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ORIGINAL ARTICLE

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Quantification of benzodiazepines in whole blood and serum

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Abstract A high-performance liquid chromatography method for the determination of benzodiazepines and their metabolites in whole blood and serum using mass spectrometry (MS) and photodiode array (PDA) detection is presented. The combination of both detection types can complement each other and provides extensive case relevant data. The limits of quantification (LOQ) with the MS detection lie between 2 and 3 μg/l for the following benzodiazepines or metabolites: 7-amino-flunitrazepam, alprazolam, desalkyl-flurazepam, desmethyl-flunitrazepam, diazepam, flunitrazepam, flurazepam, α-hydroxy-midazolam, lorazepam, midazolam, nitrazepam, nordazepam and oxazepam, respectively 5 μg/l for lormetazepam and 6 μg/l for bromazepam. The LOQ of clobazam determined with the PDA detector is 10 μg/l. A convenient approach for determining the measurement uncertainty of the presented method—applicable also for other methods in an accreditation process—is presented. At low concentrations (<10 μg/l), measurement uncertainty was estimated to be about 50%, and at concentrations >180 μg/l, it was estimated to be about 15%. One hundred and twenty-eight case data acquired over 1 year are summarised.

Keywords Benzodiazepines · Whole blood · LC–MS · HPLC–PDA · Measurement uncertainty

Introduction

Many drugs and chemicals are not volatile enough for direct analysis with gas chromatography coupled to mass spectrometry (GC–MS), the state-of-the-art in analytical toxicology. These substances need to be hydrolysed and/or derivatised to be accessible with this technique. As an alternative, liquid chromatography coupled to mass spectrometry (LC–MS) is an upcoming routine method in laboratories involved in analytical toxicology [1–4]. With LC–MS–MS, an unequivocal identification of many compounds can be carried out without previous hydrolysis and derivatisation. This also applies to benzodiazepines and their metabolites. Recently, two articles dealing with the identification and quantification of benzodiazepines by LC–MS–MS were published [5, 6].

The benzodiazepine group consists of more than 30 substances, which are degraded in vivo and some also in vitro during storage and sample preparation into active or inactive metabolites. In Switzerland the most frequently used congeners are diazepam (Valium), flunitrazepam (Rohypnol) and midazolam (Dormicum). The therapeutical range covers four orders of magnitude, from 0.5 μg/l for flunitrazepam up to 2.5 mg/l for diazepam.

In our laboratory, urine samples are screened immunochemically for legally restricted and other psychoactive drugs. This involves cases of driving under the influence of drugs (duid) as well as forensic cases with a toxicological background involving living persons and postmortem samples. If benzodiazepines are detected, a blood sample is analysed to determine the levels of specific congeners and their metabolites in blood.

A method for the identification and quantification of benzodiazepines based on a liquid–liquid extraction (LLE) with 1-chlorbutan or solid phase extraction (SPE) followed by analysis with serial photodiode array (PDA) detection and MS detection is presented. In contrast to Kratzsch et al. [5], this method utilises only one non-deuterated internal standard. High-performance liquid chromatography (HPLC) runs are performed on two independent systems (PDA/MS and PDA), because the PDA spectra in the PDA–MS system are of insufficient quality as a steep gradient is used. PDA is used for detecting substances with low ionisation affinity. Their identity is verified with the UV spectra library "UV Spectra of Toxic Compounds" [7].

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Table 1 Gradient used in the HPLC–PDA–MS system

t (min)	5 mM NH_4OAc pH 4.75 (%)	Acetonitrile (%)	Methanol (%)
0	90	5	5
7	50	25	25
27	10	45	45
30	10	45	45
31	90	5	5

The ionisation in an atmospheric pressure chemical ionisation (APCI) ion source has proved to be superior to the electrospray ionisation (ESI) for most benzodiazepines.

