benzodiazepine wide availability generally arise from legitimate sources, while clandestine manufacturing is rare, their assumption, often in combination with alcohol or illicit drugs [2] and [3], is increasingly implicated in forensic cases concerning sudden deaths [4], car accidents [5], [6] and [7], rapes and burglaries [8] and [9].

Also zolpidem and zopiclone, not containing the 5-aryl-1,4-diazepine structure, typical of benzodiazepines, have similar pharmacological properties, so that they are often preferentially prescribed as hypnotics. All these drugs may reduce the efficiency in driving a car or working at machines and may lead to addiction or severe intoxication.

Reliable, sensitive and fast analytical methods are increasingly required in forensic and clinical toxicology for the identification and quantification of the most common benzodiazepines in different biological matrices. In general, in forensic screening analysis, urine represents the primary specimen owing to the higher concentrations and longer persistence of the drugs, with respect to whole blood. Urine analysis is widely utilized in driving re-licensing or workplace drug testing, and to investigate if a crime was perpetrated through the administration of a drug, as is the case in drug-facilitated sexual assault cases. On the other hand, acute toxicity and impairing effects on driving ability have to be more appropriately correlated with the concentration levels present in blood [10].

Several procedures have been described in the literature for the determination of benzodiazepines in different biological specimens [11], [12], [13], [14], [15] and [16]. In general, immunochemical multi-residue screening methods are not suitable to selectively identify the drug and to discriminate the parent drug from their metabolites [17] and [18], while methods based on spectrophotometry are generally characterised by poor sensitivity and specificity [19]. To increase the screening efficiency, chromatographic methods have been applied [20] and [21]. Gas chromatography, coupled with mass spectrometry, is not applicable to the determination of the entire range of benzodiazepine panel, because of the thermal instability or scarce volatility shown by some of them, even after derivatization [22], [23] and [24]. In contrast, methods based on liquid chromatography (LC) hyphenated with mass spectrometry (MS) are successfully employed for all benzodiazepines [25], [26], [27] and [28]. Both electrospray ionization (ESI) and atmospheric

pressure chemical ionization (APCI), in the positive ionization mode, coupled with triple quadrupole MS/MS provide high sensitivity and selectivity [29], [30], [31], [32] and [33].

LC–MS/MS appears as the most eligible technique for the simultaneous determination of several benzodiazepines, due to its capability to recognize and quantify coeluting peaks [34]. Moreover, the recent introduction of small-sized particle LC columns allows a drastic reduction of the analysis time, without loss of resolution [35] and [36]. A summarized comparison of published methods for assaying benzodiazepines in urine is presented in Table 1.

Table 1. Comparison of the presented method with previously published LC–MS/MS methods for assaying benzodiazepines in urine.

Apparatus	Urine volume	Sample preparation	Total run time (min)[a]	Number of target analytes	Application to real cases on urine samples	Reference
LC–APCI-MS/MS	3 mL	LLE	8.0	23	329 cases (32 driving re-licensing, 268 car accidents, 29 alleged DFSA)	–
UPLC–ESI-MS/MS	0.5 mL	Diluition	4.0	13	80 patients	[36]
LC–ESI-MS/MS	0.5 mL	LLE	7.0	17	250 patients	[20]
LC–ESI-MS/MS	0.5 mL	SPE	9.1	8	Approximately 1800 (no data reported)	[30]
LC–ESI-MS/MS	1 mL	SPE	10.0	13	Not reported	[16]
LC–ESI-MS/MS	1 mL	SPE	10.0	13	205 patients	[15]
LC–ESI-MS/MS	0.5 mL	SPE	20.0	29 (8 BZDs)	108 (anonimous samples)	[28]
LC–ESI-MS/MS	2 mL	SPE	24.0	21	12 volunteers + 1 DFSA case	[31]
LC–ESI-MS/MS	0.25 mL	LLE	35.0	28	Not reported	[32]
LC–APCI-MS/MS	1 mL	Online SPE	40.0	22	3 real cases	[33]
LC–ESI-MS-TOF	1 mL	LLE	70.0	22	156 DFSA	[9]

[a]Including re-equilibration.

Aim of the present work was to develop an analytical methodology, based on fast-LC separation and triple quadrupole MS detection, for fast screening and determination of seventeen benzodiazepines, four relevant metabolites (7-aminonitrazepam, 7-aminoflunitrazepam, desalkylflurazepam and N-1-hydroxyethylflurazepam) together plus zolpidem and zopiclone in urine (Fig. 1).

