SHIMADZU

TP-728

Detection and quantitation of benzodiazepines in less than 3 min using PESI-MS and isotope dilution approach.

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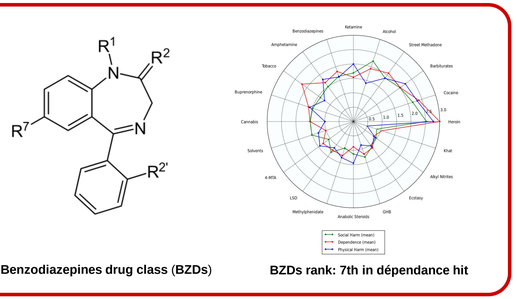
1. Introduction

Benzodiazepines (BZDs), which were the most prescribed medications globally in the 80s, remain frequently used as recreational drugs and are implicated in drug-facilitated sexual assaults or driving under influence of drugs cases. They are used for their anxiolytic, sedative, muscle relaxant and anticonvulsant properties. For that reasons, quantitative screening of BZDs is a routine work for emergency samples in a clinical toxicology context. Laboratories typically rely on immunochromatography (lateral flow test) or immunohistochemistry methods which quickly generate a result but often suffer from two major drawbacks:

- numerous false negative results
- only qualitative information

In this study, we aimed to develop an **ultra-fast** method for the measurement of BZDs by using a **Probe Electrospray Ionization (PESI) tandem mass spectrometry** system with an **isotope dilution** approach to benefit sensitivity and specificity of MS with the speed of PESI.



Benzodiazepines drug class (BZDs) **BZDs rank: 7th in dépendance hit**

Figure 1: Structure of BZDs & Recreational drugs dependence graph

2. What is PESI?

The Probe ElectroSpray Ionization (PESI) source is an ambiant ionization method. It contains a disposable solid needle that is used as a sample probe and an Electrospray Ionization that is used as an emitter. To allow the ionization, a probe needle repeatedly moves down into the sample and moves up, then the ionization occurs by applying a voltage on the needle. The compounds are so periodically ionized, and their resultant ions are pushed into the MS/MS system.

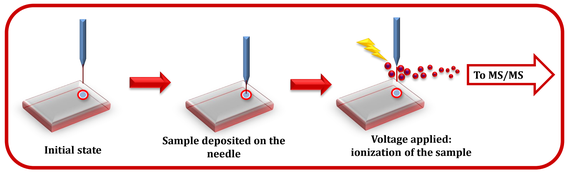


Figure 2: LCMS-8060NX with DPiMS-8060

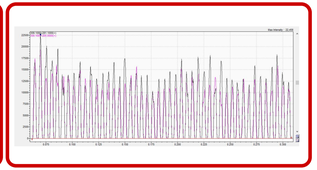


Figure 3: TIC obtained for a serum spiked with alprazolam at 100 µg/L.

3. Method

3-1. Chemicals and reagents

Bromazepam-D4 and zolpidem-D6 were supplied by Lipomed (Arlesheim, Switzerland). Alprazolam, diazepam, diclazepam, oxazepam, pyrazolam, zolpidem, zopiclone, alprazolam-D5 and zopiclone-D4 were purchased from LGC standards (Molsheim, France), and the following compounds were supplied by Cerilliant (Round Rock, TX, USA): bromazepam, nordiazepam, temazepam, diazepam-D5, nordiazepam-D5, oxazepam-D5, temazepam-D5. Clonazolam, deschloroetizolam, etizolam, flubromazepam, flubromazolam, meclonazepam nifoxipam were supplied by Chiron AS (Trondheim, Norway). Ammonium formate and ethanol were purchased from Carlo Erba. Pure water was obtained using a Millipore Integral purification system. Drug free serum was obtained from Etablissement français du sang (Limoges, France).

3-2. Sample preparation

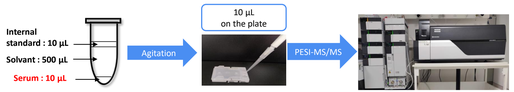
Ten µL of serum were mixed with 500 µL of an ethanol/ammonium formate 10 mM (1 :1 v/v) buffer. Then, 10 µL of a 50 µg/L of the internal standards mix were added. Finally, 10 µL of this mixture were spiked on the dedicated plastic sample plate and placed into the PESI ion-source. To perform the method validation tests, seven BZDs working solutions (5, 25, 50, 100, 500, 1000 and 2000 µg/L) were prepared in methanol and stored at -20 ° C. Each concentration levels (5, 25, 50, 100, 500, 1000 and 2000 µg/L) were prepared daily by spiking serum with working solutions. For the 10 designer BZDs, working solutions at 25, 50, 100 µg/L were prepared in methanol and stored at -20 ° C.

