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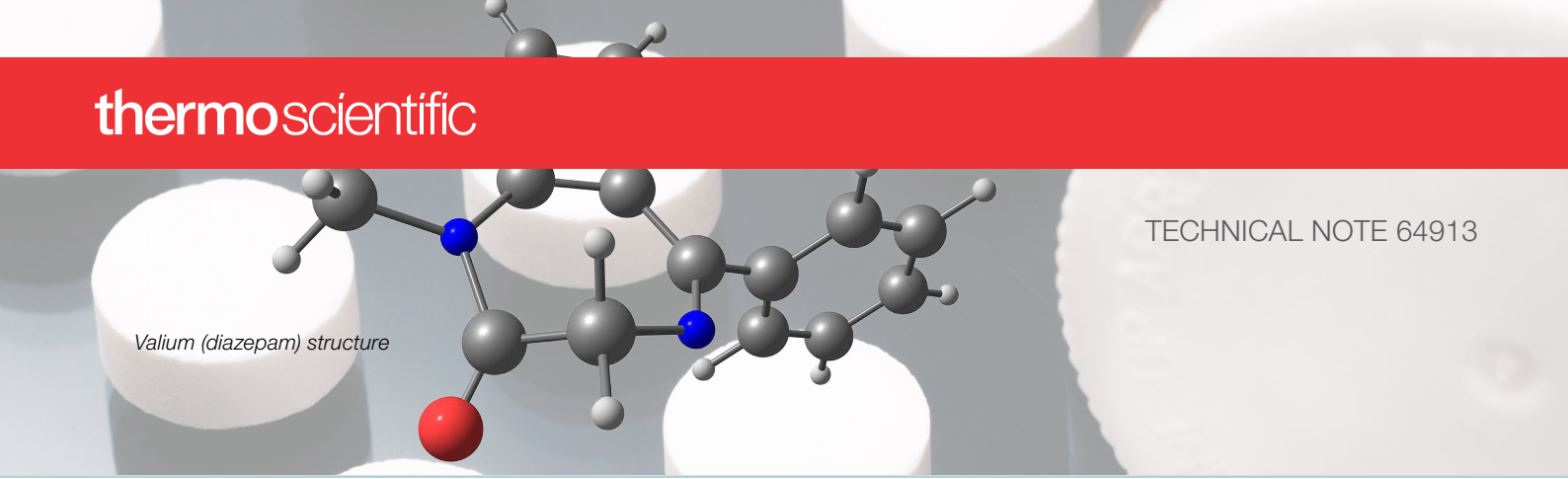

Valium (diazepam) structure

TECHNICAL NOTE 64913

Offline and online sample extraction for the quantification of benzodiazepines in human plasma or serum for clinical research

Authors

Claudio De Nardi,[1] Katharina Kern,[2] Steffen Peters[2]

[1]Thermo Fisher Scientific GmbH, Dreieich, Germany
[2]RECIPE Chemicals + Instruments GmbH, Munich, Germany

Keywords

Benzodiazepines, direct injection, offline sample preparation, plasma, serum, mass spectrometry

Application benefits

- Flexibility in sample preparation: direct injection of plasma or serum or offline sample preparation for improved sensitivity
- Analysis and quantitation of 34 benzodiazepines in a single quantitative method

Goal

Development of a robust analytical method for the quantification of 34 different benzodiazepines in human plasma or serum on a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer.

Introduction

An analytical method for clinical research for the quantification of 34 benzodiazepines (Table 1) in human plasma or serum is reported. Twenty deuterated internal standards were used for the quantification of target analytes. Two different sample preparation steps were available: automated addition of the internal standards followed by direct injection of the unextracted plasma or serum sample for minimum human intervention, or offline internal standard addition and protein precipitation for improved sensitivity. In both cases, samples were injected onto a Thermo Scientific™ Transcend™ II TLX-1 system coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with heated electrospray ionization and detection was performed by selected-reaction monitoring (SRM).

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Table 1. Concentration ranges covered by calibrators.

