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Determination of benzodiazepines in ante-mortem and post-mortem whole blood by solid-supported liquid–liquid extraction and UPLC–MS/MS☆

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ABSTRACT

A solid-supported liquid–liquid extraction ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for the determination of benzodiazepines commonly found in Norway, for use in cases with suspected driving impairment and autopsy cases by analysis of human whole blood samples. The following compounds were included: alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam (metabolite of diazepam), oxazepam and phenazepam. Aliquots of 500 μL whole blood were added 500 μL of borate buffer pH 11 and extracted by solid-supported liquid–liquid extraction on ChemElut® columns using three times 2.5 mL of methyl *tert*-butyl ether. Deuterated analogues were used as internal standards (IS) for all analytes, except for midazolam, phenazepam and bromazepam which had no commercially available deuterated analogues at the time the method was developed, and therefore used diazepam-d_5, flunitrazepam-d_7 and nitrazepam-d_5, respectively. The analytes were separated using UPLC with a 2.1 × 100 mm BEH C_{18}-column, 1.7 μm particle size, and quantified by MS/MS using multiple reaction monitoring (MRM) in positive mode. Two transitions were used for the analytes and one transition for the IS. The run time of the method was 8 min including equilibration time. The concentrations of the benzodiazepines in the method span a broad range varying from the lowest concentration of 0.005 μM for flunitrazepam to the highest of 20 μM for oxazepam. The calibration curves of extracted whole blood standards were fitted by second-order calibration curves weighted $1/x$, with R^2 values ranging from 0.9981 to 0.9998. The intermediate precision had a CV (%) ranging between 2 and 19%. Recoveries of the analytes were from 71 to 96%. The LLOQs for the analytes varied from 0.0006 to 0.075 μM and the LODs from 0.005 to 3.0 nM. Matrix effects were studied by post extraction addition and found to be between 95 and 104% when calculated against an internal standard. A comparison with two other LC–MS methods was performed during method validation. Good correlation was seen for all analytes. The method has been running on a routine basis for several years, and has proven to be very robust and reliable with good results for external quality samples. The method also meets the requirements of the legislative limits for driving under the influence of non-alcohol drugs to be introduced in the Norwegian legislative system from 2012.

1. Introduction

Benzodiazepines are amongst the most frequently prescribed psychoactive drugs world wide [1]. Due to their hypnotic, anxiolytic, anticonvulsant and muscle-relaxant properties they are used for the therapy of anxiety, convulsive attacks and sleeping disorders. The sedative and amnestic properties of some benzodiazepines are also considered useful in anaesthesia. In addition, the benzodiazepines have a rapid onset of action combined with low acute toxicity. Benzodiazepines are, however, also associated with abuse and some can be toxic at higher blood drug concentrations. Their use might lead to development of dependence, and the benzodiazepines are commonly used in combination with other substances of abuse [1]. Studies have indicated that benzodiazepines

Abbreviations: DUI, driving under the influence of drugs; UPLC–MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry; LLE, liquid–liquid extraction; LC–MS/MS, liquid chromatography with tandem mass spectrometry; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; R^2, the correlation coefficients; S/N, signal-to-noise ratio; LOD, limit of detection; LOQ, limit of quantification; ME, matrix effects; RSD, relative standard deviation; CV, coefficient of variation; NIPH, Norwegian Institute of Public Health.

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impair psychomotor, cognitive and driving performance, and especially in combination with alcohol and/or illicit drugs thus represent a risk factor in traffic safety [2–5]. Some benzodiazepines are also known to be used to facilitate sexual assault (date rape) [6,7].

In Norway benzodiazepines are frequently detected in blood samples from drivers apprehended under the suspicion of impaired driving [8,9]. In the 5-year period from 2000 to 2005 one or more benzodiazepines were found at frequencies varying between 38 and 57% of the total number of blood samples received for analysis at the Norwegian Institute of Public Health (NIPH) [9]. In a roadside study from 2005 to 2006 a sample material of 10,816 oral fluid samples provided by Norwegian motor vehicle drivers were analysed, of which a total of 1.4% were positive for benzodiazepines [10].

Due to their importance in forensic toxicological and clinical settings, there are numerous analytical procedures for the determination of benzodiazepines to be found in the literature. Gas chromatography coupled to mass spectrometry has for very many years been a method of choice in clinical and forensic toxicology. During the last 15 years, however, liquid chromatography mass spectrometry or tandem mass spectrometry has become a mature technique finding many applications in the same fields [11–13]. The use of liquid chromatography reduces the need for derivatization and is very useful for hydrophilic, thermolabile, and non-volatile substances. With the advance of columns with sub-2 μm particles and the instrumentation necessary to handle the large back-pressures that follows, i.e. ultra performance liquid chromatography (UPLC) or ultra high performance liquid chromatography (UHPLC), better separation and shorter run times are achieved as well.

Many analytical methods have been reported for the detection of benzodiazepines in various biological matrices by LC–MS or LC–MS/MS and recently by UPLC–MS(/MS) and the subject was recently extensively reviewed by Nakamura [14]. The aim of the present work is to describe a fully validated, rapid confirmation method for determination of benzodiazepines common on the Norwegian market for use in impairment cases as well as in forensic autopsy cases using solid-supported liquid–liquid extraction and UPLC–MS/MS on whole blood samples.

2. Experimental

2.1. Chemicals and reagents

The reference substances were purchased from the following manufacturers: clonazepam, flunitrazepam and nitrazepam from Alltech (Lexington, KY, USA), phenazepam from Chiron AS (Trondheim, Norway), bromazepam from Sigma–Aldrich (St. Louis, MO, USA), alprazolam, diazepam, lorazepam, midazolam, nordiazepam and oxazepam from Lipomed (Arlsheim, Switzerland). The IS alprazolam-d_5, clonazepam-d_4, diazepam-d_5, flunitrazepam-d_7, nitrazepam-d_5, nordiazepam-d_5, oxazepam-d_5 and lorazepam-d_4 were all purchased from Cerilliant Corp. (Round Rock, TX, USA).

The chemicals *di*-sodium tetra borate decahydrate (GR), sodium hydroxide (pellets, GR), ammonium acetate, acetic acid and methyl *tert*-butyl ether were provided by Merck KGaA (Darmstadt, Germany). Acetonitrile was obtained from Lab-Scan (Dublin, Ireland). Purified water was obtained with a Milli-Q system (Millipore, Billerica, MA, USA). The ChemElut™ 1 mL cartridges were obtained from Varian Inc. (Palo Alto, CA, USA).

2.2. Biological samples

For the preparation of controls and calibrators, whole blood (containing 2 g sodium fluoride, 6 mL heparin and 10 mL water per 450 mL blood) was obtained from the blood bank at Ullevål University Hospital (Oslo, Norway), and screened for drugs and alcohol before use by immunoassay and chromatographic methods. Confirmation analysis of benzodiazepines in whole blood samples at NIPH are predominantly done in impairment cases and forensic autopsy cases. The samples are then received in 4 mL BD Vacutainer® Plus Plastic Blood Collection Tubes (BD Vacutainer Systems, Frankling Lake, NJ, USA) containing 10 mg sodium fluoride and 8 mg potassium oxalate, and 25 mL Sterilin tubes (Sterilin, Caerphilly, UK) containing 200 mg potassium fluoride, respectively.

Collected samples are stored at 4 °C prior to processing. Aliquots of 500 μL are then transferred to separate 5 mL polypropylene tubes (Sarstedt AG, Rommelsdorf, Germany) which are stored at 4 °C until the time of analysis.

