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Quantitative analysis of 33 benzodiazepines, metabolites and benzodiazepine-like substances in whole blood by liquid chromatography–(tandem) mass spectrometry

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Abstract

A quantitative method using high-performance liquid chromatography–mass spectrometry (LC–MS, ion trap) after matrix supported liquid–liquid extraction is described for the simultaneous determination in whole blood of 33 benzodiazepines including metabolites and benzodiazepine-like substances. The limits of detection (LOD) range from 0.0001 to 0.0126 mg/l. Linearity is satisfactory for all compounds. The extraction recoveries for the benzodiazepines in whole blood are between 60 and 91%, desmethyldiazepam, OH-bromazepam and brotizolam excepted. Selectivity, accuracy and precision are satisfactory for clinical and forensic purposes.

Keywords: Benzodiazepines; Whole blood analysis; Drugs and driving

1. Introduction

Benzodiazepines are substances with a broad range of therapeutic uses; they have sedative-hypnotic, muscle relaxant, anxiolytic, anticonvulsant but also addictive effects [1,2]. Since the first benzodiazepines were introduced to the market in 1960, there has been an evolution in the development of these drugs toward compounds with less active metabolites, shorter action and faster clearance. There are more than twenty benzodiazepines in use in The Netherlands [3]. The benzodiazepines are relatively safe drugs with mild side effects. However, elderly patients are at increased risk of benzodiazepine induced psychomotor impairment (broken hips by falling [4]) and patients suffering a lung disease may be at increased risk of respiratory depression [5]. The use of benzodiazepines is also associated with an increased risk of a road-traffic accident [6]. Large doses are rarely fatal unless other drugs are taken concomitantly. Due to their wide variety of uses and their relative safety, benzodiazepines and benzodiazepine-like substances (e.g. zopiclone) are frequently used. Analysis of benzodiazepines, their active metabolites and benzodiazepine-like substances in blood samples may be indicated in a lot of forensic cases such as driving under the influence of drugs, cases of date-rape or violent crime and cases of unknown causes of death. In The Netherlands, autopsy cases excluded, samples are collected by physicians and shipped by mail to The Netherlands Forensic Institute in containers with sodium fluoride as preservative, which makes it impossible to obtain plasma or serum by centrifuging. As a consequence, the analytical methods must be suitable for the analysis of whole blood samples. The

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large number of possibly present benzodiazepines, metabolites and benzodiazepine-like substances must be considered. Screening methods involving high-performance liquid chromatography with diode-array detection (HPLC–DAD) or gas chromatography in combination with mass spectrometry (GC–MS) may often be not sensitive enough for forensic or clinical purposes [7].

Until now, we have used two analytical methods to analyse benzodiazepines, metabolites and benzodiazepine-like substances in blood. One method, using HPLC–DAD to determine a limited number of benzodiazepines with medium to high blood concentrations in the therapeutic range such as diazepam and oxazepam, and another method, using LC–MS to analyse benzodiazepines with low (therapeutic) blood concentrations like flunitrazepam. The aim of this study was to develop a new method that is suited for all available benzodiazepines with low limits of detection (LOD). In this article, we present a validated method that is capable of analysing 33 benzodiazepines, metabolites and benzodiazepine-like substances in whole blood samples by using LC in combination with alternate MS and MS–MS detection. The selection of the tested substances and metabolites is based on the availability in The Netherlands and the pharmacological activity [1,3].

2. Experimental

2.1. Chemicals and reagents

Chemicals were obtained from Alltech (State College, PA 16801 USA: norchlordiazepoxide), Aventis Pharma (Hoevelaken, The Netherlands: clobazam, desmethylclobazam, loprazolam), Boehringer Ingelheim (Alkmaar, The Netherlands: brotizolam), Bufa (Uitgeest, The Netherlands: diazepam, flunitrazepam, lorazepam, oxazepam, temazepam), Cerilliant (Austin TX, USA: alprazolam, hydroxy-alprazolam, bromazepam, aminoflunitrazepam, norflunitrazepam, flurazepam, desalkylflurazepam, lormetazepam, midazolam, hydroxy-midazolam, desmethyldiazepam, triazolam, hydroxy-triazolam), Hoffman-La Roche (acetamidonitrazepam, chlordiazepoxide, demoxepam, OH-ethylflurazepam, desmethylmedazepam), Lorex Synthelabo (Maidenhead, UK: zolpidem), Roche (Almere, The Netherlands: hydroxy-bromazepam, acetamidoclonazepam, aminoclonazepam) and Sigma–Aldrich Chemie B.V. (Zwijndrecht, The Netherlands: clonazepam, nitrazepam, zopiclone).