Analyte	Concentration Range (ng/mL)
7-Aminoclonazepam	5.17–78.3
7-Aminoflunitrazepam	5.24–80.2
7-Aminonitrazepam	22.1–332
α-OH-Alprazolam	5.34–83.8
α-OH-Midazolam	21.5–310
α-OH-Triazolam	4.62–65.5
Alprazolam	5.25–82.7
Bromazepam	31.5–456
Chlordiazepoxide	223–2970
Clobazam	47.7–716
Clonazepam	5.06–73.4
Demoxepam	216–3290
Desalkylflurazepam	9.67–151
Desmethylflunitrazepam	4.54–74.2
Diazepam	102–1370
Estazolam	42.8–643
Flunitrazepam	5.23–79.0
Flurazepam	8.54–128
Lorazepam	20.6–297
Lormetazepam	1.82–27.3
Medazepam	42.8–604
Midazolam	31.0–459
Nitrazepam	21.0–311
Norclobazam	266–4040
Nordiazepam	83.3–1170
Oxazepam	125–1860
Prazepam	83.3–1220
Temazepam	43.3–636
Tetrazepam	40.9–613
Trazodon	165–2520
Triazolam	4.02–60.3
Zaleplon	8.37–131
Zolpidem	42.4–684
Zopiclone	4.90–70.0

Method performance was evaluated using the MS9050 ClinMass® TDM Platform on-line with the MS9550 ClinMass Add-On Set for Benzodiazepines (for direct injection) and the MS9000 ClinMass TDM Platform with the MS9500 ClinMass Add-On Set for Benzodiazepines (for offline sample preparation), both from RECIPE, to obtain limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

The analytes and corresponding concentration ranges covered by the calibrators used are reported in Table 1.

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as 20 deuterated internal standards for the quantification.

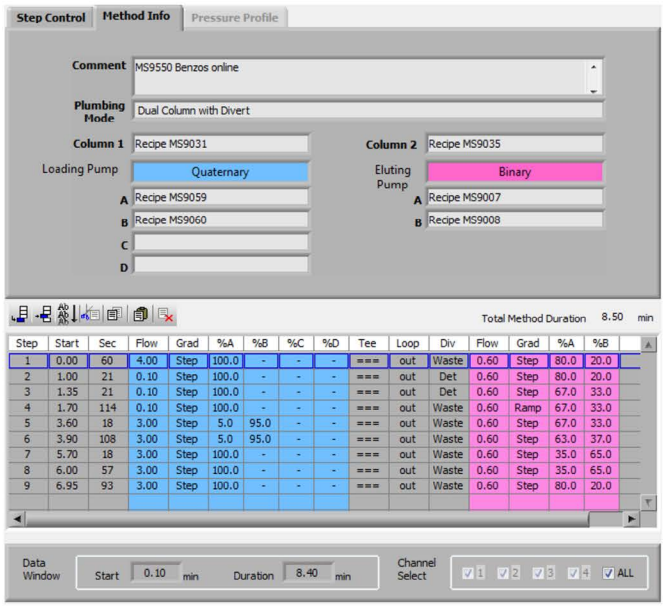
Direct injection approach: No manual sample preparation was required. The autosampler of the Transcend II TLX-1 system was used for the automated addition of the internal standards prior to injection of the unextracted plasma or serum sample onto the LC system for online SPE.

Offline protein precipitation approach: Samples of 50 µL of plasma or serum were protein precipitated using 100 µL of precipitating solution containing the internal standards. Precipitated samples were vortex-mixed and centrifuged and the supernatant was transferred to a clean plate or vial.

Liquid chromatography

The LC separation was achieved using mobile phases, an SPE cartridge (for direct injection only), and an analytical column provided by RECIPE. Details of the analytical methods for both approaches are reported in Figure 1. Total runtime was 8.5 for direct injection and 7.5 minutes for offline protein precipitation.