2.3. Standard solutions

For each compound two separate stock solutions were prepared in methanol, identified as calibration and quality control (QC), respectively. From the stock solutions aqueous work solutions were prepared containing all the benzodiazepines. Calibration and QC samples were prepared in batches adding aqueous calibration or control solution to drug free whole blood and dispensing in aliquots of 500 μL after thoroughly mixing. The aliquots were stored in 5 mL polypropylene tubes (Sarstedt AG) in a freezer at −20 °C for up to 12 months. The calibrators (n = 6–7) ranged from sub-therapeutic to high dose/toxic levels and the control samples (n = 4–5) were distributed to cover the calibration range. A mix of internal standards with concentrations ranging from 1.25 to 50 μM was prepared in water and stored at 4 °C until empty, typically 2–3 months.

2.4. Sample preparation

A 500 μL aliquot of whole blood was added 50 μL IS and 500 μL saturated borate buffer pH 11, and mixed on a multitube vortexer for 60 s. The mixture was transferred to a ChemElut™ cartridge and the analytes eluted with three aliquots of 2.5 mL methyl *tert*-butyl ether. The eluate was evaporated at 40 °C until dryness under nitrogen at a pressure of 5 psi using a Caliper TurboVap (Caliper Life Sciences, Hopkinton, MA, USA) and reconstituted in 100 μL acetonitrile:5 mM ammonium acetate buffer pH 5.0 (25:75, v/v) prior to injection into the UPLC–MS/MS-system.

2.5. UPLC conditions

A Waters Acquity UPLC module (Waters Corp., Milford, MA, USA) was used for separation. Gradient elution was performed on an Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) column with an Acquity UPLC BEH C18 VanGuard Pre-Column (2.1 × 5 mm, 1.7 μm) in front, both from Waters (Wexford, Ireland). A two level five factor full factorial design experiment studying the factors pH, temperature, flow, ion strength of the buffer and the percentage of acetonitrile at gradient starting point was used to aid in method development. The retention time of each of the benzodiazepines and number of separated peaks as found by manual inspection were used as responses. The parameters were optimized to get the maximum number of resolved peaks achievable with a retention time of less than 10 min. A flow rate of 0.6 mL/min with acetonitrile (mobile phase A) and 5 mM ammonium acetate buffer pH 5.0 (mobile phase B) as solvents was used in a convex ramp, giving a slower gradient at the beginning and a steeper at the end compared to a linear profile. The gradient is shown in Table 1. The total cycle time of the method was 8 min. The column temperature was held at 65 °C and the injection volume was 5 μL using partial loop injection with a needle overfill flush. Weak wash and strong wash

Table 1
Gradient table.[a]

Time (min)	A (%)	B (%)	Flow (mL/min)	Curve
0.00	30.0	70.0	0.600	1
7.00	40.0	60.0	0.600	7
7.01	90.0	10.0	0.600	1
7.30	30.0	70.0	0.600	1

[a] A, acetonitrile; B, 5 mM ammonium acetate buffer; pH 5.0. The curve profile 7 used for the gradient elution is a convex ramp, giving a slower gradient at the beginning and a steeper at the end compared to a linear profile. Curve profile 1 is an immediate change to the specified condition.

were performed with 600 μL acetonitrile/water (5:95) and 200 μL acetonitrile/water (90:10), respectively.

2.6. MS/MS conditions

A Waters Quattro Premier XE tandem mass spectrometer with an electrospray source (Waters Corp., Milford, MA, USA) was used for all the analyses. ESI-MS/MS-detection was performed in the multiple reaction monitoring (MRM) mode using positive ionization. The capillary voltage was set to 1.0 kV and the source block temperature to 120 °C. Nitrogen from a nitrogen generator (99.93%, OxymatN600, AGA, Norway) was used for desolvation, delivered at a temperature of 400 °C and a gas flow of 1200 L/h. The cone gas from the generator was set to 60 L/h and the collision gas (Argon, 99.999%, AGA, Norway) was maintained at approximately 6 mbar in the collision cell.

The MRM transitions, cone voltages and collision energies for the different analytes were optimized by direct infusion into the MS. Separate tuning solutions for each benzodiazepine of approximately 50 μM were infused at a flow rate of 20–50 μL/min, depending on the response of the compound, together with a constant flow of 0.2 mL/min 50:50 acetonitrile: 5 mM ammonium acetate buffer.

In the method two transitions were monitored for the analytes and one for the IS, giving a total of 30 transitions. The transitions were divided into three scan segments with 14 transitions in scan segment 1 during a time span of 1.4 min, 6 transitions in scan segment 2 during a time span of 0.6 min and 10 transitions in scan segment 3 during a time span of 3 min. The MRM transitions, cone voltages, collision energies, dwell times and scan segments used for the measurement of the benzodiazepines and the IS are provided in Table 2, in addition to typical retention times.

System operation and data acquisition were controlled using MassLynx 4.1 software (Waters Corp., Milford, MA, USA). All data were processed with the QuanLynx quantification program (Waters Corp., Milford, MA, USA). Analytes were identified by comparison of the retention times of the respective MRM transitions of the samples with the corresponding values for the QC and calibrator samples. In addition the variation of the ion ratio between the two transitions for each analyte was required to be below ±10% compared with the ion ratios of the control and QC-samples for all analytes, except bromazepam, flunitrazepam and phenazepam for which a tolerance of ±15% was allowed.

2.7. Method validation

Validation of the method included calibration model, precision, accuracy, limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), extraction recovery, specificity, matrix effects, carry-over and stability.

2.7.1. Calibration curves

The calibration curves were based on the ratio of the peak height of the analytes versus the peak height of the corresponding deuterated IS, except for midazolam, phenazepam, and bromazepam where diazepam-d_5, flunitrazepam-d_7 and nitrazepam-d_5 respectively were used. A total of seven calibrators were prepared for all compounds where the lowest calibrator corresponded to 1/10 of

Table 2
MRM transitions,[a] cone voltages, collision energies, dwell times, scan segments and retention times for analytes and internal standards (IS).

	Analyte	MRM transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)	Dwell time (s)	Scan segment	Retention time (min)	IS used
1	Alprazolam	309.1 > 205.1	45	40	0.050	2	2.67	IS1
		309.1 > 281.1	45	25	0.050	2		
2	Bromazepam	318.1 > 209.1	40	19	0.030	1	1.31	IS6
		318.1 > 290.1	40	19	0.030	1		
3	Clonazepam	316.1 > 214.1	40	37	0.010	1	2.07	IS2
		316.1 > 270.1	40	25	0.010	1		
4	Diazepam	**285.1 > 154.1**	40	27	0.055	3	4.86	IS3
		285.1 > 193.1	40	30	0.055	3		
5	Flunitrazepam	**314.1 > 239.1**	40	35	0.070	2	2.61	IS4
		314.1 > 268.1	40	25	0.070	2		
6	Lorazepam	321.2 > 275.1	35	24	0.020	1	2.22	IS5
		321.2 > 303.1	35	13	0.020	1		
7	Midazolam	326.1 > 249.1	45	40	0.055	3	4.25	IS3
		326.1 > 291.1	45	27	0.055	3		
8	Nitrazepam	282.1 > 180.1	35	40	0.030	1	1.87	IS6
		282.1 > 236.1	35	25	0.030	1		
9	Nordiazepam	**271.1 > 140.1**	40	27	0.055	3	3.27	IS7
		271.1 > 165.1	40	27	0.055	3		
10	Oxazepam	**287.1 > 104.1**	30	37	0.030	1	1.98	IS8
		287.1 > 163.1	30	37	0.030	1		
11	Phenazepam	351.0 > 179.0	40	45	0.055	3	3.83	IS4
		351.0 > 206.1	40	37	0.055	3		
IS1	Alprazolam-d_5	314.2 > 286.1	45	25	0.050	2	2.62	
IS2	Clonazepam-d_4	320.1 > 274.1	40	25	0.030	1	2.04	
IS3	Diazepam-d_5	290.2 > 154.1	40	30	0.055	3	4.77	
IS4	Flunitrazepam-d_7	321.2 > 275.2	40	25	0.060	2	2.54	
IS5	Lorazepam-d_4	325.1 > 279.1	35	24	0.030	1	2.19	
IS6	Nitrazepam-d_5	287.2 > 185.1	35	25	0.030	1	1.83	
IS7	Nordiazepam-d_5	276.2 > 140.1	35	27	0.055	3	3.21	
IS8	Oxazepam-d_5	292.1 > 246.1	35	25	0.030	1	1.95	

[a] Transitions used for quantification are in bold characters.