3-1. Instruments & Parameters

A LCMS-8060NX triple quadrupole mass spectrometer (Shimadzu Corporation) coupled with a DPiMS-8060 Shimadzu Corporation) was used in positive ionization mode for all the BZDs and the IS detection. The vertical movement of the needle was repeated with a frequence of 3.1 Hz (184/min) for a run time of 2.56 min. The parameter settings were as follows: time for probe movement = 160 ms ; probe voltage = 2.3 kV ; desolvation line temperature = 250 ° C ; heat block temperature = 30 ° C. BZDs and their IS were detected trough a multiple reaction monitoring (MRM). The main MS parameters we optimized to ensure that the first transitions (used for quantitation) exhibited the same intensity to apply an isotope dilution protocol. The selected MRM transition and MS parameters are presented in table I. BZD are analysed one after the other during 0.25 min for each one.

Compounds	Precursor ion	Product ion	MRM parameters		
	m/z	m/z	Q1 (V)	CE (eV)	Q3 (V)
Alprazolam (BZD)	309.1000	281.1000	-14.0	-28.0	-28.0
	309.1000	205.0000	-22.0	-42.0	-20.0
Bromazepam (BZD)	316.0100	182.0500	-11.0	-31.0	-11.0
	316.0100	209.0000	-11.0	-38.0	-21.0
Clonazolam	354.0800	308.0800	-13.0	-28.0	-14.0
	354.0800	280.0600	-13.0	-37.0	-13.0
Deschloroetizolam	309.1200	280.0800	-11.0	-25.0	-13.0
	309.1200	255.0950	-11.0	-23.0	-12.0
Diazepam (BZD)	285.0789	193.0886	-10.0	-33.0	-20.0
	285.0789	154.0420	-10.0	-27.0	-15.0
Diclazepam	319.3997	227.0500	-12.0	-32.0	-15.0
	319.3997	154.0400	-11.0	-28.0	-15.0
Etizolam	343.07800	313.9500	-13,0	-27.0	-14.0
	343.07800	259.9000	-12,0	-37.0	-17.0
Flualprazolam	327.1000	223.0500	-28.0	-41.0	-22.0
	327.1000	299.0500	-14.0	-29.0	-20.0
Flubromazepam	333.0000	226.0000	-12.0	-29.0	-10.0
	333.0000	183.9000	-12.0	-31.0	-18.0
Flubromazolam	371,0300	223.0700	-13.0	-46.0	-23.0
	371,0300	343.0100	-14.0	-29.0	-16.0
Meclonazepam	330.0600	283.9500	-12.0	-26.0	-13.0
	330.0600	237.9500	-12.0	-44.0	-25.0
Nifoxipam	316.0700	269.9500	-11.0	-21.0	-18.0
	316.0700	223.9500	-12.0	-30.0	-23.0
Nordiazepam (BZD)	271.0630	208.0990	-18.0	-27.0	-21.0
	271.0630	140.0260	-18.0	-27.0	-24.0
Oxazepam (BZD)	287.0600	240.9500	-10.0	-23.0	-16.0
	287.0600	43.4000	-23.0	-41.0	-16.0
Pyrazolam	354.1000	167.1000	-13.0	-35.0	-16.0
	354.1000	206.0500	-13.0	-31.0	-20.0
Temazepam (BZD)	301.0700	254.9000	-11.0	-24.0	-16.0
	301.0700	176.9000	-11.0	-41.0	-18.0
Zolpidem (BZD)	308.1760	235.1230	-20.0	-35.0	-15.0
	308.1760	92.0000	-21.0	-53.0	-20.0
Zopiclone (BZD)	389.1500	245.0500	-14.0	-20.0	-24.0
	389.1500	112.0500	-28.0	-55.0	-19.0
Alprazolam-D5	314.3000	286.1500	-17.0	-28.0	-18.0
	314.3000	210.1000	-16.0	-42.0	-20.0
Bromazepam-D4	322.1000	186.0500	-16.0	-32.0	-18.0
	322.1000	213.1000	-12.0	-28.0	-21.0
Diazepam-D5	290.2000	198.1000	-20.0	-33.0	-19.0
	290.2000	154.0000	-21.0	-28.0	-15.0
Nordiazepam-D5	276.2000	213.1000	-10.0	-30.0	-20.0
	276.2000	140.2000	-19.0	-29.0	-13.0
Oxazepam-D5	292.2000	246.1000	-14.0	-24.0	-16.0
	292.2000	45.1000	-22.0	-25.0	-20.0
Temazepam-D5	301.0700	254.9000	-11.0	-24.0	-16.0
	301.0700	176.9000	-11.0	-41.0	-18.0
Zolpidem-D6	314.3000	235.1000	-15.0	-37.0	-23.0
	314.3000	92.1000	-22.0	-52.0	-15.0
Zopiclone-D4	393.2000	245.0500	-20.0	-17.0	-24.0
	393.2000	112.1500	-20.0	-54.0	-19.0

Table I : MRM transitions and optimized parameters for 8 benzodiazepines and 10 designer drugs plus 8 internal standards.