Materials and methods

Methanol and acetonitrile were supplied in super gradient grade by Machery Nagel, Oensingen, Switzerland. Chloroform, isopropanol, 1-chlorbutan, orthophosphoric acid, acetic acid and potassium dihydrogen phosphate were supplied by VWR International, Dietikon, Switzerland. Ethylene glycol and ammonium acetate were supplied by Fluka, Buchs, Switzerland. Bond-Elut Certify extraction columns (300 mg/3 ml) were supplied by Varian, Zug, Switzerland. Certified drug-free serum for calibration and validation of the method was supplied by Bio-Rad Laboratories, Reinach, Switzerland.

All reference substances were supplied by Lipomed, Arlesheim, Switzerland, except brotizolam, which was kindly donated by F. Hoffmann-La Roche AG, Basel.

Table 2 Gradient used in the HPLC–PDA system

t (min)	50 mM KH_2PO_4 pH 2.3 (%)	Acetonitrile (%)
0	75	25
15	70	30
25	65	35
30	65	35

Sample preparation by liquid–liquid extraction

Aliquots of 1 ml whole blood or serum were spiked with 200 ng of brotizolam as internal standard. The sample was then extracted with 3 ml 1-chlorbutan by intensive vortexing. After centrifuging, the organic phase was transferred into a test tube filled with 20 µl ethylene glycol, gently evaporated with nitrogen at room temperature and redissolved in 40 µl of acetonitrile, 10 µl each was injected into the HPLC–PDA–MS and the HPLC–PDA systems.

Sample preparation with solid phase extraction

Aliquots of 1 ml whole blood or serum or 1 g of biological material were spiked with 200 ng of brotizolam as internal

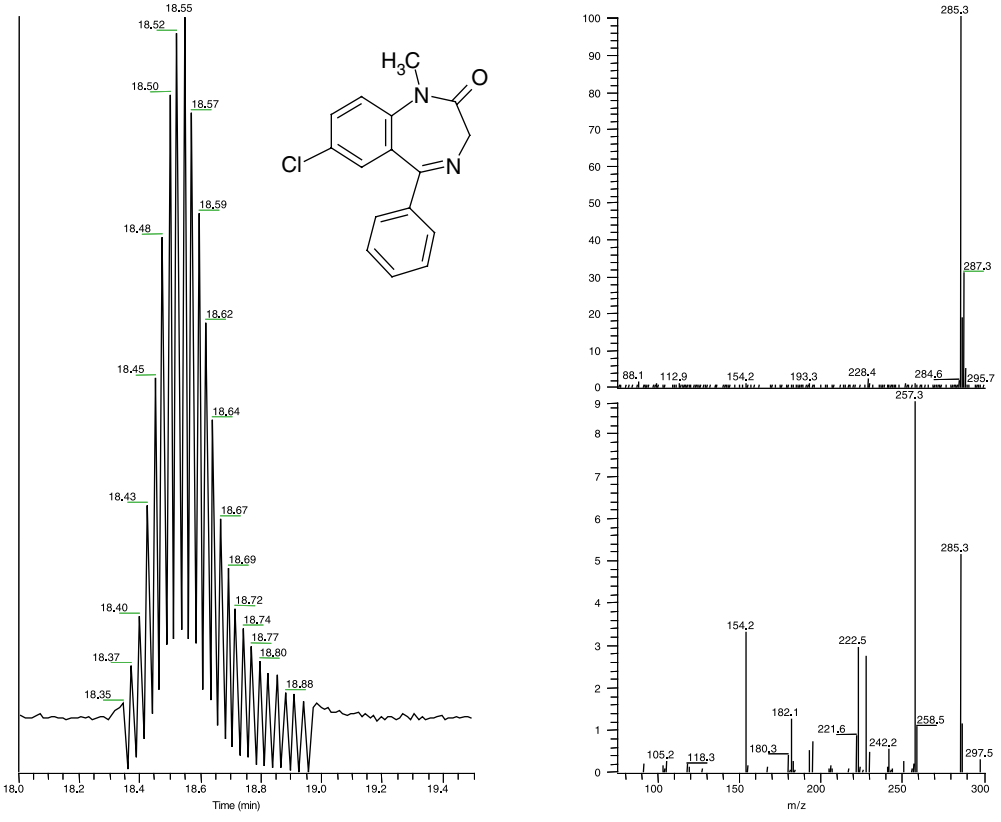

Fig. 1 *Left*, chromatogram of a combined MS and data-dependent MS–MS of diazepam, *right*, MS (*top*) and MS–MS (*bottom*) of diazepam