Table 3
Calibration range, correlation coefficient, extraction recovery, repeatability, intermediate precision and accuracy given as bias for whole blood.

	Analyte	Calibration range[a] (μM)	R^2, $n = 10$	QC sample conc. (μM)	Extraction recovery[b] (%)	Repeatability[c] CV (%)	Intermediate precision[d] CV (%)	Bias[d] (%)
1	Alprazolam	0.001–1	0.9992	0.001	–	8	12	−14
				0.006	–	2	3	15
				0.015	75	1	3	14
				0.4	88	1	2	−2
				0.8	90	2	3	−1
2	Bromazepam	(0.005)	0.9991	0.031	–	3	8	10
		0.025–3		0.075	76	4	12	0.1
				1.2	82	1	8	4
				2.4	83	2	6	3
3	Clonazepam	(0.0004)	0.9998	0.003	–	19	19	0.4
		0.002–1		0.006	71	12	14	1
				0.4	85	2	3	2
				0.8	88	1	3	1
4	Diazepam	0.02–12	0.9981	0.03	–	3	9	−19
				0.13	–	1	3	12
				0.3	76	1	3	13
				4.8	86	1	2	−5
				9.6	87	4	5	−7
5	Flunitrazepam	0.0005–0.4	0.9998	0.0006	–	13	19	−3
				0.003	–	7	11	3
				0.008	75	3	6	1
				0.16	86	2	2	2
				0.32	88	1	3	2
6	Lorazepam	(0.003)	0.9996	0.019	–	10	10	−5
		0.015–1.95		0.045	71	4	5	−8
				0.78	88	2	2	−8
				1.6	91	2	3	−7
7	Midazolam	0.009–1.8	0.9990	0.013	–	3	10	20
				0.063	–	2	6	21
				0.15	76	1	6	19
				0.8	89	3	8	14
				1.6	89	3	8	17
8	Nitrazepam	0.005–3	0.9997	0.006	–	4	9	7
				0.031	–	2	4	14
				0.075	75	2	4	12
				1.2	87	1	2	10
				2.4	87	2	3	11
9	Nordiazepam	0.02–13	0.9996	0.03	–	4	7	−11
				0.13	–	2	2	6
				0.3	75	1	3	7
				5.2	86	1	3	−2
				10.4	86	2	3	−3
10	Oxazepam	0.06–20	0.9994	0.08	–	4	7	7
				0.38	–	2	3	9
				0.9	74	2	2	8
				8	86	1	2	0.3
				16	88	1	3	−0.8
11	Phenazepam	0.003–0.5	0.9995	0.003	–	8	15	13
				0.008	79	4	10	9
				0.2	96	3	6	4
				0.4	95	2	5	4

[a] The levels in parenthesis were excluded from the calibration curves due to low signal intensity/large variability.
[b] Six replicates at each QC level.
[c] Ten replicates at each QC level. One outlier was removed for clonazepam.
[d] 21 replicates at each QC level. One outlier was removed for clonazepam.

the concentration level (cut-off) above which samples are reported as positive to the customers of NIPH, Tables 3 and 4. The calibration curves were evaluated based on ten assays with one replicate of each of the seven calibrators.

2.7.2. Precision and accuracy

Intermediate precision and accuracy was determined by extraction and analysis of 1–3 replicates of five different QC concentrations per assay. A total of ten assays were analysed differing either in day of analysis, analyst or instrument giving a total of 21 replicates at each level. Accuracy given as bias was calculated as the percent deviation of the measured mean of the QC samples from the theoretical concentration. Repeatability was estimated in a single assay by extraction and analysis of QC samples of five different concentration levels spanning from low to high concentrations, with ten replicates at each level.

Table 4
Cut-off values, LOD and LLOQ with precision and accuracy, reference concentrations for therapeutic use.

Compound	Cut-off		LOD		LLOQ		CV	Bias	Reference concentrations for therapeutic use	
	(μM)	(ng/mL)	(μM)	(ng/mL)	(μM)	(ng/mL)	(%)	(%)	(μM)	(ng/mL)
Alprazolam	0.0100	3.09	0.00001	0.003	0.0013	0.39	12	−14	<0.2	<62
Bromazepam	0.050	15.8	0.001	0.3	0.031	9.9	8	10	<0.5	<158
Clonazepam	0.0040	1.3	0.0008	0.3	0.0025	0.8	19	0.4	<0.3[a]	<95[a]
Diazepam	0.200	56.9	0.0002	0.1	0.025	7.1	9	−19	<1.5	<427
Flunitrazepam	0.0050	1.57	0.000005	0.002	0.0006	0.20	19	−3	<0.03	<9
Lorazepam	0.030	9.6	0.003	1.0	0.019	6.0	10	−5	<0.4	<129
Midazolam	0.100	32.6	0.0001	0.03	0.013	4.1	10	20	[b]	[b]
Nitrazepam	0.0500	14.1	0.00005	0.01	0.0063	1.8	9	7	<0.4	<113
Nordiazepam	0.200	54.1	0.0002	0.1	0.025	6.8	7	−11	[c]	[c]
Oxazepam	0.600	172.0	0.0006	0.2	0.075	21.5	7	7	<3.0	<860
Phenazepam	0.0050	1.7	0.00025	0.1	0.0031	1.1	15	13	<0.02	<7

[a] Clonazepam used as antiepileptikum.
[b] Midazolam is used solely as an anaesthetic in Norway.
[c] Nordiazepam is a metabolite of diazepam; not prescribed as a drug in Norway.

2.7.3. Limit of quantification and detection

LLOQ was determined as the QC concentration where the variation (RSD) and bias were within ±20% based on the transition used for quantification and with signal to noise >10 for both transitions. ULOQ was set to the highest calibrator. LOD was determined by extracting dilutions of a low calibrator and evaluation of signal to noise (S/N > 3) for both transitions.

2.7.4. Extraction recovery

The extraction recovery was determined at three concentration levels with six replicates of each concentration. Recovery was calculated by comparison of the peak heights obtained when the analytes were added before extraction and the internal IS were added after ($n = 6$), with those obtained when both the analytes and IS were added after the extraction step ($n = 6$).

2.7.5. Specificity

The specificity of the method was tested by analysis of blank matrix and zero (blank matrix added IS) samples for blood provided by the blood bank and authentic autopsy samples. In addition the method was tested using high concentrations of frequently found drugs in analysis of impairment and autopsy cases in Norway ($n = 100$). Drug free whole blood was fortified in a concentration equivalent to high therapeutic concentration. The drugs tested were antidepressants, analgesics, antipsychotics, cardiac drugs, antiepileptics, opioids, amfetamines, and other compounds evaluated in forensic samples at our laboratory. A listing of the concentrations of the tested drugs is given in Table 5. The analytical LC gradient was applied and the chromatograms evaluated for interfering peaks at the same retention time as the analytes.

2.7.6. Carry-over

Carry-over was investigated by preparing a calibrator with a concentration 3 times the highest calibrator and evaluating the chromatograms of two extracted matrix blanks run consecutively after this calibrator.