A



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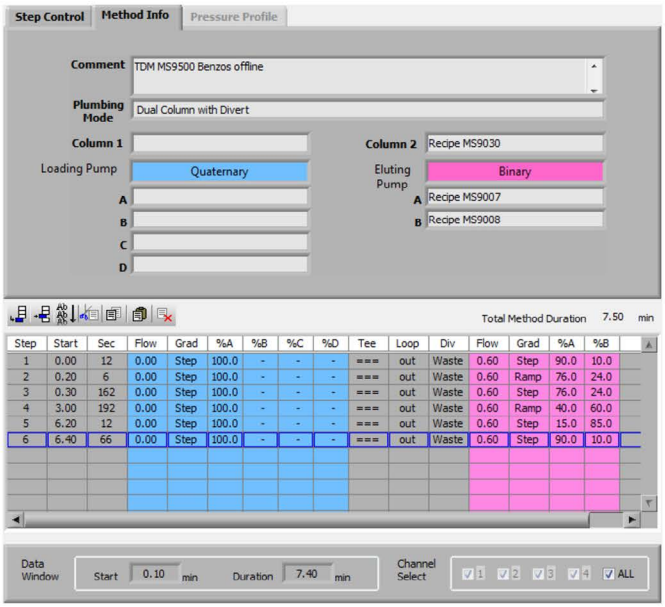

Figure 1. LC method description for (a) direct injection and (b) offline protein precipitation.

Mass spectrometry

Analytes and internal standards were detected by SRM on a TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. Two SRM transitions for each analyte are included in the acquisition method for quantification and confirmation, respectively.

Method evaluation

The method performance was evaluated by limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision for each analyte. Analytical accuracy was evaluated for 13 analytes in terms of percentage bias between nominal and average back-calculated concentrations using the quality control samples BZF #1/15A and BZF #1/15B from Arvecon GmbH prepared and analyzed on five different days in a single run each day. Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the controls from RECIPE at two different levels in replicates of eight (n=8) prepared and analyzed in one batch. Inter-assay precision was evaluated on the same controls in replicates of three (n=3) prepared and analyzed on five different days.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 3.3 software.

Results and discussion

The method proved to be linear not only in the calibration ranges covered by the calibrators but also in wider ranges obtained by diluting the lowest calibrator up to 20-fold. The obtained linearity ranges are reported in Table 2 for both approaches. Representative chromatograms for the lowest calibrator for 7-aminoflunitrazepam, midazolam, and their corresponding calibration curves are reported (Figures 2 and 3).

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -3.1% and 15.6% for direct injection and between -7.3% and 12.4% for offline sample preparation (Table 3).

The %CV for intra-assay precision was less than 14.0% for all the analytes excluding zoplicone (Table 4). The maximum %CV for inter-assay precision including all the analytes was 12.6% excluding zoplicone (Table 5). In both cases, zoplicone exhibited some stability issues in the extracted samples, thereby affecting the precision results.

Table 2. Linearity ranges for both direct injection and offline protein precipitation approach.

	Concentration Range (ng/mL)	
Analyte	**Direct Injection**	**Offline Protein Precipitation**
7-Aminoclonazepam	1.72–78.3	1.72–78.3
7-Aminoflunitrazepam	0.52–80.2	0.52–80.2
7-Aminonitrazepam	4.42–332	1.17–332
α-OH-Alprazolam	1.78–83.8	1.78–83.8
α-OH-Midazolam	2.15–310	2.15–310
α-OH-Triazolam	1.54–65.5	1.54–65.5
Alprazolam	0.530–82.7	1.75–82.7
Bromazepam	10.5–456	3.15–456
Chlordiazepoxide	22.3–2970	11.2–2970
Clobazam	4.77–716	2.39–716
Clonazepam	1.69–73.4	1.69–73.4
Demoxepam	21.6– 290	10.8–3290
Desalkylflurazepam	0.970–151	3.22–151
Desmethylflunitrazepam	1.51–74.2	1.51–74.2
Diazepam	10.2–1370	5.10–1370
Estazolam	4.28–643	2.14–643
Flunitrazepam	0.520–79.0	1.74–79.0
Flurazepam	0.854–128	0.427–128
Lorazepam	2.06–297	6.87–297
Lormetazepam	0.182–27.3	0.182–27.3
Medazepam	4.28–604	2.14–604
Midazolam	3.10–459	1.55–459
Nitrazepam	2.10–311	2.10–311
Norclobazam	88.7–4040	88.7–4040
Nordiazepam	8.33–1170	8.33–1170
Oxazepam	12.5–1860	6.25–1860
Prazepam	8.33–1220	4.17–1220
Temazepam	4.33–636	2.17–636
Tetrazepam	4.09–613	2.05–613
Trazodon	16.5–2520	8.25–2520
Triazolam	0.40–60.3	1.34–60.3
Zaleplon	8.37–131	0.837–131
Zolpidem	4.24–684	4.24–684
Zopiclone	1.63–70.0	1.63–70.0