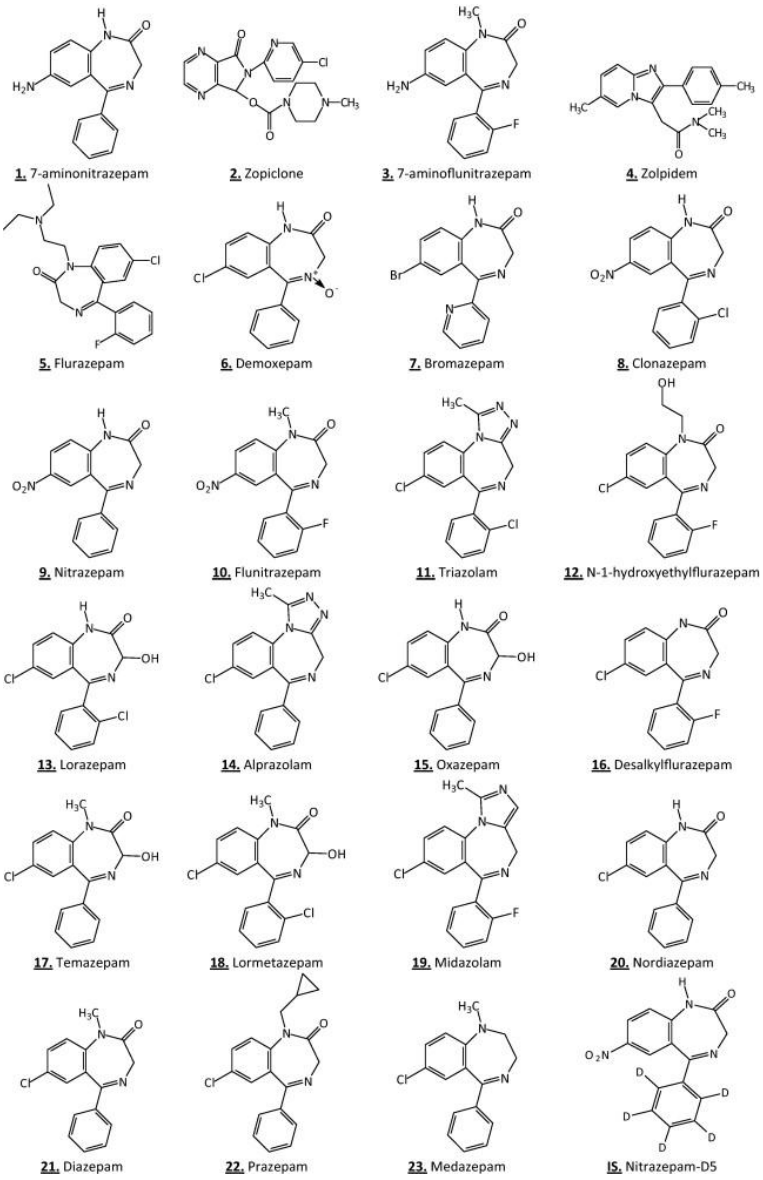

Fig. 1. Molecular structures of benzodiazepines, metabolites, zolpidem and zopiclone.

The protocol was fully validated according to UNI EN ISO/IEC 17025:2005 rules and 2006 SOFT/AAFS guidelines for toxicological analysis. In particular, selectivity, linearity range, detection (LOD) and quantitation (LOQ) limits, precision, accuracy, and recovery were evaluated [37]. Finally, the whole method was successfully applied in the routine analysis of 329 forensic samples

from driving re-licensing, car accidents and alleged sexual violence cases, in order to investigate the diffusion of a wide range of benzodiazepines and analogues in our territory.

2. Experimental

2.1. Chemicals, reagents and standard solutions

Flunitrazepam, diazepam, demoxepam, medazepam, clonazepam, and desalkylflurazepam were purchased from S.A.L.A.R.S. S.p.A. (Como, Italy). Oxazepam, lormetazepam, alprazolam, lorazepam, nitrazepam, midazolam, prazepam, temazepam, triazolam, zolpidem, nordiazepam, flurazepam, N-1-hydroxyethylflurazepam, 7-aminonitrazepam, 7-aminoflunitrazepan and nitrazepam-D_5 were acquired from LGC Promochem (Milan, Italy). Bromazepam and zopiclone were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Beta-glucuronidase enzyme with secondary aryl-sulfatase activity and various other chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA). Water was produced by a Milli-Q System (Millipore Corporate Headquarters, Billerica, USA).

Fresh blank urinary samples obtained from laboratory personnel (10 subjects) were stored at 4 °C and used as surrogate matrix.

Standard solutions of demoxepam, diazepam, flunitrazepam, zopiclone and bromazepam were prepared in methanol at 1.0 mg/mL concentration. All the other standards were purchased in ampoules at 1.0 mg/mL concentration. Nitrazepam-D5, used as the internal standard (IS), was available at 0.1 mg/mL concentration. The standard solutions were stored at −20 °C. Working water solutions were prepared by progressive dilution of the standard solution. Testing and calibration samples were obtained by spiking the blank urine samples with the working solutions.

Phosphate buffer solution at pH 6.0 was prepared by dissolving 5.23 g of KH_2PO_4 and 2.06 g of Na_2HPO_4 in water up to a final 1.0 L volume. Phosphate buffer solution at pH 7.5 was prepared by adding 3.06 g of KH_2PO_4 and 4.90 g of Na_2HPO_4 into 1.0 L of water solution. Carbonate buffer (pH 9.6) was prepared by dissolving Na_2CO_3 (2.12 g) and $NaHCO_3$ (6.72 g) in 1.0 L of water solution.