2.7.7. Matrix effects

Matrix effects were tested using the post extraction addition approach [15,16] for two different concentration levels. Samples from eight different lots of human blood were used, with four of them being from autopsy cases and four from the local blood bank. Two sets of samples were prepared. Set A consisted of eight extracts of the blank matrices with the analytes of interest added post extraction and set B of five replicates of neat solutions containing equivalent amounts of analytes of interest prepared in the solution used for reconstitution. IS was added after the extraction, but prior to evaporation. The matrix effect (ME) in percent was calculated by referring the mean peak height for the samples spiked after extraction (A) with the mean peak height found for the neat solutions (B): $ME = (A/B) \times 100\%$. A value above 100% indicates ion enhancement, and a value below 100% indicates ion suppression. The relative matrix effects were calculated as the variability in matrix effects expressed as CVs (%).

2.7.8. Stability

QC samples were prepared in whole blood and kept in a freezer at −20 °C for 12 months to evaluate the long term stability. To evaluate the stability of extracted samples, standards and QC samples were kept for a week in an autosampler at 10 °C as well as in a freezer at −20 °C and then reanalyzed and calculated with freshly prepared calibrators.

2.8. Comparison with other methods

The previously used confirmation method for benzodiazepines at NIPH was a whole blood protein precipitation LC–MS method [17] run on a Waters ZQ MS instrument with a 2695 Alliance pump (Waters, Milford, MA, USA). Separation was performed with a Waters Symmetry C18-column or X-terra MS-column (2.1 × 150 mm, 3.5 μm) with gradient elution at a flow rate of 0.3 mL/min and a total cycle time of 16 min. Diazepam-d_5 was used as IS. 100 samples consisting of driving under the influence of drugs (DUI) cases, autopsy cases and external quality control samples were analysed on both methods, and the results were compared. In addition, during method validation of a new screening method introduced in the routine at NIPH in 2009 the results for the screening analysis and the confirmation method presented herein were compared [18].

3. Results and discussion

To our knowledge only three earlier papers describe the use of UPLC–MS/MS for benzodiazepine analysis in whole blood. Ishida et al. reported a validated quantitative screening method for 43 benzodiazepines, their metabolites, zolpidem and zopiclone in human plasma [19]. Extraction was done from 1 mL plasma by solid phase extraction. The method used scan mode for high dose benzodiazepines and selected ion recording mode for low dose benzodiazepines and had a total run time of 17 min using formic acid in water and acetonitrile as mobile phase. Diazepam-d_5 was used as IS for all the benzodiazepines. The injection volume was 5 μL. Validation data was given for plasma, but the method was also said to work well for whole blood samples.

Table 5
Compounds tested for evaluation of specificity.

Analyte	Blood concentration (mg/L)	Protonated molecular mass, MH+	Analyte	Blood concentration (mg/L)	Protonated molecular mass, MH+
7-Aminonitrazepam	0.2	252	Tramadol	2.6	264
7-Aminoclonazepam	0.4	286	Atenolol	1.6	267
7-Aminoflunitrazepam	0.08	284	Sotalol	1.6	273
Venlafaxine	1.4	278	Metoprolol	1.6	268
Doxepine	1.4	280	Propranolol	1.6	260
Fluvoxamine	0.8	319	Losartan	2.5	423
Mianserin	0.9	265	Flecainide	2.5	415
Amitriptyline	1.4	278	Bisoprolol	2	326
Valsartan	5	436	Carvedilol	2.4	407
Fluoxetine	1.5	310	Diltiazem	2.5	415
Clomipramine	1.6	315	Amlodipine	2.5	409
Moklobemide	5.4	264	Verapamil	2.7	455
Mirtazepine	0.5	266	Telmisartan	3	515
Citalopram	1.3	325	Irbesartan	5	429
Reboxetine	0.6	314	Morphine 3-glucuronide	11.5	462
Paroxetine	0.7	330	Morphine 6-glucuronide	1.2	462
Sertraline	0.6	307	6-MAM	1.6	328
Nortriptyline	1.1	264	Morphine	1.6	286
Trimipramine	1.2	295	Codeine	2.1	300
Amisulpride	0.7	370	Ethylmorphine	1.6	314
Haloperidol	0.06	376	Oxycodone	1.6	316
Perphenazine	0.06	405	Buprenorphine	0.02	468
Zuclopenthixol	0.2	402	Cocaine	0.2	304
Cis-flupentixol	0.1	435	Benzoylecgonine	1.4	290
Olanzapine	0.2	313	Diltiazem	0.4	415
Risperidon	0.3	411	Methamphetamine	3.7	150
Clozapine	0.9	327	MDMA	3.9	194
Gabapentin	17	172	MDA	3.6	180
Levomepromazine	0.3	329	MDEA	4.1	208
Alimemazine	0.2	299	Ephedrine	3.3	166
Pregabalin	159	160	Pseudoephedrine	4.0	166
Chlorprotixene	0.3	316	Norpseudoephedrine	3.8	152
Quetiapine	0.4	384	Cathinone	3.7	150
Theophylline	180	181	PMMA	1.2	180
Paracetamol	151	152	PMA	0.08	166
Salicylic acid	28	139	mCPP	1.2	197
Lamotrigine	5	257	MDPV	5.6	276
Phenobarbital	47	233	2C-B	0.05	260
Carbamazepine	24	237	Ethyl glucuronide	22.0	221
Phenytoin	50	252	Ethyl sulfate	7.0	125
Ketamine	6	238	Zolpidem	0.02	308
Pethidine	2.5	248	Zopiclone	0.03	389
Promethazine	1.4	285	Meprobamate	1.09	219
Dextropropoxyphene	1.7	340	Fentanyl	0.002	337
Ketobemidone	0.6	248	Methadone	0.1	310
Amphetamine	0.2	136	Methylphenidate	0.01	234
Hydroxyzine	0.7	375	Carisoprodol	1.3	261
Biperiden	0.6	312	Hydromorphone	0.01	286
Baclofen	1.1	214	THC	0.003	315
Duloxetine	0.3	298	Hydroxybupropion	0.3	256
Ziprasidone	0.2	413	Varenicline	0.2	212
Fenazon	18.8	189	Dexchlorpheniramine	0.1	275
10-OH-carbazepine	12.7	255	Aripiprazole	0.2	449
Norpropoxyphene	0.7	326	Prochlorperazine	0.07	374
Clomethiazole	0.3	162	9-OH-risperidone	0.2	427

Gunn et al. reported a method for detection and quantification of 12 benzodiazepines in serum or whole blood [20]. Protein precipitation of 250 μL serum was done with 1 mL cold acetonitrile mixed with ten deuterated IS. The method used two MRM transitions for each benzodiazepine and had a total run time of 7.5 min using formic acid in water and acetonitrile as mobile phase. The injection volume was 5 μL. Very little validation data was, however, presented.

Simonsen et al. [21] described a validated method for screening and quantification of 23 benzodiazepines and metabolites together with zopiclone and zaleplon in human whole blood. Extraction was done on 0.2 g whole blood using ethyl acetate at pH 9. The method used two MRM transitions for each benzodiazepine and had a total run time of 5.5 min. Ten deuterated benzodiazepines and zopiclone-d_8 were used as IS. The mobile phase consisted of a system with aqueous ammonia and methanol and the injection volume was 10 μL.

Our method has many similarities with the method of Simonsen et al. but represents a different selectivity due to the use of an acidic mobile phase system with acetonitrile, as compared to the basic mobile phase with methanol. According to the forensic toxicology guidelines of the American Academy of Forensic Sciences (AAFS) and the Society of Forensic Toxicologists (SOFT) screening and confirmation methods should, whenever possible, be based on different chemical principles in two independent extracts [22]. The very good specificity of mass spectrometry makes it the preferred technique of forensic toxicological analysis. Utilizing different pH and organic modifiers to give a difference in selectivity is one

important way of fulfilling the principles of the guidelines while still using the same mass spectrometric equipment. In addition we present full validation data for phenazepam, which is not presented by any of the other papers.