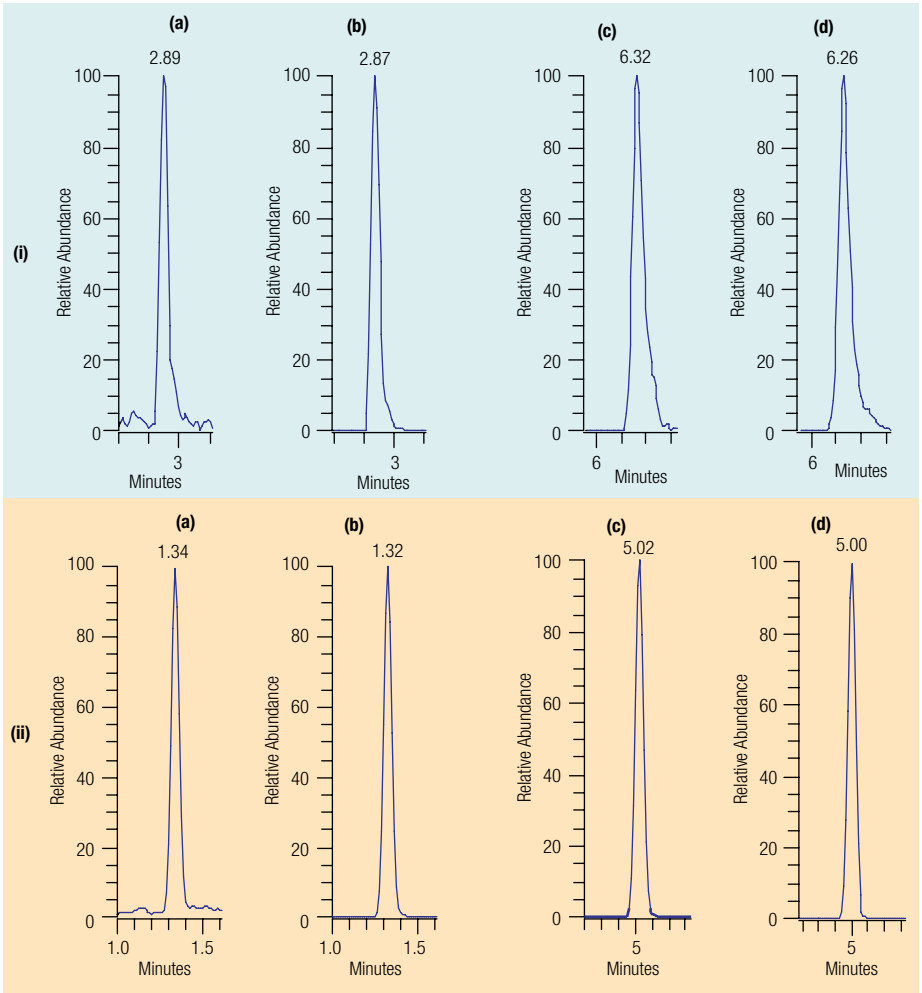

Figure 2. Representative chromatograms for the lowest calibrator for (a) 7-aminoflunitrazepam, (b) d7-7-aminoflunitrazepam, (c) midazolan and (d) d4-midazolam for (I) direct injection and (II) offline sample preparation.