2.2. Biological specimens pretreatment

Urine samples (3.0 mL) were added with 6 µL of a 100 µg/mL nitrazepam-D_5 solution, used as the internal standard (IS) and 2.0 mL of phosphate buffer at pH 6.0. The samples were then treated with 50 µL of β-glucuronidase from *Helix pomatia* and incubated for 1 h at 55 °C. After cooling at room temperature, the pH was adjusted to 7.5 by adding 3.0 mL of phosphate buffer at pH 7.5. A liquid–liquid extraction was performed for 5 min in a vortex multimixer (Tecnovetro, Monza, Italy) by adding 5.0 mL of a 85:15 (v:v) dichloromethane and propan-2-ol mixture. After centrifugation at 3000 rpm for 3 min, the lower organic layer was transferred into a tube, dried under a gentle stream of nitrogen at 40 °C in a Techne Sample Concentrator (Barloworld Scientific, Stone, UK)

and the residue was dissolved in 100 µL of methanol. An aliquot of 3 µL was injected into the fast-HPLC/MS–MS system.

2.3. LC–MS/MS method

All the analyses were performed using an Agilent Technologies (Milan, Italy) HPLC 1100 liquid chromatograph interfaced to an Applied Biosystem API 3200 triple-quadrupole mass spectrometer (Applied Biosystems Division Headquarters, Foster City, USA) operating in APCI-positive ion mode. LC separation was performed using a Eclipse XDB C18 column (50 mm × 4.6 mm i.d.), with particle size of 1.8 µm (CPS Analitica, Milan, Italy). The elution solvents were water (solvent A) and methanol (solvent B). The mobile phase eluted under the following linear gradient conditions: (a:b; v/v) from 80:20 to 0:100 in 7.5 min, isocratic elution at 100% B for 0.5 min, fast linear gradient to 80:20 and then isocratic elution for 1.50 min for re-equilibration. The flow rate was 1.0 mL/min. The APCI source was held at 300 °C.

3 µL of sample extract was injected and the data were acquired at unit mass resolution in selected-reaction monitoring (SRM) mode, using the mass transitions listed in Table 2. Three MS/MS transitions were utilized for identifying and determining each analyte and internal standard. To maximize the fragment ion signals while maintaining comparable precursor ion abundance, for each analyte a different collision energy was optimized.

Table 2. Retention time precision and MS characteristic transitions of the tested benzodiazepines.

	Compound	RT (min)	RT precision (*n* = 10) CV%			SRM transitions (m/z)	CE (V)
			100 ng/mL	300 ng/mL	1000 ng/mL		
1	7-Aminonitrazepam	3.60	0.22	0.23	0.16	252 → 252	16
						252 → 224	29
						252 → 208	38
2	Zopiclone	3.92	0.29	0.24	0.20	391 → 247	23
						389 → 245	20
						389 → 217	41
3	7-Aminoflunitrazepam	4.16	0.13	0.12	0.14	284 → 227	30
						284 → 236	30
						285 → 285	10
4	Zolpidem	5.17	0.26	0.16	0.09	308 → 235	40
						308 → 236	35
						308 → 263	31

	Compound	RT (min)	RT precision (*n* = 10) CV%			SRM transitions (m/z)	CE (V)
			100 ng/mL	300 ng/mL	1000 ng/mL		
5	Flurazepam	5.20	0.15	0.10	0.63	388 → 315	29
						390 → 390	8
						390 → 317	29
6	Demoxepam	5.41	0.10	0.07	0.18	287 → 287	9
						287 → 207	45
						289 → 289	8
7	Bromazepam	5.44	0.11	0.09	0.18	316 → 209	33
						318 → 318	9
						318 → 209	33
8	Clonazepam	5.68	0.12	0.09	0.17	316 → 270	33
						316 → 241	45
						316 → 214	47
9	Nitrazepam	5.70	0.11	0.09	0.14	282 → 236	32
						282 → 207	45
						282 → 180	52
10	Flunitrazepam	5.72	0.09	0.07	0.17	314 → 314	10
						314 → 268	33
						314 → 240	39
11	Triazolam	5.96	0.08	0.57	0.55	343 → 308	33
						345 → 345	14
						345 → 317	33
12	N-1-hydroxyethylflurazepam	5.97	0.09	0.08	0.09	333 → 333	11
						333 → 315	29
						333 → 305	28
13	Lorazepam	6.01	0.09	0.14	0.08	323 → 277	25
						321 → 275	30
						321 → 229	39
14	Alprazolam	6.02	0.11	0.14	0.08	309 → 281	35
						309 → 274	35
						311 → 283	35
15	Oxazepam	6.05	0.10	0.13	0.10	289 → 269	20
						287 → 241	30
						287 → 231	28

	Compound	RT (min)	RT precision (*n* = 10) CV%			SRM transitions (m/z)	CE (V)
			100 ng/mL	300 ng/mL	1000 ng/mL		
16	Desalkylflurazepam	6.14	0.09	0.13	0.10	289 → 226	40
						289 → 140	38
						291 → 142	42
17	Temazepam	6.19	0.10	0.13	0.15	301 → 283	16
						303 → 257	27
						303 → 285	18
18	Lormetazepam	6.25	0.07	0.08	0.18	335 → 289	24
						335 → 317	14
						337 → 291	30
19	Midazolam	6.46	0.09	0.08	0.15	326 → 291	33
						326 → 249	52
						328 → 291	33
20	Nordiazepam	6.48	0.11	0.08	0.09	271 → 271	13
						271 → 208	39
						271 → 226	33
21	Diazepam	6.62	0.09	0.08	0.09	285 → 222	35
						285 → 193	44
						287 → 193	43
22	Prazepam	7.21	1.29	0.11	0.13	325 → 271	26
						327 → 273	29
						325 → 208	52
23	Medazepam	7.36	0.07	0.07	0.08	271 → 271	39
						271 → 180	28
						273 → 207	39
IS	Nitrazepam-D5	5.67	0.10	0.17	0.17	287 → 287	9
						287 → 241	35
						287 → 185	47

2.4. Method validation

The method was validated by investigating the following parameters: selectivity, linearity range, identification and quantitation limits (LOD and LOQ), precision, accuracy and recovery. Carry-over and matrix effect phenomena were also evaluated.