standard, and 9 ml 0.1 M phosphate buffer pH 6.0 was added. The Bond-Elut Certify column was conditioned with 2 ml methanol and 2 ml 0.1 M phosphate buffer pH 6.0. After loading the centrifuged sample solution, the column was rinsed successively with 2 ml deionised water, 2 ml 20% methanol in aqua dest, 3 ml 0.1 M phosphate buffer pH 1.0, 3 ml methanol and 2 ml chloroform. After drying with air for 15 min, elution was performed with 2 ml of a freshly prepared mixture of chloroform, isopropanol and concentrated ammonia solution (69:29:2 v/v). The eluate was collected in a test tube filled with 20 µl ethylene glycol, gently evaporated with nitrogen at room temperature and redissolved in 40 µl of acetonitrile. 10 µL each was injected into the HPLC–PDA–MS and the HPLC–PDA systems.

Chromatography with the HPLC–PDA–MS system

Chromatography was performed using a LCQ Duo system from Thermo Electron—a quaternary HPLC pump (P4000), an autosampler (AS3000) and a PDA detector (UV6000LP)—with a Restek Allure C18 (150×3.2 mm, 5 µm) column and Xcalibur 1.2 software. APCI source settings are vaporizer temperature, 450°C; sheath gas flow rate, 30 arb; discharge current, 5 µA; capillary temperature, 150°C; capillary voltage, 41 V; tube lens offset, 55 V. HPLC solvent flow is adjusted to 0.45 ml/min. The gradient used is shown in Table 1.

Chromatography with the HPLC–PDA system

Chromatography was performed using a Thermo Separation Products system from Thermo Electron—a quaternary HPLC pump (P4000), an autosampler (AS3000) and a PDA detector (UV6000LP)—with a VWR superspher 60 RP select B (125×3 mm, 4 µm) column and ChromQuest Version 4.0 software. Solvent flow is adjusted to 0.8 ml/min. The gradient used is shown in Table 2.

Results and discussion

Brotizolam is a benzodiazepine well proven to be a suitable internal standard with extraction behaviour comparable to the other benzodiazepines. In Switzerland the drug containing brotizolam (Lendormin) was only rarely prescribed and has no longer been available since 2003.

A working group of the Society of Toxicological and Forensic Chemistry (GTFCH) has shown that the recovery of benzodiazepines from liquid–liquid extraction is better than 85% and frequently reaches 100% [8]. Therefore, the recoveries were not again determined in our laboratory. However, in postmortem toxicological analyses, often only putrefied blood or tissue samples such as liver are available. In these cases, a solid phase extraction leads to cleaner extracts with less interfering substances during chromatography.

The addition of ethylene glycol to the extract prior to evaporation suppresses adsorption of the analytes onto glass surfaces and has been shown to be especially helpful when lorazepam or flunitrazepam are present in the sample [9].

High-performance liquid chromatography–photodiode array–mass spectrometry

The gradient used in HPLC–PDA–MS detection is capable of separating most of the benzodiazepines and their metabolites within the retention time window of 9–20 min and is a compromise between rapid elution and good separation of the analytes. In contrast to PDA detection, the baseline in MS detection does not increase dramatically during gradient elution. Therefore, sensitivity is not negatively influenced.