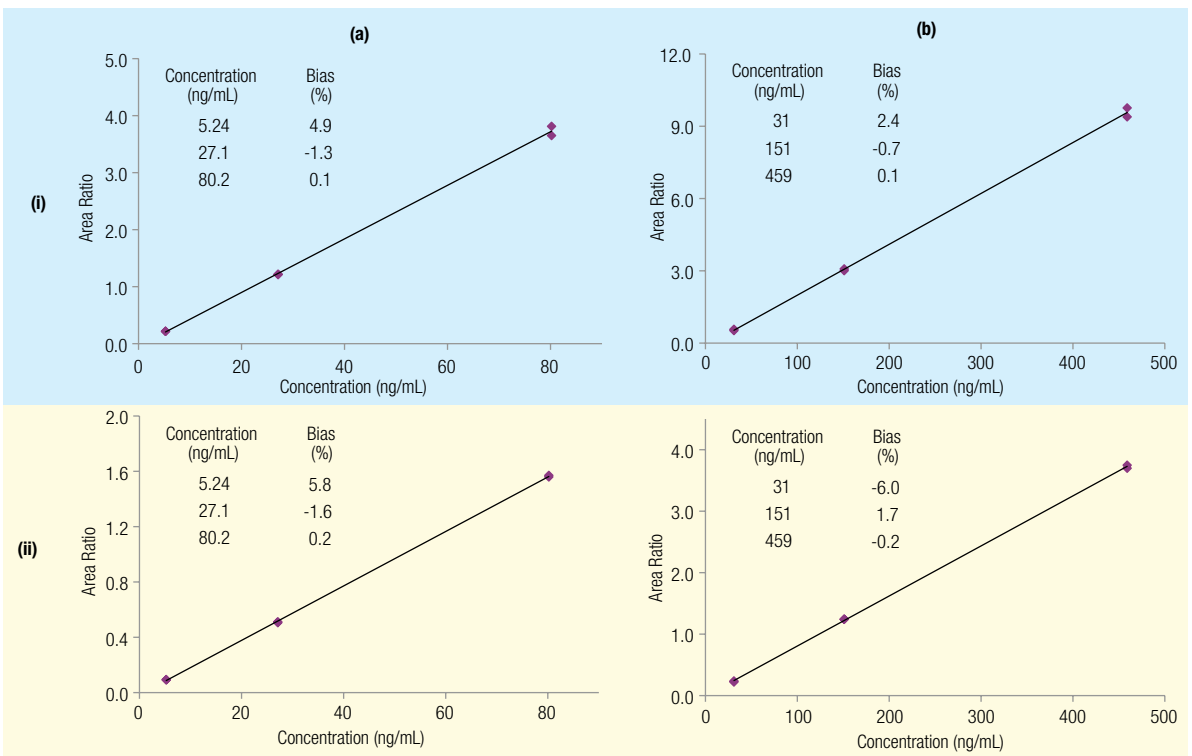

Figure 3. Representative calibration curves for (a) 7-aminoflunitrazepam and (b) midazolan for (I) direct injection and (II) offline sample preparation.

Table 3. Analytical accuracy results (n=5).

Analyte	Control	Nominal Conc. (ng/mL)	Direct Injection		Offline Protein Precipitation	
			Measured Conc. (ng/mL)	Bias (%)	Measured Conc. (ng/mL)	Bias (%)
7-aminoflunitrazepam	1/15 B	18.3	19.5	6.4	19.3	5.5
Alprazolam	1/15 A	34.4	39.8	15.6	38.7	12.4
Bromazepam	1/15 A	141	157	11.3	147	4.1
Clonazepam	1/15 A	56.1	54.3	-3.1	58.7	4.6
Desmethylflunitrazepam	1/15 B	12.4	13.3	6.9	11.5	-7.3
Diazepam	1/15 A	449	445	-0.9	441	-1.7
Flunitrazepam	1/15 B	7.00	6.81	-2.7	7.20	2.9
Lorazepam	1/15 B	94.3	99.0	4.8	102	8.6
Midazolam	1/15 B	136	144	5.7	139	2.2
Nordiazepam	1/15 A	384	408	6.3	421	7.3
Oxazepam	1/15 B	163	184	12.8	174	6.5
Temazepam	1/15 A	153	171	11.7	168	9.6
Zolpidem	1/15 B	151	162	7.1	164	8.7

Table 4. Intra-assay precision results (n=8).