3.1. Method validation

A full validation was conducted during the original method development. In 2011 legislative limits for driving under the influence of non-alcohol drugs were recommended by an expert panel [23], to be introduced in the Norwegian legislative system from 2012. The benzodiazepines in the method, except bromazepam, lorazepam, midazolam and nordiazepam, were amongst the 20 non-alcoholic drugs with proposed blood drug concentration limits. For diazepam, flunitrazepam and nitrazepam the limits were fulfilled, but for alprazolam, clonazepam, oxazepam and phenazepam the limits were lower than the present cut-off values used at NIPH. A new full validation was therefore conducted to ensure that the method were in accordance with the new requirements.

Baseline separation of most of the compounds minimizes possible interferences between the compounds of the method itself. Some of the deuterated internal standards and the analytes have the same transitions, and care was taken to quantitate on transitions being unique for each analyte. Fig. 1 shows the chromatograms for a medium high standard.

3.1.1. Calibration curves

Calibration curves were made for each of the benzodiazepines in the concentration ranges listed in Table 3. Seven calibrators were prepared initially. In the case of bromazepam, clonazepam, lorazepam and phenazepam the signal of the lowest calibrator was of low intensity giving rise to a rather large variability for the calibration curves, and this calibrator was therefore excluded from the standard curves. The concentrations for the second lowest calibrator thus define the lower end of the calibration range for these compounds. The calibration range was large, and a weighted $(1/x)$ second-order regression line, excluding the origin, was found to fit the observed data points. Weighted $(1/x)$ residual plots and the squared correlation coefficients were evaluated. The residuals were found to spread randomly around zero for all the compounds except alprazolam, diazepam and nordiazepam, for which the residuals of the lowest calibrator was low compared to the other residuals. As the accuracy and bias for the QC sample in this area meets the criteria of ±20% this was deemed acceptable. The cut-offs used in routine applications is also ten times higher than this level. The resulting correlation coefficients for the whole blood calibrators (Table 3) were above 0.998 for all the compounds ($n = 10$).

3.1.2. Precision, accuracy, extraction recovery, limit of detection and quantification

The repeatability, intermediate precision, accuracy given as bias and extraction recovery are provided in Table 3. For bromazepam, clonazepam, lorazepam and phenazepam the lowest calibrator was excluded and the lowest QC sample thus fell outside the calibration range and is not included in table.

Repeatability and intermediate precision given as coefficient of variation (%) were calculated for 4–5 concentration levels. In all cases the repeatability was found to be ≤13% and the intermediate precision ≤15%, except for the lowest QC concentration of clonazepam for which both parameters where 19%, and the lowest QC concentration of flunitrazepam which had an intermediate precision of 19%. Bias for the QC samples varied between −19 and 21%. Recoveries of the analytes were from 71 to 96%.

The found LLOQs with corresponding precision and bias are presented together with the LODs in Table 4. The LLOQs varied from

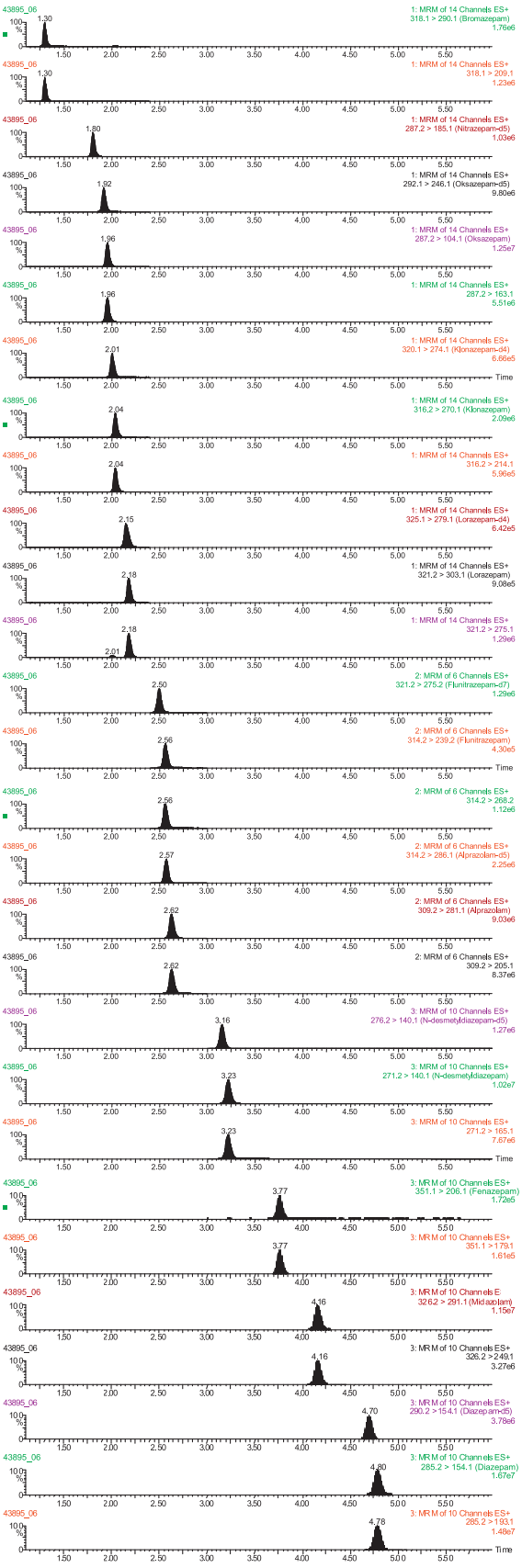

Fig. 1. Ion chromatograms for a medium high calibrator.

Table 6
Evaluation of matrix effects.

	Analyte	QC conc.	ME (%)	Relative ME CV %	ME corrected with IS (%)	Relative ME corr. with IS CV (%)
1	Alprazolam	0.015	100	1	100	1
		0.8	100	1	101	1
2	Bromazepam	0.075	99	3	101	3
		2.4	102	2	104	2
3	Clonazepam	0.006	94	8	96	7
		0.8	100	2	102	2
4	Diazepam	0.3	86	8	98	2
		9.6	93	3	102	2
5	Flunitrazepam	0.0075	98	4	100	4
		0.32	96	2	98	1
6	Lorazepam	0.045	99	5	100	5
		1.56	100	2	100	1
7	Midazolam	0.15	91	5	104	3
		1.6	94	4	103	2
8	Nitrazepam	0.075	98	3	100	2
		2.4	100	1	101	2
9	Nordiazepam	0.3	92	5	101	1
		10.4	94	3	100	1
10	Oxazepam	0.9	99	1	99	1
		16	100	1	99	1
11	Phenazepam	0.0075	95	9	97	10
		0.4	93	5	95	4