Stock solutions were prepared by dissolving 10 mg (accurately weighed) of the compound in 10 ml of methanol. These solutions were stored at −20 °C. Working standards were diluted in methanol. The concentrations of the working standards were approximately 0.1 mg/l methanol for all compounds.

The solution of the internal standard (methylbromazepam), was obtained from Chromsystems (Munchen, Germany). Solvents used were methanol (Rathburn, Brunschwig Chemie B.V., Amsterdam, The Netherlands, HPLC-grade), acetonitrile (Merck, VWR Amsterdam, The Netherlands, HPLC-grade Lichrosolv), ammonia solution 25% (m/v) (Merck, pro analyse), formic acid (Merck, pro analyse) and *t*-butylmethylether (Merck, Suprasolv). The ammonia solution (approximately 0.005 M) was made up by diluting 0.75 ml ammonia 25% (m/v) to 2000 ml with distilled water. The formic acid solution (approximately 0.006 M) was made up by diluting 0.45 ml formic acid 100% (v/v) to 2000 ml with distilled water.

ChemElut® cartridges (Varian, Middelburg, The Netherlands: pre-buffered, pH 9) were used for the sample preparation of whole blood.

Human blood was obtained after informed consent, from donors of the blood bank in Leiden and screened for the presence of benzodiazepines by HPLC–DAD and LC–MS(MS).

2.2. Sample preparation

Blood samples, spiked with all compounds and 50 μl of the internal standard solution, were applied onto the ChemElut® cartridges, followed by 1.5 ml water. The samples were eluted from the cartridges with 4 ml *t*-butylmethylether and after a pause of 20 min with another 2 × 4 ml *t*-butylmethylether. Evaporation of the eluate was performed by using an Automatic Environmental Speedvac AES2000 (Savant) with drying rate "high" (50 °C) for 35 min. The residue was redissolved in 100 μl methanol:water (40:60).

2.3. Equipment

The LC–MS system consisted of a TSP Spectra SYSTEM (Finnigan, Breda, The Netherlands), including a SN4000 controller, a vacuum degasser (SCM 1000), a pump (P4000) and an auto sampler (AS3000), connected to an ion trap mass spectrometer (LCQ, Finnigan).

In order to determine the influence of the interface on the sensitivity, standards (1000 mg/l diluted in acetonitrile:water = 10:90) were analyzed by using atmospheric pressure chemical ionization (APCI)–LC–MS as well as ESI–LC–MS. From all tested benzodiazepines, the signal-to-noise ratios from the different interfaces were compared. The interface used in the final procedure is atmospheric pressure chemical ionization.

For the analysis of benzodiazepines, conditions were optimized. Chromatographic data were acquired and processed using X-calibur™ 1.2 software (Finnigan).

2.4. Chromatography

In order to optimize the separation, two different column materials and four different solvents were tested. Eight column–solvent combinations were tested by analysing standards in methanol in order to achieve the best separation of the compounds. The columns tested were the Xterra MS C-18 (150 mm × 2.1 mm, 3.5 μm PS, Waters) and the

Xterra RP C-18 (150 mm × 2.1 mm, 3.5 μm PS, Waters). The injection volume was 50 μl. Chromatography was performed by using a flow-rate of 0.2 ml/min and a column temperature of 30 °C. The linear gradients used were acetonitrile/ammonia approximately 0.005 M (pH 10, 25–65% (v/v) acetonitrile), acetonitrile/formic acid approximately 0.006 M (pH 3, 25–60% (v/v) acetonitrile), methanol/ammonia approximately 0.005 M (pH 10, 45–90% (v/v) methanol) and methanol/formic acid approximately 0.006 M (pH 3, 30–60% (v/v) methanol).