4. Validation

For 8 BZDs (alprazolam, bromazepam, diazepam, nordiazepam, oxazepam, temazepam, zolpidem, zopiclone), 7 concentration levels (5, 25, 50, 100, 500, 1000 and 2000 µg/L) were considered for the validation of the method. Each concentration level was determined according to the isotope dilution protocol. Therefore, the concentrations were calculated with the ratio between the peak area of molecules and that of their IS multiplied by its concentration, which was 50 µg/L in our method.
Each concentration was prepared and analysed each day for 6 days to determine the inter-day precision (coefficient of variation, CV) and accuracy (bias) using the ID.
The intra-day precision and accuracy were assessed for each level (n=6 for the same day). The method's validation parameters are summarized in table II. With the isotope dilution protocol, inter-day and intra-day precision and relative biases lower than 20 % were systematically obtained for all the molecules and each concentration level.

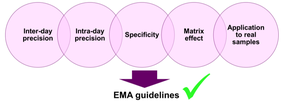
		Alprazolam		Bromazepam		Diazepam		Nordiazepam	
		CV (%)	Biais	CV (%)	Biais	CV (%)	Biais	CV (%)	Biais
Intra-day precision	5	12.41	-10.58						
	25	10.02	-8.00			13.86	-9.77	18.85	-0.24
	50	10.44	4.12	11.20	-14.49	11.13	-13.28	18.66	-0.06
	100	12.88	2.83	18.39	-9.73	13.39	-8.18	17.54	10.57
	500	13.99	2.19	19.37	-10.96	5.88	-13.63	19.11	7.92
	1000	9.23	9.21	11.86	-4.95	10.71	-5.01	11.87	7.36
	2000	5.32	10.32	11.64	-6.33	9.50	-1.37	11.21	11.24
Inter-day precision	5	9.20	1.19						
	25	8.69	-3.21			4.56	-15.99	8.33	-1.05
	50	10.40	-8.80	3.95	4.73	5.69	-0.43	15.10	2.69
	100	4.12	9.26	17.67	0.37	9.04	-18.95	13.40	2.29
	500	5.28	9.44	6.12	2.35	5.35	-12.61	6.78	4.88
	1000	4.24	8.53	6.95	5.22	2.24	-19.07	8.03	2.04
	2000	6.13	1.25	10.86	8.47	6.37	-17.53	14.01	10.13

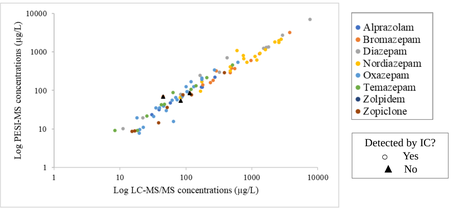
		Oxazepam		Temazepam		Zolpidem		Zopiclone	
		CV (%)	Biais	CV (%)	Biais	CV (%)	Biais	CV (%)	Biais
Intra-day precision	5					10.15	-7.53		
	25	16.02	5.90	11.96	7.15	8.15	-5.30	10.54	14.47
	50	14.83	-0.63	8.99	0.98	3.87	2.68	8.47	-2.34
	100	8.78	-6.74	12.93	-5.05	5.12	4.30	11.33	-5.33
	500	10.52	-7.29	4.19	-12.55	5.45	0.58	10.09	-17.74
	1000	16.47	-11.78	9.17	-10.61	2.90	2.82	14.63	-7.83
	2000	17.32	-9.70	9.20	-10.13	4.14	12.10	11.11	-1.67
Inter-day precision	5					3.80	7.34		
	25	9.35	5.33	10.51	-11.36	6.94	-4.80	14.87	-6.13
	50	13.91	-2.74	9.50	12.39	5.29	11.58	12.50	-4.35
	100	10.62	1.11	4.33	-14.27	3.45	7.92	14.15	-2.48
	500	8.90	6.01	4.28	-14.23	4.99	9.85	8.20	-1.27
	1000	9.19	-7.75	4.28	-14.70	3.62	4.27	12.47	-14.24
	2000	4.46	-13.47	6.41	-13.97	4.04	0.46	8.57	-2.67

Table II: Validation parameters of the method

5. Real Samples

For the present study, 40 samples sent to our Lab for determination of BZDs (i.e. routine activity) were analyzed on the same day using by a LC-MS/MS method, by an immunoassay and by the new developed PESI-MS/MS method. The immunoassay was installed on an Architect Ci8100 (Abbott, France). The LC-MS/MS methods needed 100 µL of serum and was based on a QuEChERS salts extraction. It was developed using a LCMS-8050 (Shimadzu Corporation) for the analysis of 35 traditional and designer BZDs with calibration ranges from 5 to 2000 µg/L. Both the immunoassay and the LC-MS/MS, employed in routine in the Lab, were fully validated for clinical practice according to European Medicines Agency and ISO15189 guidelines.





Regression analysis for BZD concentrations measured with PESI-MS and LC-MS/MS

6. Results

Among the 40 real samples, 100 % of the molecules detected by LC-MS (n=89) were also detected by PESI-MS and regression analysis reported an excellent agreement between the two methods (r^2=0.93). With IC, three false negative cases were observed: two cases with bromazepam and one with zolpidem. Among these real samples, no designer BZD was detected.

7. Conclusions

A method for an ultrafast measurement of 18 BZDs in serum was developed and validated using a PESI-MS approach combine with an isotope dilution protocol. Using only 10 µL of sample. This method provides an accurate determination of commercially available BZDs and detection of designer BZDs in about 2.5 minutes.