2.4.1. Selectivity

Ten different blank urine samples were deconjugated, extracted, and analyzed as described above, to test the selectivity of the whole analytical procedure. For each sample and all analytes the signal to noise (S/N) ratio was measured for the corresponding mass transitions at the expected retention time windows.

2.4.2. Identification criteria and repeatability of diagnostic fragment ions relative abundances

Identification criteria for the analytes were established according to 2006 SOFT/AAFS guidelines [37]. The repeatability of relative peak intensities for the transitions of each analyte was determined on ten spiked urine samples at three concentration levels (100, 300 and 1000 ng/mL). Retention time precision at each concentration was also determined.

2.4.3. Linearity

The linear calibration model was checked by analyzing (three replicates) blank urine samples spiked with standard solutions at final concentration of 0, 50, 100, 250, 500 and 1000 ng/mL. The linear calibration parameters were obtained using the least squares regression method. The correlation coefficient (R^2) was utilized to estimate linearity. The quantitative results from area counts were corrected using the IS signal areas.

2.4.4. Matrix effect evaluation

Matrix effects possibly due to ion-enhancement or ion-suppression, occasionally observed in APCI when target analytes and matrix components coelute, were evaluated by comparing the slopes of the calibration curves obtained by spiking the blank urine samples with the slopes of those arising from water standard solutions. A *t*-test at 95% confidence level was used to compare each couple of slopes (external calibration in water *vs.* standard addition in urine matrix) and to establish if the slope differences were statistically significant.

2.4.5. Limit of detection (LOD)/limit of quantification (LOQ)

The limit of detection (LOD) was calculated as the concentration of the analyte that gives a signal (peak area) equal to the average background of the blank (S_{blank}) plus three times its standard deviation (LOD = $S_{blank} + 3s_{blank}$), while the LOQ was calculated as LOQ = $S_{blank} + 10s_{blank}$ [38]. For each analyte, LOQ generally corresponds to the lowest concentration that provides a useful signal along the calibration curve. The noise was measured from −0.05 min before the peak onset till the beginning of the peak for each analyte. The LOD values were experimentally confirmed by analysing blank urine samples spiked with all the target analytes at concentrations equal or slightly (<10%) higher than estimated LODs.

2.4.6. Precision and accuracy

For all analytes, intra-assay precision (%) and accuracy (expressed as bias %) were evaluated by extracting and analyzing ten urine samples spiked at three concentration levels (100, 300 and 1000 ng/mL). Inter-assay precision and accuracy were evaluated by preparing and analyzing for five consecutive days one set of urine spiked with the analytes at 300 ng/mL final concentration. Standard criteria designated satisfactory assay precision when CV% values were below 25% for concentrations of 100 and 300 ng/mL and below 15% for 1000 ng/mL [37], [39] and [40]. Since the acceptance criteria for accuracy are not fixed by internationally standardized rules, we choose that satisfactory accuracy was achieved when the experimentally determined concentrations lied within ±25% from the expected values. The parameters most commonly changing in everyday toxicological analysis, namely sample volume, reagent batch and operator, were deliberately varied to test if satisfactory accuracy was maintained.

2.4.7. Extraction recovery

The extraction recoveries were calculated by comparing the experimental results of two sets of solutions at three concentrations. In the first set, ten blank urine samples were spiked with all analytes at 100, 300 and 1000 ng/mL final concentration before the extraction step, while in the second set the standard solution spikings (at the same concentrations) were made on the blank urine extracts.

2.4.8. Carry-over

The background chromatographic profiles for each analyte main transitions were monitored during the analysis of blank urine samples injected for five times after a chromatographic run where a blank urine sample was spiked with all analytes at 1000 ng/mL concentration. To assure the absence of carry-over, for each transition, the signal to noise ratio (S/N) had to be lower than 3.

3. Result and discussion

3.1. HPLC molecules separation and detection

The optimized HPLC–MS/MS method allowed the determination of the twenty-three analytes and the internal standard in less than 8 min, with retention times ranging from 3.60 min to 7.36 min. The whole chromatographic run, comprehensive of the time required for column re-equilibration before the following injection, was completed in less than 10 min. Fig. 2 shows a typical fast HPLC–MS/MS chromatograms recorded from a urine sample spiked with a mixture of the twenty-three analytes at concentration of 300 ng/mL and the IS nitrazepam-D_5 at 200 ng/mL concentration. In all cases, the acceptance criteria were respected, with negligible interference from the matrix.