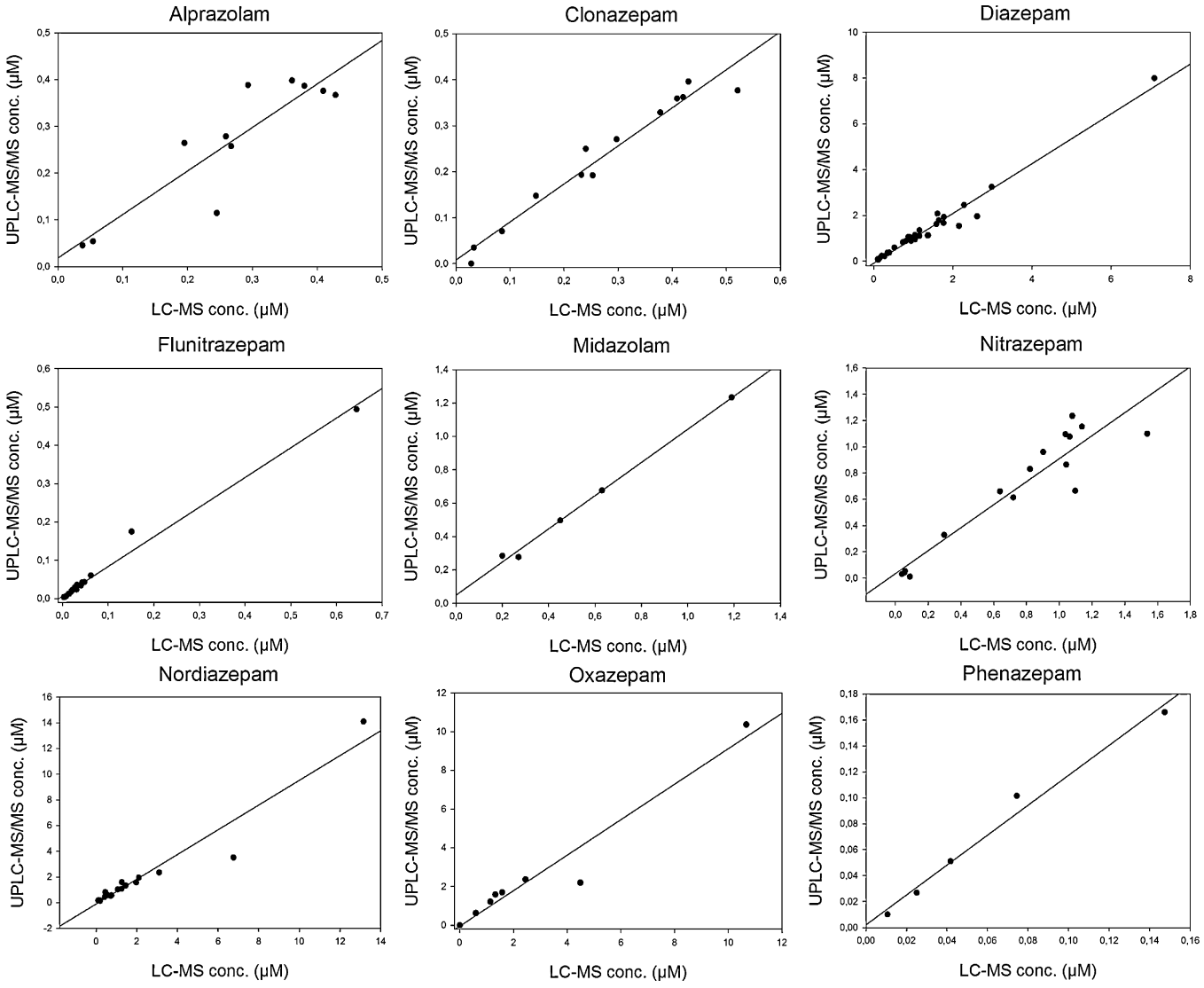

Fig. 2. Comparison between the UPLC–MS/MS method and the LC–MS method for the nine benzodiazepines common to the two methods.

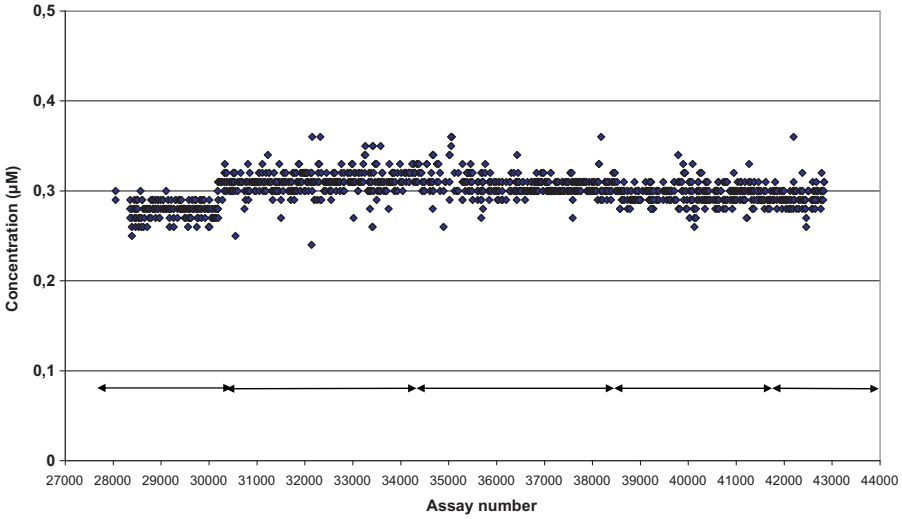

Fig. 3. Measured concentrations for a low diazepam QC sample (theoretical value 0.300 µM) from June 2007 until December 2010. The arrows show the start and end point of the five different QC batches used during this period.

0.0006 to 0.075 µM and the LODs from 0.005 to 3.0 nM. Table also provides upper reference concentration levels for therapeutic use.

3.1.3. Matrix effects and specificity

The data for the matrix effect studies are given in Table 6. Matrix effects were studied by post extraction addition and found to be between 86 and 102%. The relative matrix effects given as CVs (%) were between 1 and 9%. The matrix effects varied between 95 and 104% when calculated against an IS. Deuterated analogues used as IS will, at least to some extent, correct for matrix effects, as they will be affected in a similar fashion depending on the analyte and the deuterated analogue co-eluting. Retention time will vary with both the age of the column and from batch to batch and the investigation of matrix effects thus reflects the situation at the time of the experiments.

Two parallels of a low QC sample were in addition spiked with compounds commonly found in autopsy samples and compared to a neat QC sample of the same concentration. No significant differences in concentrations indicating ion suppression were found.

Of the 100 substances tested for interfering peaks (Table 5) no interfering peaks were found at or close by the retention times of the analytes for the MRM transitions in the method. This was also the case for extracted matrix blank samples or zero samples (blanks added IS) from 6 different blood bank blood lots and 6 autopsy samples.

3.1.4. Carry-over

No false-positive result due to carry-over was found from a sample fortified with a concentration 3 times higher than the highest QC sample. In addition, for routine samples two separately prepared aliquots are always run on two different assays and the mean value calculated for all the positive findings. If the highest concentration found for one of the benzodiazepines is more than 15% higher than the mean value from the two analyses, a new aliquot is analysed and the result compared with the previously obtained results. This further minimizes the risk of any carry-over going unnoticed. Even though the possibility of carry-over must always be kept in mind, as concentrations can be uncommonly high e.g. in autopsy cases

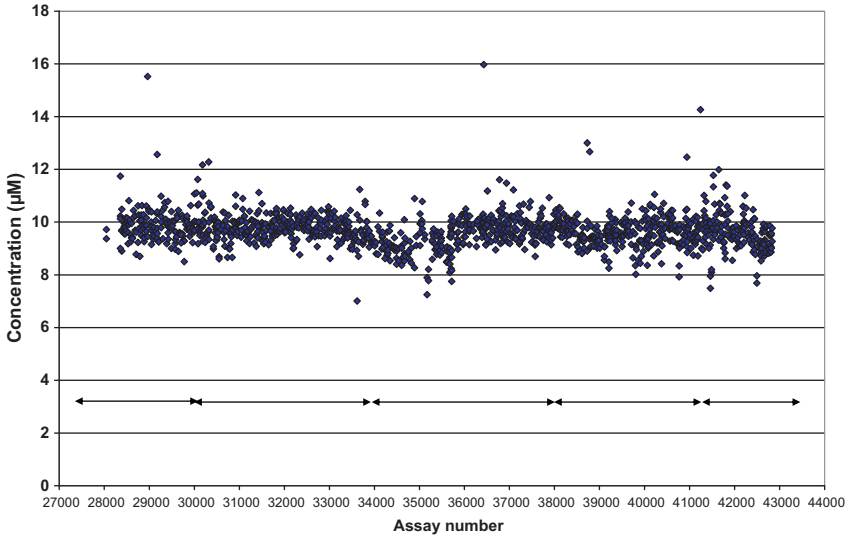

Fig. 4. Measured concentrations for a high diazepam QC sample (theoretical value 9.60 µM) from June 2007 until December 2010. The arrows show the start and end point of the five different QC batches used during this period.