Analyte	Direct Injection				Offline Protein Precipitation			
	MS6080 #434		MS6081 #434		MS6080 #434		MS6081 #434	
	Average Conc. (ng/mL)	CV (%)	Average Conc. (ng/mL)	CV (%)	Average Conc. (ng/mL)	CV (%)	Average Conc. (ng/mL)	CV (%)
7-Aminoclonazepam	13.5	7.4	44.1	8.5	14.7	8.0	49.7	3.3
7-Aminoflunitrazepam	16.2	1.2	51.9	2.4	16.3	2.6	52.9	3.3
7-Aminonitrazepam	76.2	12.7	227	4.9	78.2	5.1	261	3.1
α-OH-Alprazolam	16.5	11.9	57.8	8.4	17.3	8.5	53.8	3.2
α-OH-Midazolam	70.3	7.3	235	5.3	70.9	3.5	230	2.7
α-OH-Triazolam	13.2	11.4	42.7	9.4	13.6	9.6	43.0	5.2
Alprazolam	16.2	3.2	55.5	4.2	15.3	6.6	54.0	5.3
Bromazepam	96.6	8.1	312	10.7	92.1	4.8	319	4.5
Chlordiazepoxide	642	1.7	2133	1.6	656	3.5	2067	2.9
Clobazam	150	5.7	506	6.7	143	2.8	471	2.3
Clonazepam	15.6	9.4	52.0	6.5	15.1	8.5	50.9	4.3
Demoxepam	722	10.0	2386	7.6	679	3.4	2251	3.3
Desalkylflurazepam	31.5	9.3	104	7.1	32.1	4.3	104.9	3.2
Desmethylflunitrazepam	15.1	11.3	49.7	8.8	15.5	6.6	49.9	8.6
Diazepam	270	3.2	897	3.5	283	2.0	913	1.8
Estazolam	131	2.0	439	1.6	135	2.5	445	3.3
Flunitrazepam	15.7	6.8	52.4	3.6	17.1	5.9	55.5	5.5
Flurazepam	25.9	12.9	84.5	14.0	25.7	2.2	83.1	2.3
Lorazepam	60.9	6.5	198	6.8	62.3	5.3	198	2.8
Lormetazepam	7.15	10.8	23.3	5.6	6.92	5.6	22.0	3.0
Medazepam	119	6.6	390	5.8	113.7	2.0	377	2.7
Midazolam	91.0	4.5	309	4.0	87.9	2.6	292	2.5
Nitrazepam	60.8	8.7	200	8.9	68.7	2.3	220	4.6
Norclobazam	849	7.4	2930	6.6	831	4.3	2803	3.8
Nordiazepam	247	2.5	803	2.8	244	2.5	795	1.9
Oxazepam	374	2.5	1244	2.7	377	1.2	1248	2.4
Prazepam	260	1.7	836	1.9	263	1.8	829	2.5
Temazepam	135	3.8	430	2.6	131	2.0	425	2.4
Tetrazepam	125	11.2	429	10.9	122	2.2	408	2.9
Trazodon	539	7.9	1696	8.7	520	4.0	1661	2.7
Triazolam	11.9	62	40.8	5.2	11.7	7.6	42.1	3.4
Zaleplon	28.1	9.5	90.0	7.5	26.6	6.5	86.8	4.9
Zolpidem	128	2.6	448	3.3	135	2.5	447	2.3
Zopiclone	17.3	15.2	55.5	15.1	15.5	14.5	53.6	3.8

Table 5. Inter-assay precision results (n=15).