For the mass spectral analysis of benzodiazepines, APCI proved to be superior to ESI. When using ESI ionisation, suppression caused by coeluting compounds may even prevent some analytes from being detected [10]. This effect is less pronounced when using APCI. This is shown by comparing the areas of the internal standard brotizolam of 20 spiked whole blood extracts. The coefficient of variation

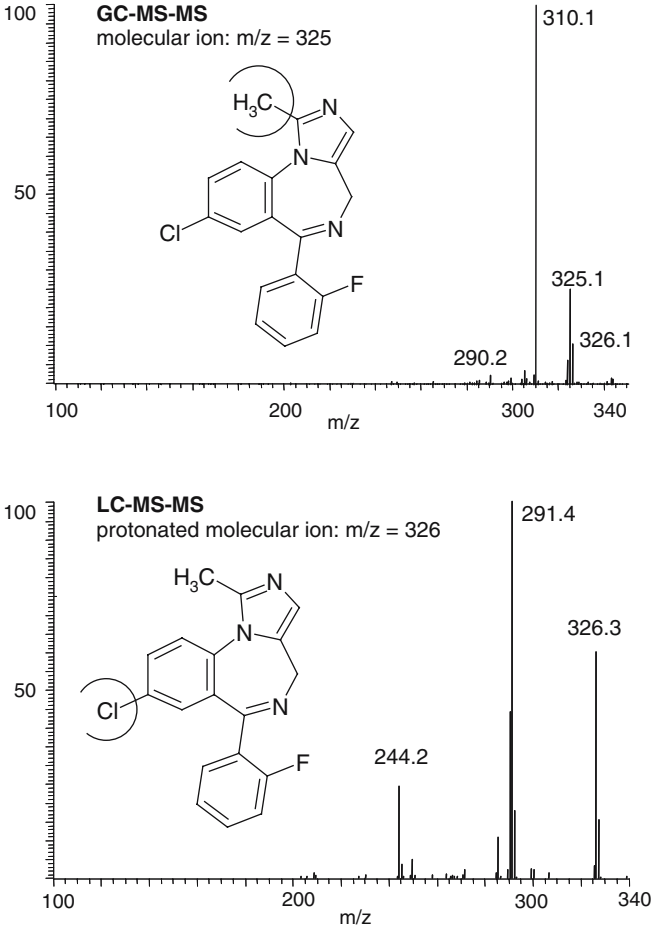

Fig. 2 Comparison of the MS–MS spectra of midazolam collected in a trap after GC and LC separation

was 17%, indicating only a small effect of ionisation suppression or enhancement. This gives us thus the possibility of using a single internal standard without having to use deuterated standards, which would otherwise interfere with PDA detection.

The MS system is tuned with diazepam in the positive ionisation mode for maximal response. This tuning turned out to be suitable for all benzodiazepines yielding an acceptable response with the exception of clobazam.

Unequivocal identification with data-dependent MS–MS

The ionisation at atmospheric pressure is a very mild process that produces the protonated molecular ion nearly without further fragmentation. For unequivocal identification, the molecular ion needs to be activated to dissociate into specific fragments. This can be done by MS–MS if an ion trap or a triple stage quadrupole is used in a so-called collision-induced dissociation (CID) [11].

The software used allows MS–MS to be automatically performed on any m/z ratio above a defined threshold. This feature is called data-dependent MS–MS. When screening the MS–MS, spectra obtained may be identified with the help of an MS–MS library.

The programming of two scan events, full scan and data-dependent MS–MS, helps to identify the analytes in the extracted sample without needing to define the masses to perform the MS–MS experiment prior to analysis (Fig. 1). Besides benzodiazepines, other substances present in the extract can also be identified except for very low-dosed compounds such as buprenorphine or lysergic acid diethylamide (LSD).

Contrary to GC–MS, where spectra obtained by electron impact ionisation do not depend on the make of the detector, LC–MS–MS spectra can vary enormously between detectors of different manufacturers. It is therefore not surprising that spectra obtained in an LC–MS–MS system do not match corresponding library spectra of a different system and especially not of a GC–MS library [2, 12–14]. An example is shown in Fig. 2 where midazolam fragmented in GC–MS–MS gives a main fragment at m/z 310 which indicates the loss of a methyl group. In LC–MS–MS, the activated protonated midazolam has a main fragment at m/z 291, indicating the loss of the chloride atom.