The optimized method used a Xterra MS C-18 column (150 mm × 2.1 mm, 3.5 μm PS, Waters, Etten-Leur, The Netherlands). The gradient used for this LC–MS(MS) method is methanol/formic acid approximately 0.006 M (pH 3, 30–60% (v/v) methanol). The time intervals were t = 0–5 min (30–40% (v/v) methanol), t = 5–25 min (40–50% (v/v) methanol), t = 25–30 min (50–60% (v/v) methanol), t = 30–35 min (60% (v/v) methanol), t = 35–36 min (60–30% (v/v) methanol), t = 36–45 min (30% (v/v) methanol). The runtime was 45 min, including a re-equilibration time of 10 min.

2.5. Mass spectrometry

In order to detect benzodiazepines with low as well as high concentrations, the mass spectrometer acquires data alternately in MS (full scan) and MS–MS (product ion scan) mode. Daughter spectra of compounds analysed by MS–MS were obtained from the parent (molecular) ions or from a suitable fragment ion, by collisionally activated dissociation in the ion trap, using a collision energy of 30–50%, depending on the substance (see Section 3.2).

Due to restrictions of the equipment in MS–MS mode concerning the number of scans per segments, it was not possible to use the MS–MS mode for all tested substances. The MS–MS product ion scan mode was used for the benzodiazepines with low concentrations and for benzodiazepines that co-elute. The alternate detection mode reduces the sensitivity of the detection. Therefore, the sensitivity was optimised by dividing the chromatographic run into segments. The segments were chosen in such a way that each contained a small number of benzodiazepines at low concentrations. The choice of the segments allows for small fluctuations in the retention times.

The final aquisition mode used MS detection for 15 substances and MS–MS detection for 21 substances, including the internal standard. The compounds were identified by the presence of the characteristic ions, listed in Table 1.

2.6. Assay validation

Validation of the assay included the lower limit of detection (LOD), the lower limit of quantification (LOQ), extraction recovery, linearity, specificity, precision and accuracy. The limit of detection was estimated at a signal-to-noise ratio equal to three in spiked whole blood. The limit of quantification was calculated for a signal-to-noise ratio of 10. Extraction recovery, expressed as a percentage, was determined by assaying spiked blood samples 10 times at a concentration level of 1.0 mg/l. The extraction recovery was determined by comparing the peak heights of extracted blood samples with the peak heights of standards prepared in methanol. Linearity was estimated by assaying calibration curves consisting of a blank blood sample and 10 spiked blood samples (0.005; 0.01; 0.02; 0.05; 0.1; 0.2; 0.5; 1.0; 2.0; 5.0 mg/l). Linear regression has been used to estimate the slopes and the intercepts. Deviation from linearity was investigated by consecutively including an extra datapoint in the calibration curve and performing linear regression. Non linearity was concluded when the slope differed by more than 20%. Accuracy was estimated by analysing a quality control serum sample (n = 6), spiked with oxazepam, temazepam, nordazepam and diazepam, obtained from the KKGT (Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology, The Hague, The Netherlands). No quality control samples were available to estimate the accuracy of all the tested substances in whole blood. The concentrations of the analytes were calculated using calibration curves in whole blood. Intra-day precision was determined by the analysis of the quality control serum sample (n = 6) and spiked blood samples at three concentration levels (Table 4, n = 10). The precision was expressed as the relative standard deviation (RSD).

3. Results

3.1. Chromatography

Eight different column material–solvent combinations were tested to achieve the best separation. The methanol/formic acid gradient in combination with the Xterra MS C-18 150 mm × 2.1 mm, 3.5 μm PS resulted in the best separation and was selected. Fig. 1 shows the overlaid ion chromatograms of the tested benzodiazepines and benzodiazepine-like substances. A run of 45 min, including a re-equilibration time of 10 min was selected.