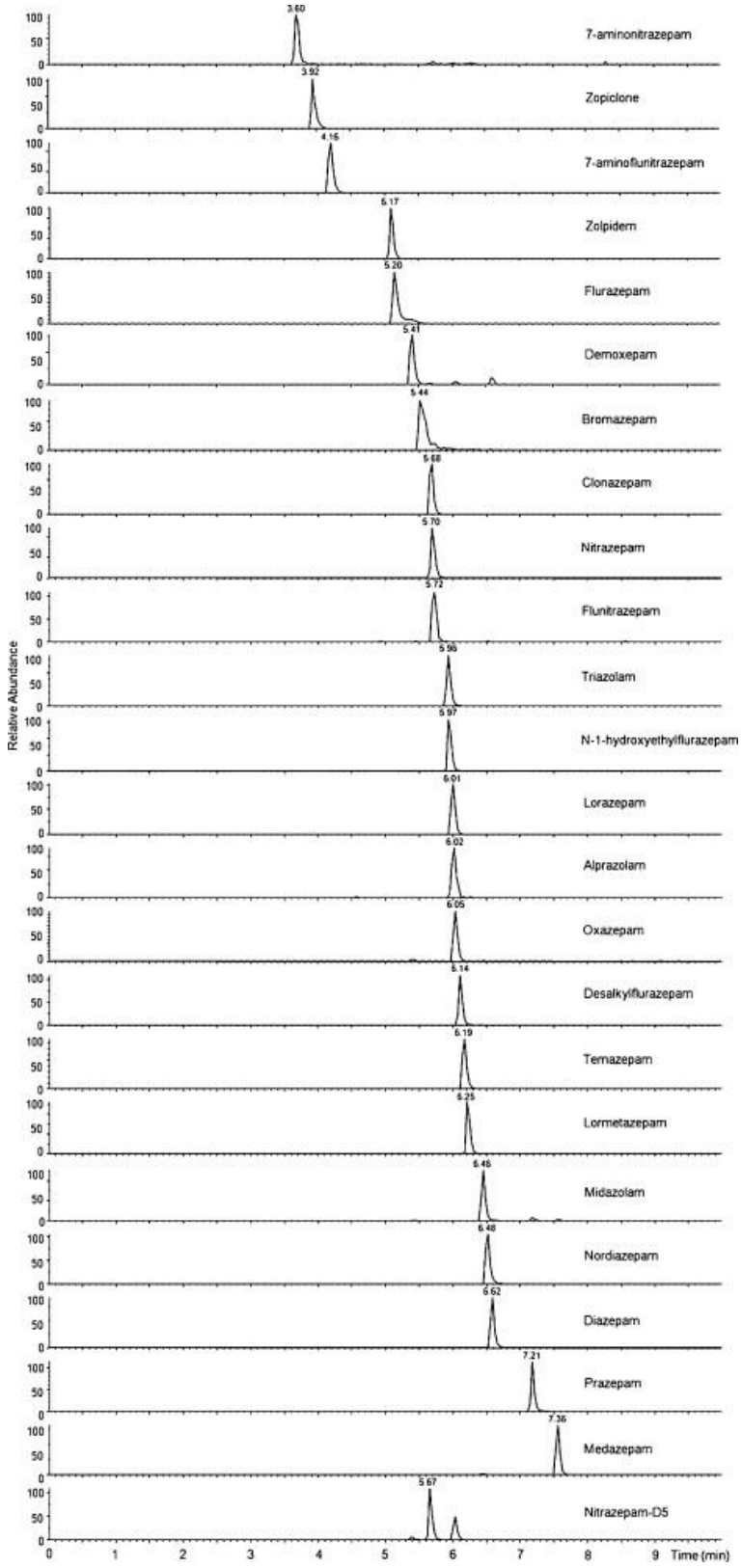

Fig. 2. Selected ion chromatograms recorded from a urine sample spiked with the tested analytes at concentration of 300 ng/mL and the IS nitrazepam-D5 at 200 ng/mL concentration.

3.2. Validation

3.2.1. Identification criteria and selectivity

For each analyte, the characteristic SRM transitions, the retention times with their intra-assay precision, expressed as CV%, are presented in Table 2. The intra-assay precision values for retention times measured at 100, 300 and 1000 ng/mL concentration randomly ranged from 0.07% to 1.29%, confirming that retention times are repeatable (CV% < 2%) and not affected by the analytes concentration.

The three SRM transitions selected for each analyte provided at least 4 identification points while the substantial stability of their relative abundance proved compliant for the unambiguous identification of all analytes included in the assay, in agreement with CE/2002/657 decision and 2006 SOFT/AAFS guidelines criteria.

SRM chromatograms from 10 negative urine samples showed no interfering signals (i.e., S/N ratio minor than 3) at the retention time where each analyte is expected to elute. This demonstrated that the method is selective for all tested compounds and free from positive interference from urine components.

3.2.2. Linearity and evaluation of LOD and LOQ

The SRM protocol described in Table 2 was used to build the calibration plots for all twenty-three analytes from spiked blank urine. Table 3 reports the resulting R^2 values, that range from 0.9900 and 0.9995 indicating good fit and linearity of the calibration curves. Table 3 also reports LOD and LOQ values, calculated from the analysis of multiple blank samples and confirmed (LODs) experimentally. LOD values lay between 0.5 and 30.0 ng/mL.