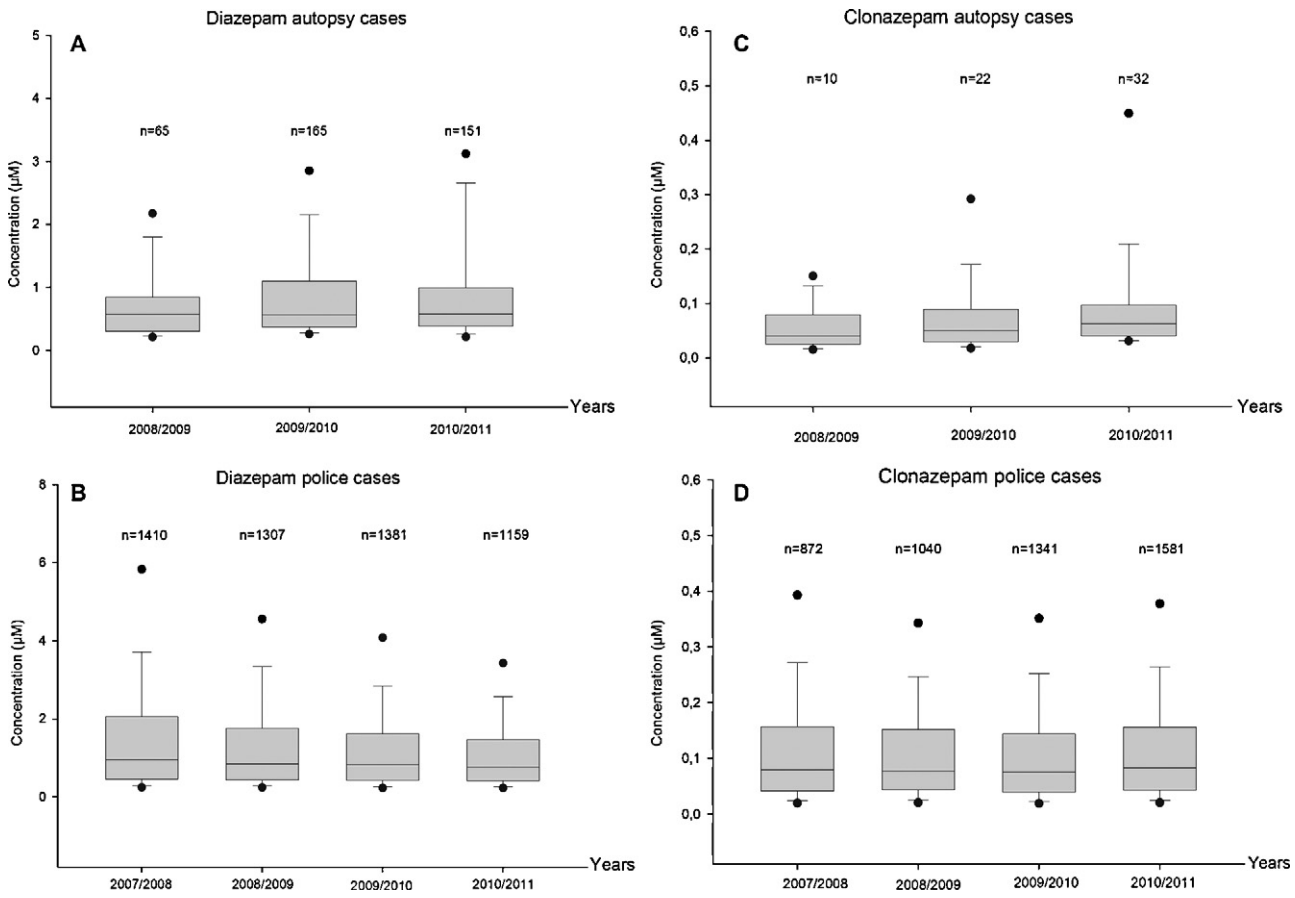

Fig. 5. Box-plot of the concentrations for diazepam and clonazepam in autopsy cases and police cases (DUI and violence cases). (A) Diazepam in autopsy cases, (B) diazepam in police cases, (C) clonazepam in autopsy cases, (D) clonazepam in police cases. As the routine analysis of autopsy samples started in 2009 only some of the autopsy samples found positive for diazepam and clonazepam at NIPH in the time period 2008/2009 were analysed by the method presented herein, which accounts for the low number of positive findings for this period. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. The 5th and 95th percentiles are marked with a black circle.

with death due to an overdose, this has so far not been observed as a problem in the routine use of the method.

3.1.5. Stability

QC samples prepared in whole blood and kept in a freezer at −20 °C were stable for up to 12 months. Extracted samples, standards and QC samples were stable for a week in a freezer at −20 °C. Samples should not be kept in an autosampler at 10 °C any longer than a week, as bromazepam then showed signs of degradation.

3.2. Method comparison

Method comparison was performed by analyzing 100 blood samples by the former LC–MS method and by the developed UPLC–MS/MS method. No false positive or negative results where found for the UPLC–MS/MS method when compared to the previous LC–MS method.

Fig. 2 compares results found with the UPLC–MS/MS method with results from the LC–MS method for the nine benzodiazepines that were determined in the old method. In general good correspondence was found for the concentrations examined, with R^2 values ranging from 0.88 to 0.98. For alprazolam and nitrazepam, the results show a larger variation than the other benzodiazepines. The LC–MS method made use of diazepam-d_5 as internal standard only, and the use of alprazolam-d_5 and nitrazepam-d_5 in the UPLC–MS/MS method is expected to correct better for possible interferences in the samples or variations in the experimental conditions.

The use of the UPLC–MS/MS method resulted in shorter runtimes, better separation, cleaner extracts, improved specificity and lower LLOQs compared to the LC–MS method. Deuterated IS are used for eight of the benzodiazepines, thus improving the robustness of the quantitative determination with respect to variation in experimental conditions and reducing possible effects of ion suppression.

The correspondence between our UPLC–MS/MS screening method published last year and the confirmation method presented herein was found to be good in the low and median range. Larger differences are found for the high concentration, due to nonlinear calibration curves for the screening method [18].

3.3. Performance of the method

A study of the long term precision of the method was undertaken for diazepam. Calculated concentrations for a low and a high QC sample were plotted from the time the method was implemented in routine use at NIPH in June 2007 and until December 2010 as shown in Figs. 3 and 4. The long term precision was found to be very good. For the high QC sample the mean value was found to be 9.69 ± 0.65 μM with a CV = 6.7% and bias = 0.9%. For the low QC sample the mean value was found to be 0.300 ± 0.015 μM with a CV = 5.1% and bias = 0.1%. During this period of time new calibrators

Table 7
Results from proficiency testing rounds.