3.2. Mass spectrometry

Although the interface (ESI or APCI) was not optimal for each substance, satisfactory sensitivities were obtained for all compounds by using the APCI interface. For APCI, optimal conditions were found to be vaporizer temperature, 400 °C; discharge current, 4 μA; capillary voltage, 45.00 V; capillary temperature 150 °C. Table 1 shows the ratio S/N (using APCI): S/N (using ESI) ratios at a concentration of 10 mg/l using ESI and APCI sources and the parameters for the APCI–LC–MS(MS) method. For the compounds analysed by MS, retention time, MS molecular ion, MS fragment ions and scan range are presented. For the compounds analysed by MS–MS, retention time, MS molecular ion, MS–MS

Table 1
Parameters for the LC–MS(MS) method

Compound	Comparison of the sources ratio S/N (APCI):S/N (ESI)	Retention time (min)	MS molecular ion	Acquisition mode	MSMS precursor/parent ion	Collision energy (%)	MS or MS–MS product ions				Scan range (m/z)	MS–MS segment[a]
Aminoclonazepam	4.2	5.64	286	MS–MS	250	40	222	121			75–500	1
Acetamidonitrazepam	5.8	6.77	294	MS–MS	207	46	266	163			80–500	1 + 2
7-Aminoflunitrazepam	2.5	7.90	284	MS–MS	264	42	256	163			75–500	1 + 2
Flurazepam	0.4	9.91	388	MS–MS	315	48	317	288	143		105–500	1 + 2
Loprazolam	1.7	9.91	465	MS–MS	408	48	381	300			125–500	1 + 2
Acetamidoclonazepam	3.3	10.04	328	MS–MS	292	46	241	205			90–500	2
OH-midazolam	1.1	15.78	342	MS–MS	324	36	313	203			90–500	2 + 3
Bromazepam	2.7	17.16	316	MS–MS	288	38	261	209			85–500	3 + 4
OH-Bromazepam	1.5	18.33	332	MS–MS	314	32	286	275			90–500	3 + 4
Internal standard	n.d.	18.75	330	MS–MS	302	42	273	223	194		90–500	3 + 4
N-Desmethylflunitrazepam	4.8	19.15	300	MS–MS	254	42	272	214	176		80–500	4 + 5
Nitrazepam	3.4	20.88	282	MS–MS	236	42	254	208	176		75–500	5 + 6
Clonazepam	4.5	22.39	316	MS–MS	270	42	288	251	223	176	85–500	5 + 6
OH-triazolam	2.3	22.72	359	MS–MS	331	42	341	313	261		95–500	5 + 6
Flunitrazepam	5.4	22.96	314	MS–MS	268	42	286	193			85–500	5 + 6
α-OH-alprazolam	5.1	23.83	325	MS–MS	297	40	279	227	176		85–500	6
Alprazolam	3.3	27.16	309	MS–MS	281	42	274	206			85–500	7 + 8
OH-ethylflurazepam	3.9	27.44	333	MS	[b]	[b]	315	305	194		50–500	[b]
Triazolam	3.4	27.93	343	MS–MS	308	42	279	206	165		90–500	7 + 8
Lorazepam	6.3	28.07	321	MS–MS	303	30	335	275	166		85–500	7 + 8
Brotizolam	2.9	29.81	395	MS–MS	314	42	316	279	177		105–500	7 + 8
Desalkylflurazepam	3.9	29.95	289	MS	[b]	[b]	291	261	226	140	50–500	[b]
Lormetazepam	3.0	32.05	335	MS–MS	316	30	289	214	166		90–500	7 + 8
Zolpidem	2.0	5.35	308	MS	[b]	[b]	263	235			50–500	[b]
Desmethylmedazepam	1.6	9.46	257	MS	[b]	[b]	240	193			50–500	[b]
Norchlordiazepoxide	8.1	9.88	287	MS	[b]	[b]	271	257	237	223	50–500	[b]
Midazolam	2.7	10.57	326	MS	[b]	[b]	286	270	163		50–500	[b]
Chlordiazepoxide	0.4	12.49	300	MS	[b]	[b]	284	269	227		50–500	[b]
Demoxepam	14.7	18.31	287	MS	[b]	[b]	271	269	194		50–500	[b]
Zopiclone	60.5	17.47	277	MS	[b]	[b]	263	245	217		50–500	[b]
Desmethylclobazam	32.1	21.73	287	MS	[b]	[b]	245	219			50–500	[b]
Clobazam	34.4	25.04	301	MS	[b]	[b]	259	233	224		50–500	[b]
Oxazepam	3.4	27.45	287	MS	[b]	[b]	269	241	231	163	50–500	[b]
Temazepam	3.3	29.97	301	MS	[b]	[b]	283	255	228		50–500	[b]
Nordazepam	1.2	32.35	271	MS	[b]	[b]	243	208	140		50–500	[b]
Diazepam	0.6	34.82	285	MS	[b]	[b]	257	228	212		50–500	[b]