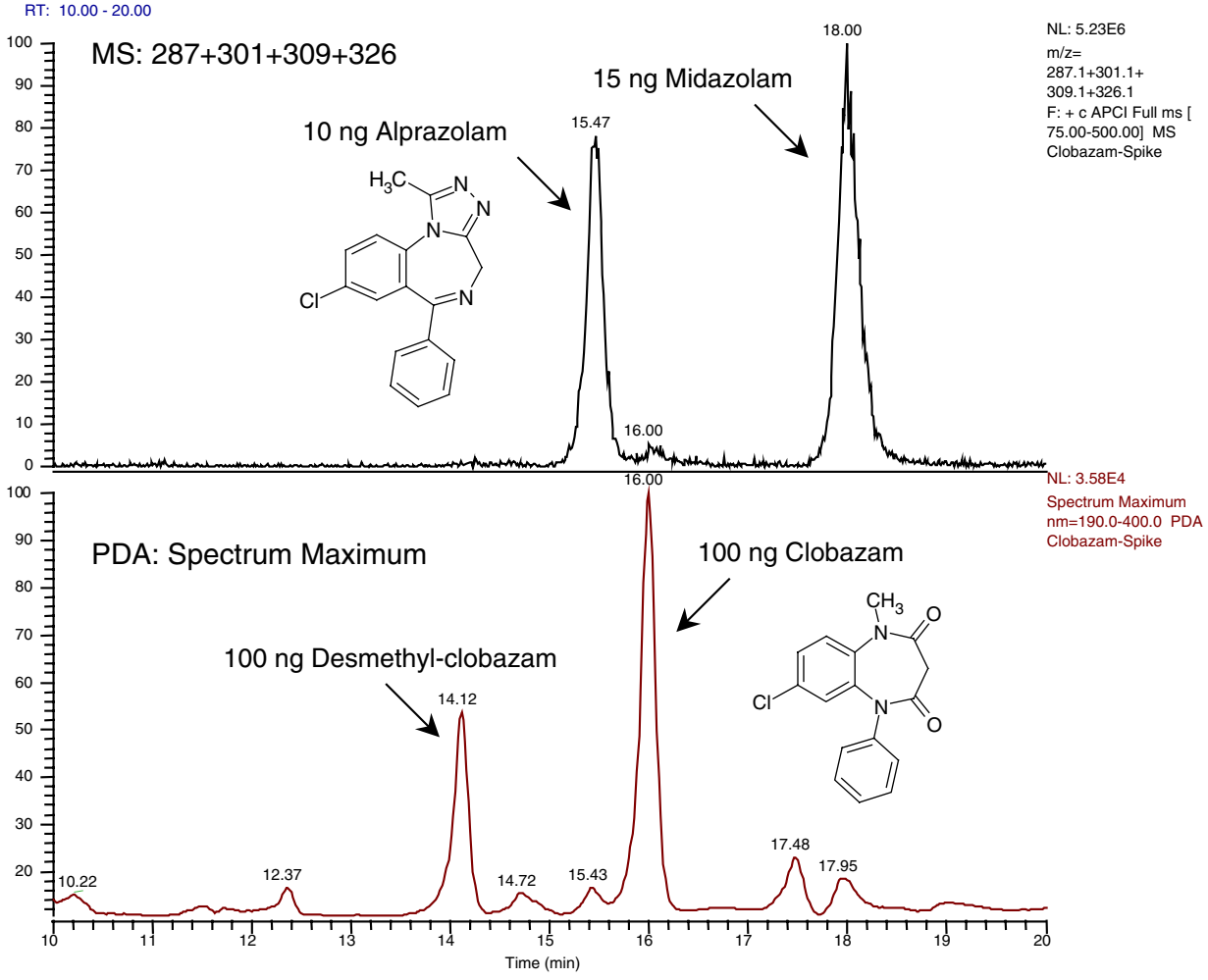

Fig. 3 Comparison of a reference standard mix detected by MS (trace of the corresponding masses) and PDA (spectrum maximum trace, absorbance at the maximum of every recorded spectrum)

Table 3 The validation parameters LLOQ and linearity determined with LC–MS compared to the lower therapeutic concentration and the toxic threshold concentration