Proficency testing round	Compound	Theoretical value (μM)	NIPH result (μM)	*Z*-Score
Nordquant 2009-1	Alprazolam	0.55	0.57	−0.24
	Clonazepam	0.44	0.42	−0.13
	Diazepam	1.09	1.12	−0.04
	Flunitrazepam	0.17	0.18	−0.54
	Midazolam	0.67	0.91	−1.07
	Nitrazepam	1.63	1.59	−0.08
	Oxazepam	1.34	1.22	−0.72
	Phenazepam	0.67	0.64	−0.87
Nordquant 2009-2	Alprazolam	0.21	0.19	0.00
	Clonazepam	0.23	0.23	0.10
	Diazepam	0.34	0.45	0.00
	Flunitrazepam	0.068	0.071	0.00
	Nitrazepam	0.84	0.78	−0.96
Nordscreen 2009-1	Diazepam	0.27	0.18	−0.72
	Nordiazepam	0.34	0.15	−0.72
Cardiff tox quant 2009-01	Diazepam	17.6	19.1	0.8
	Nordiazepam	7.42	7.9	0.7
	Oxazepam	8.74	8.4	−0.6
Cardiff tox quant 2009-04	Lorazepam	0.16	0.14	−1.5
Cardiff tox quant 2009-10	Diazepam	0.71	0.75	0.7
	Nordiazepam	1.11	1.07	−0.4
Nordquant 2010-1	Alprazolam	1.21	1.09	−1.01
	Clonazepam	1.13	1.03	−0.86
	Diazepam	4.15	4.07	−0.30
	Flunitrazepam	0.109	0.080	0.07
	Nitrazepam	0.19	0.18	0.00
Nordquant 2010-2	Alprazolam	0.49	0.46	−1.30
	Clonazepam	0.76	0.63	−0.42
	Diazepam	2.09	2.25	−1.05
	Flunitrazepam	0.059	0.051	−0.01
	Nitrazepam	0.59	0.54	0.00
Cardiff tox quant 2010-01	Alprazolam	0.270	0.295	0.46
Cardiff tox quant 2010-04	Alprazolam	0.097	0.118	−0.89
Cardiff tox quant 2010-07	Diazepam	5.27	4.71	−0.75
	Nordiazepam	1.93	1.97	0.08
Quartz 2010-41	Nordiazepam	1.98	1.99	−0.25

and QC samples were prepared several times. As far as possible the preparations were done independently with approximately 3–6 months in between, thus minimizing the possibility that any stability problems would go unnoticed due to concurrent changes in solutions made at the same period of time.

Fig. 5 presents box-plots of the concentrations for diazepam and clonazepam in autopsy cases and police cases (DUI and violence cases) analysed with the method. For both compounds the median value is quite stable for the whole period although the number of samples varies. As the routine analysis of autopsy samples started in 2009 only some of the autopsy samples found positive for diazepam and clonazepam at NIPH in the time period 2008/2009 were analysed by the method presented herein, which accounts for the low number of positive findings for this period in Fig. 5.

Our laboratory participates in several proficiency testing rounds each year, and benzodiazepines are often present in the external quality control samples we receive. Table 7 lists our results for benzodiazepines the last 2 years. All the benzodiazepines in the method were present in at least one external QC sample, with the exception of bromazepam. *Z*-Scores were calculated as the difference between our result and the consensus mean or median of the results of the participating laboratories and divided by the combined SD for our method and the inter-laboratory variation. The *Z*-Scores were $|Z| \leq 1.5$ for all the measurements, thus indicating good accuracy for the quantitative results obtained with the method.

3.4. Analytical findings

The UPLC–MS/MS method has been used for confirmation and quantitative analysis of benzodiazepines in whole blood samples at the NIPH since June 2007. The method has been used for investigations of suspected impairment – primarily in DUI-cases, and from 2009 also for analysis of autopsy samples. All together a total of 12,000–13,000 cases have been analysed by the method per June 2011.

The distribution of the positive findings of benzodiazepines in whole blood analysed in autopsy cases since July 2009 and in DUI-cases since 2007 is shown in Fig. 6. For the autopsy cases diazepam is by far the most frequently found benzodiazepine, followed by alprazolam, oxazepam and clonazepam. In 2009 diazepam was the third most frequently occurring drug (medicinal or illegal) found at NIPH in DUI cases and in 2010 the fourth. Clonazepam are also found increasingly often, and was the fifth most found drug in both 2009 and 2010, and often in high concentrations. On several occasions we have observed that a pronounced rise in the findings of one specific benzodiazepine during a year is seen in combination with increased concentrations of the same compound in the positive samples.

Phenazepam is not prescribed in Norway, and appears sporadically in our material, most often in samples being taken by the police in the south-western part of Norway. Lorazepam (not marketed in Norway, but prescribed in special cases) and bromazepam

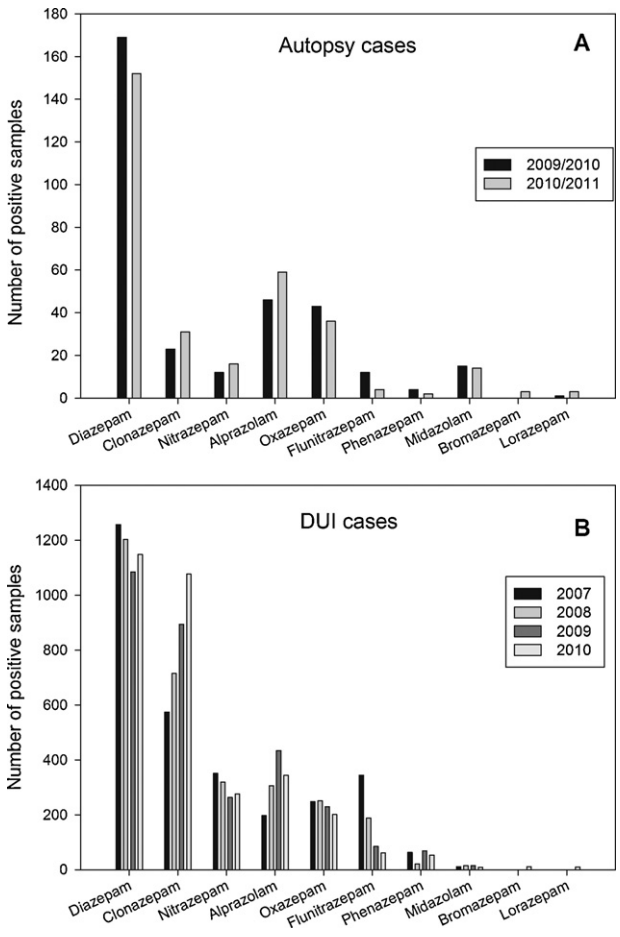

Fig. 6. Positive findings of benzodiazepines at NIPH from 2007 to 2010. Nordiazepam is not included in the figure. (A) Autopsy cases and (B) DUI cases.

(illegal use only) were included for routine screening during 2009. The metabolite of diazepam, nordiazepam, is omitted from the figure.

Altogether five benzodiazepines can be found on the top-ten list of findings in DUI cases at NIPH in 2010, with alprazolam, oxazepam and nitrazepam being the other three. In 2002 flunitrazepam was the benzodiazepine most often found. The change in findings of benzodiazepines at NIPH during the last 10 years correlates well with the number and kind of illegal tablets seized by the Norwegian police and customs, suggesting that many of the incidences correspond to illegal use. Increased availability of illegal benzodiazepines correlates both with increased occurrence as well as high concentrations above therapeutic levels in our material.

4. Conclusion

A fast, selective and robust UPLC–MS/MS method for the determination of alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam (metabolite of diazepam), oxazepam and phenazepam extracted from post-mortem and ante-mortem whole blood by solid-supported liquid–liquid extraction has been developed and validated. Deuterated IS are used for eight of the benzodiazepines, thus improving the robustness of the quantitative determination with respect to variation in experimental conditions and reducing possible effects of ion suppression.

The quantitative results obtained by the method have shown good accuracy for external quality samples in proficiency testing. Very importantly, the method meets all the requirements of the legislative limits to be introduced in the Norwegian legislative system from 2012 for driving under the influence of non-alcohol drugs.

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