Note: Ketazolam was excluded from the tested substances, due to rapid in vitro decomposition to diazepam: n.d.: not determined.

[a] Time intervals of the segments: segment 1: 0.00–8.25 min; segment 2: 8.25–15.00 min; segment 3: 15.00–17.75 min; segment 4: 17.75–19.75 min; segment 5: 19.75–21.50 min; segment 6: 21.50–25.25 min; segment 7: 25.25–29.00 min; segment 8: 29.00–37.00 min; segment 9: 37.00–45 min.

[b] Analysis in MS mode.

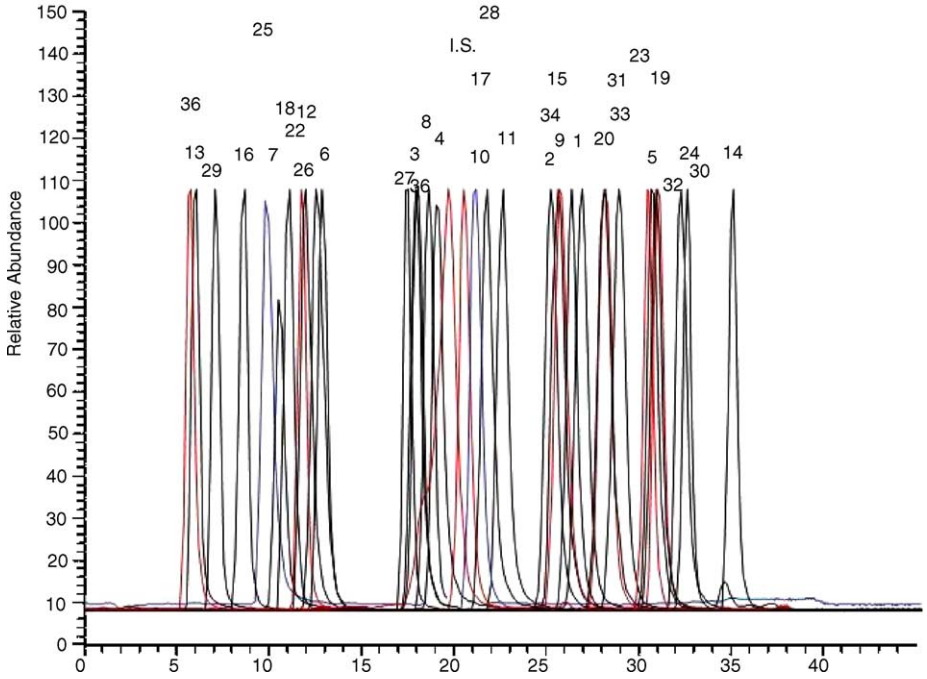

Fig. 1. Overlaid ion chromatograms of the tested benzodiazepines and benzodiazepine-like substances: (1) alprazolam; (2) α-OH-alprazolam; (3) bromazepam; (4) OH-bromazepam; (5) brotizolam; (6) chloordiazepoxide; (7) norchlordiazepoxide; (8) demoxepam; (9) clobazam; (10) desmethylclobazam; (11) clonazepam; (12) acetamidoclonazepam; (13) aminoclonazepam; (14) diazepam; (15) flunitrazepam; (16) 7-aminoflunitrazepam; (17) *N*-desmethylflunitrazepam; (18) flurazepam (19) desalkylflurazepam; (20) OH-ethylflurazepam; (21) internal standard; (22) loprazolam; (23) lorazepam; (24) lormetazepam; (25) desmethylmedazepam; (26) midazolam; (27) 1-OH-midazolam; (28) nitrazepam; (29) acetamidonitrazepam; (30) nordazepam; (31) oxazepam; (32) temazepam; (33) triazolam; (34) OH-triazolam; (35) zolpidem; (36) zopiclone.

mother ion and collision energy, MS–MS fragment ions and scan segments are shown.