Benzodiazepines and metabolites	LLOQ (μg/l)	Low therapeutic concentration [16] (μg/l)	Toxic [16] (μg/l)	Linearity LLOQ up to (μg/l)
Flunitrazepam	3	5	50	100
7-Amino-flunitrazepam	2			100
Desmethyl-flunitrazepam	3			100
Flurazepam	2	20	200	1,000
Desalkyl-flurazepam	2			1,000
Bromazepam	6	50	300	2,000
Lorazepam	3	20	300	1,000
Diazepam	2	200	3,000	3,000
Nordazepam	2	20	1,500	3,000
Oxazepam	2	200	2,000	3,000
Midazolam	2	40	1,000	1,500
α-Hydroxy-midazolam	3			1,500
Alprazolam	3	5	100	1,000
Nitrazepam	3	30	200	3,000
Lormetazepam	5	5		1,000
Clobazam[(a)]	10	100		2,000

[(a)]LLOQ and linearity determined with the HPLC–PDA system

Serial detection with PDA and MS detectors

Although LC–MS is a very powerful tool for identification, the PDA detector is much more sensitive for some compounds. Clobazam (Urbanyl), a 1,5-benzodiazepine derivative used as sedative, anticonvulsant and anxiolytic agent, is an atypical representative of the benzodiazepine group. We recently noticed that this compound and its metabolite are not detected with the presented LC–MS procedure. However, identification and quantitation of clobazam in the same blood extract was smooth when using the LC–PDA system. According to Weinmann (personal communication) LC–MS with turbo ion spray ionisation also performs well regarding clobazam detection. This underlining the fact that LC–MS features depend on the make of the detector. As real samples with clobazam are detected only rarely, the above mentioned effect is shown on a reference sample mix with clobazam, desmethyl-clobazam, alprazolam and midazolam (Fig. 3).

PDA detection alone on the other hand would lack the necessary selectivity and sensitivity for positively identifying many of the other target compounds. Relevant compounds could therefore be missed if screening is carried out only with MS or PDA detection.

Validation parameters and analytical limits

Validation parameters such as selectivity, lower limit of quantification (LLOQ) and linearity were determined according to Peters and Maurer [15]. The LLOQ for the tested benzodiazepines and their metabolites are well below the lowest therapeutic concentrations. If information on a specific benzodiazepine is requested, a forced MS–MS experiment on the corresponding molecular ion is performed in the specific time frame. In this way, limits of detection below 1 μg/l can be achieved. Table 3 lists the LLOQ and the linear range determined with the presented LC–MS method and compares them to the lowest therapeutic concentrations and the toxic threshold concentrations [16].