Analyte	Direct Injection				Offline Protein Precipitation			
	MS6080 #434		MS6081 #434		MS6080 #434		MS6081 #434	
	Average Conc. (ng/mL)	CV (%)	Average Conc. (ng/mL)	CV (%)	Average Conc. (ng/mL)	CV (%)	Average Conc. (ng/mL)	CV (%)
7-Aminoclonazepam	14.8	7.3	47.6	11.3	14.9	5.7	47.5	4.3
7-Aminoflunitrazepam	15.8	3.4	52.9	5.2	15.9	3.7	52.6	2.4
7-Aminonitrazepam	73.5	8.8	260.7	9.2	75.2	4.1	249	3.1
α-OH-Alprazolam	16.2	12.6	53.2	9.0	17.3	10.6	54.4	5.9
α-OH-Midazolam	69.7	5.9	224.6	3.5	67.9	2.9	219	4.1
α-OH-Triazolam	14.7	11.1	44.9	9.7	13.7	8.4	44.1	4.1
Alprazolam	16.6	5.8	55.1	5.1	16.4	7.6	54.1	4.0
Bromazepam	93.0	7.5	282.0	5.6	96.3	5.5	309	8.7
Chlordiazepoxide	637	2.6	2071	2.6	680	4.4	2151	4.3
Clobazam	150	7.2	466	6.4	144	4.3	470	4.7
Clonazepam	15.6	8.5	50.4	5.1	14.9	5.5	50.5	4.5
Demoxepam	687	6.7	2189	9.0	670	4.1	2276	3.2
Desalkylflurazepam	32.9	9.6	105.1	7.7	31.0	6.4	98.9	5.1
Desmethylflunitrazepam	15.8	10.4	52.9	7.4	15.8	10.4	52.6	5.2
Diazepam	277	2.9	927	4.1	272	3.0	900	2.1
Estazolam	132	3.9	440	2.6	131	5.3	432	3.1
Flunitrazepam	15.7	7.5	53.6	5.7	16.5	7.6	53.0	7.0
Flurazepam	25.7	10.6	90.5	8.2	25.0	2.4	83.7	2.2
Lorazepam	63.0	9.2	205.8	6.3	61.0	5.4	200	5.7
Lormetazepam	7.25	9.1	22.3	8.5	6.73	4.6	22.4	5.1
Medazepam	113.9	5.5	363.4	2.3	117.3	3.6	388	2.5
Midazolam	90.1	5.4	294.2	3.4	86.4	2.8	290	1.7
Nitrazepam	676	9.0	220.1	6.3	65.9	5.7	220	5.9
Norclobazam	872	9.7	2766	6.5	813	9.6	2689	5.3
Nordiazepam	248	3.6	808	3.7	247	4.0	788	3.0
Oxazepam	387	5.6	1220	3.5	378	3.9	1249	1.9
Prazepam	263	2.2	845	4.0	260	2.6	829	1.4
Temazepam	132	3.4	424	3.5	132	3.5	432	3.5
Tetrazepam	129	12.5	405	8.4	122	4.7	408	1.4
Trazodon	556	4.9	1781	9.9	523	2.7	1703	3.6
Triazolam	12.0	6.5	40.9	4.1	11.9	10.3	39.8	6.4
Zaleplon	26.5	11.0	88.1	10.0	25.9	8.3	87.8	5.5
Zolpidem	132	3.2	448	2.7	132	3.7	443	2.6
Zopiclone	19.9	14.0	74.7	9.4	18.4	21.7	67.6	29.8

Conclusions

A liquid chromatography-tandem mass spectrometry method for quantification of 34 different benzodiazepines in human plasma or serum was developed in this study. Two different sample preparation techniques were implemented. For direct injection, the MS9050 ClinMass TDM Platform on-line with the MS9550 ClinMass Add-On Set for Benzodiazepines from RECIPE was used. For offline sample preparation, the MS9000 ClinMass TDM Platform with the MS9500 ClinMass Add-On Set for Benzodiazepines also from RECIPE was used. The method was analytically validated on a Transcend II TLX-1 system coupled to a TSQ Endura triple quadrupole mass spectrometer. The method offers the flexibility of two possible sample preparation approaches, with direct injection for minimal human intervention, and offline sample precipitation, for improved sensitivity. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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