3.3. Limit of detection, limit of quantification, linearity and recovery

The LOD, LOQ, linearity and recovery on whole blood samples are presented in Table 2. The LOD ranges from 0.0001 mg/l for triazolam to 0.0068 mg/l for OH-bromazepam, with the exception of norchlordiazepoxide (LOD 0.0126 mg/l). In general, the LODs of compounds detected by MS–MS are lower than the LODs of substances submitted to MS. Taking into account therapeutic and toxic concentrations, linearity was studied in the range 0–0.200 mg/l, 0–0.500 mg/l, 0–1.000 mg/l, 0–2.000 mg/l or 0–5.000 mg/l in whole blood. Table 2 shows the regression coefficients, the slopes and the intercepts of the calibration curves in the defined concentration ranges. Linearity was satisfactory for all compounds.

The extraction recoveries for the different benzodiazepines in whole blood were between 60 and 91%, with the exception of desmethylmedazepam (43%), OH-bromazepam (52%) and brotizolam (114%).

Results were calculated without correction for the recovery of the internal standard; the relative standard deviation in the peak height of the internal standard was 40% in whole blood, compared to 7% in methanol. As a result, calibration curves that used relative responses were worse than calibration curves that used absolute responses. The concentration of methylbromazepam in the solution is unknown; we therefore obtained relative low responses and a relative high variation in the peak height of this compound. The extraction recovery may be problematic. No experiments have been performed to optimize the recovery of the internal standard.

3.4. Specificity

Specificity is defined as the ability of the bioanalytical method to measure a substance unequivocally and to discriminate between the analyte(s) and other components, that may be present [8]. In order to screen for interfering substances, three replicate analyses of six different blank blood samples were analyzed by using the developed LC–MS(MS) method. No interfering substances were detected.

3.5. Accuracy and precision

Accuracy and precision of four benzodiazepines, were calculated from six replicate analyses of a quality control serum sample. The mean, accuracy and precision of oxazepam, temazepam, nordazepam and diazepam are presented in Table 3. Intra-day precision for all benzodiazepines was calculated from the results of 10 replicate analyses at