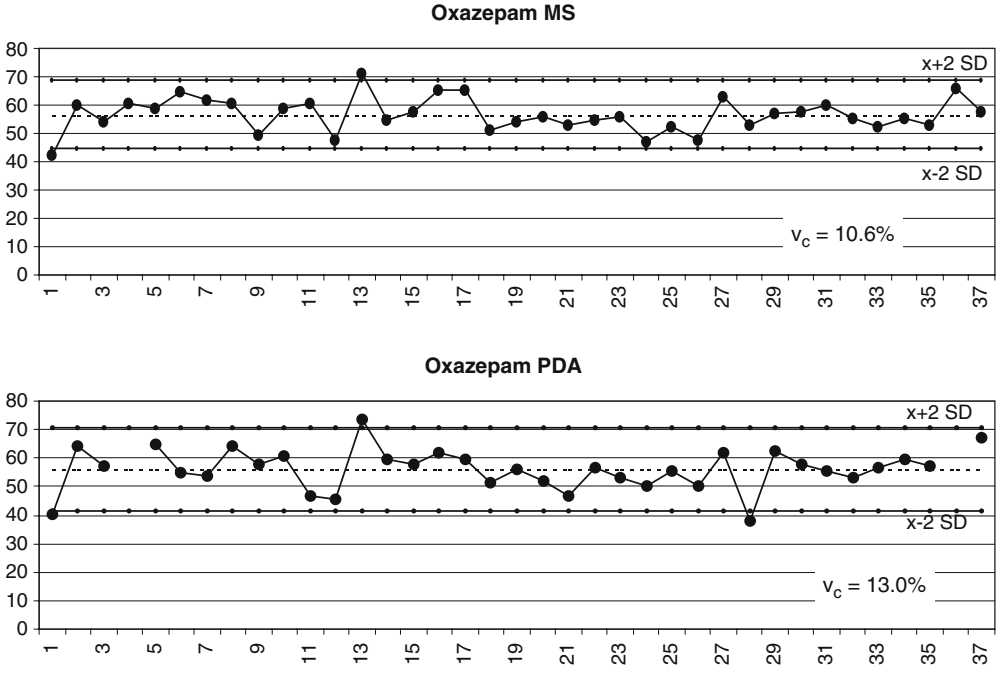

Fig. 4 Control charts for oxazepam with lyophilised serum

Table 4 Determined and certified benzodiazepine concentrations of commercial control samples used for the evaluation of the measurement uncertainty

Benzodiazepine	Determined concentration including SD (µg/L)		Certified concentration (µg/L)
	MS	PDA	
Flunitrazepam	20.1±2.8	18.2±2.7	15.7
1-Amino-flunitrazepam	23.4±2.2	21.9±3.1	18.0
Desmethyl-flunitrazepam	28.7±4.8	27.2±2.3	26.0
Oxazepam	56.6±6.0	56.1±7.3	65.9
Nordazepam	187.4±14.4	186.2±12.6	190.1
Diazepam	213.5±17.1	203.9±14.2	211.0
Bromazepam	98.5±9.8	103.7±7.9	89.9

It is highly advisable to use a quality control sample drawn from spiked whole blood or serum samples in every series and to monitor the obtained results in control charts. The data of the control samples are plotted together with the limits of acceptance (mean concentration of benzodiazepine±2 SD). As a rule, of 20 measurements only 1 may lay outside these limits. Otherwise, the method used must be revised [17]. The control charts can be used to monitor the quality of the current sample series and to estimate the measurement uncertainties. Control charts used for this method showed that results obtained with the MS detection are comparable to results obtained when using the PDA detector as is shown for oxazepam in Fig. 4. The quality control sample used consisted of a commercially available lyophilised spiked serum with a certified oxazepam concentration of 66 µg/l. The measured oxazepam contents are spread around an average of 57 µg/l, with most data lying between 45 and 70 µg/l. Better results were obtained with a pool of sera spiked in our laboratory and stored in 1-ml vials at −20°C. With a spiked concentration of 65 µg/l oxazepam, an average of 64 µg/l (MS 62 µg/l, PDA 65 µg/l) was obtained with all values lying between 54 and 70 µg/l. The data of other benzodiazepines (Table 4) also gave better results when using our frozen spiked serum than with most of the commercially available lyophilised serum samples. This may be due to the same origin of the reference solutions for the preparation of calibration solutions and quality control sera.

Table 5 Estimated measurement uncertainties pooled into classes

Concentration (µg/l)	$u_{95\%,\ \text{relative}}$ (%)
LOQ-10	50
>10–20	40
>20–40	30
>40–70	25
>70–180	20
>180–500	15

As an assumption for a pragmatic estimation of measurement uncertainty, precision should not differ from one benzodiazepine to another over the whole concentration range. Results from proficiency tests show that this assumption is correct.

In a first step, the coefficients of variation v_c of each benzodiazepine was plotted vs the mean value of the benzodiazepine concentration x. The v_c fit to a function of the type $v_c=a\times x^b$. The standard deviation (SD) was calculated for each point as $SD=v_c\times x/100$. Finally, the measurement uncertainty $u_{95\%}$ was calculated as $u_{95\%}=2\times SD$ (Fig. 5) [18]. For routine work, measurement uncertainties were pooled into classes instead (Table 5).

The accuracy of the results depended on the concentration: a concentration closer to the LLOQ meant a higher measurement uncertainty. This finding has already been described by Horwitz [19].