Table 3. Calibration levels, R^2 values for calibration curves (urine) slopes (urine and water) and matrix effect; LODs and LOQs values of the 23 investigated benzodiazepines.

	Compound	Calibration levels (ng/mL)	Correlation coefficient (R^2) (urine)	Slope (urine)	Slope (water)	Matrix effect (±%)	LOD (ng/mL)	LOQ[a](ng/mL)
1	7-Aminonitrazepam	50–1000	0.9917	0.262	0.276	–5.1%	5	17
2	Zopiclone	50–1000	0.9986	0.114	0.0557	+105%	2	7
3	7-Aminoflunitrazepam	50–1000	0.9971	0.332	0.354	–6.2%	1	3
4	Zolpidem	50–1000	0.9994	0.664	0.640	+3.7%	3	10
5	Flurazepam	50–1000	0.9959	0.865	0.829	+4.3%	10	33
6	Demoxepam	50–1000	0.9990	0.670	0.640	+4.7%	5	17
7	Bromazepam	50–1000	0.9920	0.464	0.434	+6.9%	5	17
8	Clonazepam	50–1000	0.9992	0.297	0.274	+8.4%	1	3
9	Nitrazepam	50–1000	0.9974	0.571	0.566	+0.9%	0.5	2
10	Flunitrazepam	100–1000	0.9900	0.0462	0.0502	–8.0%	25	83

	Compound	Calibration levels (ng/mL)	Correlation coefficient (R^2) (urine)	Slope (urine)	Slope (water)	Matrix effect (±%)	LOD (ng/mL)	LOQ[a](ng/mL)
11	Triazolam	100–1000	0.9924	0.178	0.195	–8.7%	25	83
12	Hydroxyethylflurazepam	50–1000	0.9965	0.828	0.738	+12.2%	8	27
13	Lorazepam	50–1000	0.9909	0.0983	0.0904	+8.7%	5	17
14	Alprazolam	50–1000	0.9995	0.0999	0.125	–20.1%	10	33
15	Oxazepam	100–1000	0.9952	0.184	0.157	+17.2%	30	100
16	Desalkylflurazepam	50–1000	0.9941	0.446	0.452	–1.3%	2	7
17	Temazepam	50–1000	0.9942	0.40505	0.417	–2.9%	2	7
18	Lormetazepam	50–1000	0.9935	0.918	0.873	+5.1%	5	17
19	Midazolam	50–1000	0.9988	0.284	0.286	–0.7%	8	27
20	Nordiazepam	50–1000	0.9986	0.177	0.186	–4.8%	8	27
21	Diazepam	50–1000	0.9965	0.346	0.326	+6.1%	2	7
22	Prazepam	50–1000	0.9983	0.496	0.587	–15.5%	1	3
23	Medazepam	50–1000	0.9982	1.13	1.27	–11.0%	0.5	2

[a]Calculated LOQ.

3.2.3. Matrix effect evaluation

The slopes of the calibration curves obtained by spiking the blank urine samples and the ones arising from water standard solutions are reported in Table 3, together with percent difference, taking the second ones as the reference. The effect of the real urine matrix appears modest (<10%) and statistically not significant for most of analytes tested. Accordingly, the differences are equally shared between positive and negative. Slightly larger negative effect (signal suppression) is evident for alprazolam, prazepam and medazepam, whose slope variations are statistically significant at 95% confidence level. A large signal enhancement (+105%) was recorded for zopiclone, when the urine matrix is used to build the calibration curve, instead of pure water. A significant positive matrix effect was detected also for oxazepam, although the variation is considerably smaller (+17%). To prevent as much as possible the matrix effects, possibly present when a specific urine sample has to be analyzed, all calibrations and validation tests were conducted on a mixture of human urine samples, spiked with the analytes standard solutions.

3.2.4. Precision and accuracy

Intra- and inter-assay data on precision and accuracy are reported in Table 4. The results show a satisfactory repeatability, as the percent variation coefficient (CV%) is lower than 15% for all the analytes spiked at low, medium and high concentration. In particular, intra-assay precision exhibit CV% values below 12% for the samples spiked at 100 and 300 ng/mL and below 5% for the samples spiked at 1000 ng/mL.

Table 4. Intra/inter-assay precision (CV%), accuracy (bias%) and recovery (%) for each analyte tested.