Table 2
Validation data on whole blood samples

Benzodiazepine	LOD (ng/ml)	LOQ (ng/ml)	$y = ax + b$ (R^2)	a, slope (l/mg)	b, intercept (a.u.)	Linearity range tested (mg/l)	Recovery	
							%	RSD%
Aminoclonazepam	0.6	2.1	0.99812	4.52E+07	−3.39E+06	0.002–5.0	62	15
Acetamidonitrazepam	0.4	1.4	0.99953	1.85E+07	−4.51E+05	0.001–5.0	68	14
7-Aminoflunitrazepam	0.8	2.6	0.99476	1.74E+07	−2.41E+05	0.003–5.0	62	17
Flurazepam	1.6	5.5	0.98042	2.34E+06	−3.41E+04	0.006–1.0	67	15
Loprazolam	0.4	1.4	0.99537	9.77E+06	1.72E+04	0.001–0.5	78	7
Acetamidoclonazepam	0.2	0.9	0.99737	1.71E+07	−1.73E+05	0.001–1.0	88	7
Bromazepam	1.3	4.3	0.99876	1.72E+07	−8.03E+05	0.004–5.0	84	15
OH-Bromazepam	6.8	22.8	0.99793	3.58E+07	−2.64E+05	0.023–2.0	52	26
N-Desmethylflunitrazepam	0.2	0.7	0.99867	3.21E+07	−1.82E+05	0.001–1.0	83	7
Nitrazepam	0.2	0.7	0.99979	2.03E+07	−9.33E+04	0.001–1.0	70	6
Clonazepam	0.3	0.9	0.99970	2.89E+07	−2.29E+04	0.001–1.0	72	6
OH-triazolam	0.2	0.6	0.99703	1.40E+07	−6.62E+03	0.001–2.0	87	6
Flunitrazepam	0.2	0.7	0.99932	2.71E+07	3.33E+04	0.001–1.0	75	6
α-OH-alprazolam	0.4	1.2	0.99924	3.20E+07	−2.27E+05	0.001–0.5	74	6
Alprazolam	0.4	1.3	0.99896	3.67E+07	−1.11E+05	0.001–0.5	88	6
OH-ethylflurazepam	1.8	5.9	0.99925	3.93E+07	−3.98E+04	0.006–0.5	86	2
Triazolam	0.1	0.4	0.99921	4.91E+07	−2.32E+05	0.0004–0.5	78	5
Lorazepam	0.2	0.6	0.99904	3.27E+07	−2.41E+03	0.001–1.0	84	2
Brotizolam	0.3	0.9	0.99906	2.19E+07	−9.13E+04	0.001–0.5	114	8
Desalkylflurazepam	0.9	2.9	0.99702	5.30E+07	−4.13E+05	0.003–2.0	82	7
Lormetazepam	0.6	1.8	0.99917	1.95E+07	−2.26E+05	0.002–5.0	87	8
Desmethylmedazepam	2.3	7.7	0.99477	2.19E+07	−1.51E+05	0.008–0.2	43	29
Norchlordiazepoxide	12.6	41.9	0.99846	3.46E+06	−3.67E+04	0.042–1.0	60	17
Midazolam	1.0	3.5	0.99607	6.86E+07	−7.62E+05	0.005–2.0	65	8
Chlordiazepoxide	1.7	5.8	0.99180	2.14E+07	−5.99E+05	0.006–2.0	61	11
Demoxepam	1.0	3.3	0.99888	2.99E+07	−1.88E+04	0.003–0.5	91	7
Zopiclone	4.6	15.3	0.99818	1.27E+07	−1.92E+05	0.015–1.0	88	11
Desmethylclobazam	2.9	9.6	0.99954	2.52E+07	−3.32E+04	0.010–0.5	82	6
Clobazam	0.6	2.0	0.99948	1.06E+08	−2.89E+03	0.002–1.0	82	4
Oxazepam	2.2	7.7	0.99952	3.02E+07	−3.32E+04	0.008–1.0	83	5
Temazepam	1.6	5.2	0.99974	3.70E+07	−2.97E+05	0.005–2.0	81	6
Nordazepam	4.3	14.3	0.99871	6.01E+07	−2.38E+05	0.014–1.0	76	5
Diazepam	4.0	13.3	0.99898	7.23E+07	−1.52E+04	0.013–1.0	73	4

LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation. *Note* 1: OH-midazolam was not determined due to insufficient substance available. *Note* 2: zolpidem was excluded because the results were not satisfactory.

three concentration levels. Table 4 shows the intra-day precision for all compounds.

4. Discussion

In literature, several methods for the analysis of benzodiazepines are described, generally consisting of GC–MS methods as well as methods using HPLC–DAD [9–12]. However, high-performance liquid chromatography with mass spectrometric detection is becoming increasingly the method of choice for simultaneous quantification and confirmation of many polar and/or chemically instable drugs, including benzodiazepines [13–17]. The analysis of benzodiazepines can be performed by using APCI–LC–MS [7,18,19] as well as electro spray ionization (ESI)–LC–MS [20,21].