Case data

The presented method has been in use in our laboratory since 2000. Between July 2003 and June 2004, for in-

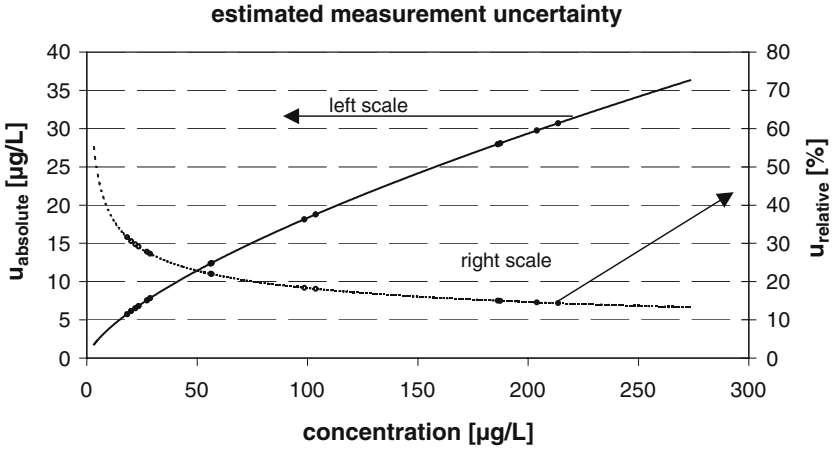

Fig. 5 Absolute and relative measurement uncertainty for benzodiazepines as estimated with the presented method

Table 6 Samples analysed during 1 year (mid 2003 to mid 2004)

	Duid	Forensic cases		Custom analysis	Total	Concentration range (μg/l) from LLOD up to
		Postmortem	Living persons			
No. of cases	30	46	9	43	128	
Diazepam	14	22	5	14	55	5,200
Nordazepam	16	23	5	20	64	4,300
Oxazepam	11	16	2	17	46	1,550
Alprazolam	2	5	0	5	12	530
Midazolam	3	3	1	10	17	200
Flurazepam	0	4	0	2	6	1,100
Bromazepam	4	6	1	0	11	450
Lorazepam	3	12	1	14	30	190
Lormetazepam	0	0	0	2	2	40
Flunitrazepam	7	5	0	5	17	50
Nitrazepam	1	0	0	0	1	60
Negative	0	2	1	4	7	
Total	61	96	15	89	261	

stance, a total of 128 samples were analysed (Table 6), 30 samples related to driving under the influence of drugs (duid), 55 postmortem samples and 43 samples obtained from other forensic laboratories for benzodiazepine analysis. In all cases where the concentration of the analyte was higher than the upper limit of the linear range, a second determination was carried out using less material. The positive immunotest result in urine could not be confirmed for the blood sample in two cases, and in one case no blood was available to confirm the positive immunotest result. In four blood samples obtained from other forensic laboratories, no benzodiazepine was detected. No case with clobazam was encountered. In addition to the benzodiazepines, 42 other substances of toxicological interest could be identified in the 128 specimen analysed (Table 7). In the same time frame, our laboratory participated successfully in three proficiency tests that covered nine target compounds.

Table 7 Further substances identified (mid 2003 to mid 2004)

Further substances identified		
Amitryptiline	Ketamine	Papaverine
Bisoprolol	Lamotrigine	Paroxetine
Buflomedil	Laudanosine	Pipamperone
Carbamazepine	Lidocaine	Promethazine
Chlorprothixene	Metamizole	Propyphenazone
Citalopram	Methadone	Sertraline
Clomethiazole	Methaqualone	Thioridazine
Cocaine	Metoclopramide	Tramadol
Diphenhydramine	Metoprolol	Trimethoprim
Doxepine	Mianserin	Trimipramine
Doxylamine	Mirtazapine	Venlafaxine
Fentanyl	Noscapine	Verapamil
Fluconazole	Olanzapine	Zolpidem
Fluoxetine	Omeprazole	Zopiclone

Summary

A procedure for the reliable identification and quantification of benzodiazepines in whole blood and serum by means of LC–MS and HPLC–PDA is presented. The procedure is easily applicable, it does not require deuterated internal standards and proved to be successful in practice. Furthermore, a simple method for estimating measurement uncertainty is demonstrated.

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