	Compound	Intra-assay (*n* = 10)						Inter-assay (*n* = 5)		Mean recovery % (*n* = 10)		
		Precision (CV%)			Accuracy (Bias%)			Precision (CV%)	Accuracy (Bias%)			
		100 ng/ mL	300 ng/ mL	1000 ng/ mL	100 ng/ mL	300 ng/ mL	1000 ng/ mL	300 ng/ mL	300 ng/ mL	100 ng/ mL	300 ng/ mL	1000 ng/ mL
1	7-Aminonitrazepam	8.4	5.2	2.2	−14.9	−9.1	+20.5	4.1	−7.2	54.7	56.2	55.7
2	Zopiclone	6.9	2.5	4.2	−11.9	−6.8	−12.8	1.2	−7.0	62.3	68.8	77.4
3	7-Aminoflunitrazepam	9.0	3.0	3.0	−10.6	−7.0	−1.2	2.9	−8.1	75.7	77.4	80.8
4	Zolpidem	6.3	3.4	3.0	−13.6	−5.8	+4.0	4.4	−3.8	78.0	81.2	85.2
5	Flurazepam	7.2	1.7	3.0	−13.1	−3.6	−5.6	1.1	−3.2	89.0	88.8	88.6 0
6	Demoxepam	5.0	2.7	3.1	−1.6	−6.1	−11.3	3.3	−5.0	75.4	75.2	75.1
7	Bromazepam	7.1	6.4	3.0	−7.2	+4.0	−1.9	7.2	+1.8	81.1	85.5	88.6
8	Clonazepam	6.1	2.4	2.7	−6.6	+5.9	−10.8	1.8	−2.2	89.3	91.3	90.7
9	Nitrazepam	6.1	2.3	2.7	−1.4	−2.7	−13.0	2.0	−8.5	86.9	90.2	91.1
10	Flunitrazepam	10.0	4.6	4.4	−21.1	−6.5	+20.4	5.2	−9.2	76.4	87.8	88.7
11	Triazolam	7.9	3.5	2.3	−8.4	+1.1	−3.1	4.1	+1.0	80.2	82.2	84.6
12	N-1-hydroxyethylflurazepam	7.2	3.4	2.5	+13.5	+10.2	−21.9	2.9	+7.5	95.2	98.8	100.8
13	Lorazepam	7.2	5.6	2.6	−8.4	−9.7	−8.1	4.0	−6.6	90.0	91.6	93.0
14	Alprazolam	11.8	1.5	1.1	−7.8	−2.6	+9.5	1.0	−4.0	68.0	71.1	72.6
15	Oxazepam	5.4	2.9	2.0	−4.6	−12.4	−11.3	2.9	−13.1	91.7	91.0	92.0
16	Desalkylflurazepam	5.5	3.5	2.8	−6.4	−2.3	−14.1	3.4	−4.6	88.4	90.4	94.1
17	Temazepam	5.7	4.4	3.1	−5.8	−5.9	−12.7	3.5	−6.0	92.9	93.5	91.0

	Compound	Intra-assay (*n* = 10)						Inter-assay (*n* = 5)		Mean recovery % (*n* = 10)		
		Precision (CV%)			Accuracy (Bias%)			Precision (CV%)	Accuracy (Bias%)			
		100 ng/mL	300 ng/mL	1000 ng/mL	100 ng/mL	300 ng/mL	1000 ng/mL	300 ng/mL	300 ng/mL	100 ng/mL	300 ng/mL	1000 ng/mL
18	Lormetazepam	5.2	2.4	4.0	−1.2	−2.0	−14.1	2.0	−5.2	88.4	89.6	91.6
19	Midazolam	6.8	4.8	3.1	−12.0	−14.7	−6.8	5.0	−15.2	89.9	87.2	86.5
20	Nordiazepam	5.8	5.4	3.1	−0.5	−11.9	−14.1	5.0	−10.8	89.0	90.1	83.6
21	Diazepam	7.7	7.9	3.9	−10.8	−12.6	−11.0	6.6	−14.3	88.4	87.3	85.2
22	Prazepam	9.9	11.0	3.2	−13.5	−12.9	−6.4	9.2	−9.9	88.4	88.5	87.1
23	Medazepam	7.6	10.4	2.3	−7.0	+10.2	−21.9	8.9	+9.2	90.7	93.3	95.2

Intra-assay accuracy expressed as percent bias varies from excellent (nordiazepam −0.5%) to acceptable (flunitrazepam −21.1%) at the lowest concentration tested (100 ng/mL). At intermediate spiking concentrations (300 ng/mL) more uniform results were obtained, ranging from +1.1% (triazolam) to −14.7% (midazolam), whereas at the highest concentration level the determination of a few analytes showed a slightly higher bias, including 7-aminonitrazepam (+20.5%), flunitrazepam (+20.4%), N-1-hydroxyethylflurazepam (−21.9%) and medazepam (−21.9%). On the whole, all the experimental bias values were below the acceptable limit of ±25% at the extreme concentrations, and below a satisfactory ±15% at intermediate concentration.

At the same concentration level (300 ng/mL), also the inter-assay precision proved very satisfactory, as the CV% values ranged from 1.0% for alprazolam to 9.2% for prazepam, likewise the intra-assay accuracy, ranging from +1% to −15.2.

3.2.5. Extraction recovery and carry-over effect

Total extraction recovery values for each analyte are given in Table 4, at three concentration levels (100, 300 and 1000 ng/mL) and ranged from 54.7% to 100.8%. Recovery values homogeneously exceeding 75% were recorded for all target analytes, with the exclusion of zopiclone, 7-aminonitrazepam, and alprazolam. Given that the recovery repeatability was good, for forensic applications absolute recoveries above 50% have to be considered as perfectly acceptable, which was the case even for 7-aminonitrazepam at all concentration levels.

The background chromatographic profiles for the main transitions of each analyte, which monitored during the analysis of blank urine injected after highly spiked samples, did not show the presence of any significant signal (i.e., the signal to noise ratio was always <3) at the retention times of the tested analytes. The presence of carry-over effect was therefore excluded.