The LC–MS(MS) method described in this article allows us to analyze 33 benzodiazepines, metabolites and benzodiazepine-like substances in whole blood and to reach detection limits lower than those observed with conventional LC–DAD or GC–MS methods. Low dose as well as high dose benzodiazepines can be determined in one run. The results of

Table 3
Precision and accuracy

Compound	Quality control sample KKGT 2003, The Netherlands concentration in serum (mg/l)	Analytical results mean, $n = 6$ (mg/l)	Accuracy % deviation	Precision intra-day RSD (%)
Oxazepam	0.5063	0.4720	−3.4	3.5
Temazepam	0.1044	0.1067	2.2	5.5
Nordazepam	0.2107	0.2298	9.1	3.6
Diazepam	0.3445	0.4139	20.1	5.6

Table 4
Precision of benzodiazepines in whole blood samples

Benzodiazepine	0.01 mg/l (RSD%)	0.1 mg/l (RSD%)	1 mg/l (RSD%)
Aminoclonazepam	0	8	10
Acetamidonitrazepam	3	22	10
7-Aminoflunitrazepam	3	18	12
Flurazepam	7	23	10
Loprazolam	35	12	5
Acetamidoclonazepam	3	15	5
OH-midazolam	n.d.	n.d.	n.d.
Bromazepam	3	7	10
OH-Bromazepam	17	30	18
N-Desmethylflunitrazepam	5	10	5
Nitrazepam	5	17	4
Clonazepam	8	13	5
OH-triazolam	9	15	4
Flunitrazepam	11	11	5
α-OH-alprazolam	4	14	4
Alprazolam	9	15	4
OH-ethylflurazepam	10	11	2
Triazolam	4	14	4
Lorazepam	4	14	2
Brotizolam	9	13	6
Desalkylflurazepam	6	11	5
Lormetazepam	4	13	6
Desmethylmedazepam	11	35	20
Norchlordiazepoxide	8	14	12
Midazolam	3	11	5
Chlordiazepoxide	3	14	8
Demoxepam	11	16	5
Zopiclone	8	12	8
Desmethylclobazam	8	13	4
Clobazam	8	12	3
Oxazepam	14	14	3
Temazepam	5	16	4
Nordazepam	8	10	3
Diazepam	16	11	3

RSD: relative standard deviation: n.d.: not determined due to insufficient substance available.

this study show the advantages of LC–MS(MS): higher specificity, lower detection limits, simultaneous measurement of many substances as well as satisfactory validation characteristics. The alternation of MS and MS–MS detection allows us to measure high and low blood levels of different substances simultaneously; no interference has been noticed.

Extraction was performed with pre-buffered columns at pH 9. This is in agreement with other methods, where high recoveries were found by adjusting the pH to alkaline before extraction [7]. The extraction procedure we use allows us to reach lower limits of detection by concentrating the compounds by a factor 10. The low limits of detection make this method suitable for the analysis of benzodiazepines in whole blood as well as in other biological fluids such as oral fluid. This may be an advantage in research projects concerning road-side drug testing.

In other methods described, the pH of the mobile phase varied from acidic to slightly alkaline [7,11,15]. In our protocol, best separation was achieved by using a solution at pH 3.

The range of linearity of most benzodiazepines was satisfactory, with respect to the therapeutic range for forensic and clinical purposes. In case of (suspected) concentrations higher than the range of linearity, repeated analysis is required after dilution. For most compounds, extraction recoveries were more than 60%.

For some benzodiazepines and metabolites, extraction recoveries were relatively low. For those substances, adding a suitable internal standard or optimizing the extraction procedure may improve the results. However, an internal standard for each benzodiazepine, metabolite or benzodiazepine-like substance, is impractical and sometimes not available. For compounds having a relatively low recovery or higher relative standard deviation, our method gives semi-quantitative results. Accuracy has been determined for oxazepam, temazepam, nordazepam and diazepam and was found to be satisfactory. A variation of 20% is generally considered as acceptable [22]. Results may be biased by matrix effects since the quality control sample was drug spiked serum, whereas results were calculated on calibration curves in whole blood samples. Intra-day precision of the benzodiazepines in the quality control sample and in the spiked whole blood samples was sufficient for forensic purposes [22]. Unfortunately, quality control blood samples were not available for all tested substances.

Inter-day precision was not determined: in forensic case work, calibration curves are included in each analysis.

5. Conclusions

This sensitive and selective method offers the opportunity for simultaneous screening and quantification of almost all benzodiazepines and benzodiazepine-like substances, which are available in The Netherlands and that are relevant in clinical and forensic cases. Low dose as well as high dose benzodiazepines can be measured simultaneously, with low detection limits, low limits of quantification and satisfactory validation characteristics.

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