4. Application to real cases

The fully validated method was applied to 329 authentic urine samples requiring confirmation for benzodiazepines, zolpidem and zopiclone. All samples were collected from either: (i) drivers convicted for driving under influence (DUI) going through re-licensing examination ($n = 32$, group A), (ii) police controls on drivers involved in car accidents ($n = 268$, group B), and (iii) alleged victims of sexual assaults ($n = 29$, group C). Results for the positive samples ($n = 278$) are presented in Table 5, showing all identified molecules ($n = 354$), some of which arise from the simultaneous presence of more drugs in the same sample. Due to the extensive and complex metabolism of benzodiazepines in human, leading to possible structural interconversions among them, the identification of the administrated parent drug is occasionally not completely clear. For example, it is known that diazepam administration can produce positive results also for nordiazepam, temazepam and oxazepam, all of which are commercial drugs themselves. Similarly, a positive test for lorazepam could alternatively indicate the ingestion of lorazepam itself or may

arise from the administration of lormetazepam or delorazepam (chlordesmethyldiazepam), a drug commonly marketed in Italy under the trade name of EN.

Table 5. Comparison of positive findings for benzodiazepines among driving license commission (group A), car accidents (B) and alleged sexual violence cases (C).

Detected molecule	Group A	Group B	Group C	Total findings
2-Hydroxyethylflurazepam	–	23	–	23
Alprazolam	8	42	1	51
Bromazepam	–	13	1	14
Clonazepam	3	8	1	12
Demoxepam	–	1	–	1
Diazepam	4	4	–	8
Lorazepam	5	79	2	86
Lormetazepam	2	18	–	20
Midazolam	–	47	–	47
Nitrazepam	–	1	–	1
Nordiazepam	5	20	–	25
Oxazepam	8	27	–	35
Temazepam	6	20	–	26
Triazolam	–	1	–	1
Zolpidem	–	4	–	4

The prevalence distribution in the samples of the pharmaceutical drugs included in the screening are illustrated in Fig. 3. In 24.8% of positive samples ($n = 69$), the only drug detected was lorazepam, while a combination of lorazepam and lormetazepam was detected in 7.2% of cases ($n = 20$). Alprazolam was found to be of widespread use in local territory, as the positive findings reached 18.3% of total positive samples ($n = 51$). The high number of midazolam positive samples (16.9%, $n = 47$) is possibly associated to its use as a premedication for sedation, not to medical prescription. Accordingly, all midazolam positive samples were found in subjects undergoing hospitalization after being involved in car accidents (see again Table 5). The administration of diazepam (8.6%, $n = 24$) was ascertained only in the cases when all metabolites were simultaneously detected (nordiazepam, oxazepam and temazepam), in the absence of the parent drug. In contrast, the presence of either oxazepam (2.5%, $n = 7$) or temazepam together with oxazepam (1.4%, $n = 4$), can be attributed to the administration of different benzodiazepins, since these molecules are common metabolites from several parent drugs. Other drugs were also identified, including flurazepam (8.3%, $n = 23$), bromazepam (5.0%, $n = 14$), clonazepam (4.3%, $n = 12$), zolpidem (1.4%) or others (1.1%).

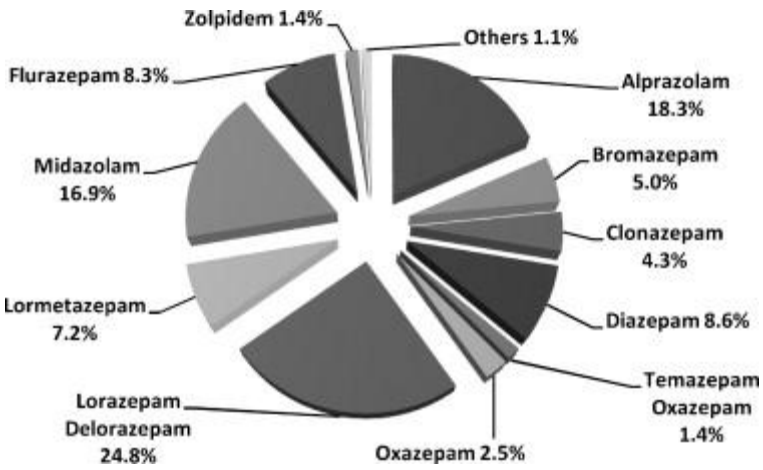

Fig. 3. Prevalence of the investigated pharmaceutical drugs among the positive samples.

5. Conclusions

A fast HPLC–tandem mass spectrometry method was developed and validated for the determination of seventeen benzodiazepines and four key metabolites in human urine, plus zolpidem and zopiclone. The method was used to investigate the prevalence of these molecules in our territory. The introduction of fast-LC in HPLC–MS/MS drastically reduced the analysis time required for carrying out our toxicological procedures, without sacrificing chromatographic resolution, accuracy and precision. Good sensitivity, selectivity and optimal linear response were observed, together with good repeatability and accuracy for quantitative determinations. Since the extraction recoveries are comparatively high and the analytical performances are relatively uniform for all the studied analytes, the method can find easy application in routine analysis for toxicological investigation. In particular, the present method proved to be profitably applied to driving re-licensing, car accidents and forensic cases involving drug-facilitated sexual assault (DFSA).

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