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TECHNICAL NOTE 65135

Quantification of benzodiazepines in human plasma or serum by liquid chromatography-tandem mass spectrometry for clinical research

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Keywords

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

Goal

Implementation of an analytical method for the quantification of a panel of 35 different benzodiazepines in human plasma or serum on a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer.

Application benefits

- Simple offline sample preparation by protein precipitation
- 35 benzodiazepines in a single quantitative method

Introduction

An analytical method for clinical research for the quantification of 35 benzodiazepines in human plasma or serum is reported; the panel includes 3-hydroxybromazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, α-OH-alprazolam, α-OH-midazolam, α-OH-triazolam, alprazolam, bromazepam, chlordiazepoxide, clobazam, clonazepam, demoxepam, desalkylflurazepam, desmethylflunitrazepam, diazepam, estazolam, flunitrazepam, flurazepam, lorazepam, lormetazepam, medazepam, midazolam, nitrazepam, norclobazam, nordiazepam, oxazepam, prazepam, temazepam, tetrazepam, trazodone, triazolam, zaleplon, zolpidem, and zopiclone. Twenty deuterated internal standards were used for the quantification. Sample preparation was performed by simple offline internal standard addition and protein precipitation. Extracts were injected onto a Thermo Scientific™ Vanquish™ Flex Binary system connected to a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer with heated electrospray ionization. Detection was performed by selected-reaction monitoring (SRM). Method performance was evaluated using the ClinMass® TDM Platform with the ClinMass Add-On Set for Benzodiazepines from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of

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linearity of response within the calibration ranges, accuracy, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

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

Table 1. Concentration ranges covered by calibrators.

Analyte	Concentration (ng/mL)
3-Hydroxybromazepam	15–246
7-Aminoclonazepam	4.98–69.9
7-Aminoflunitrazepam	5.26–74
7-Aminonitrazepam	21.7–306
alpha-Hydroxyalprazolam	5.67–80.6
alpha-Hydroxymidazolam	20.1–277
alpha-Hydroxytriazolam	4.4–63
Alprazolam	5.28–81
Bromazepam	34.2–465
Chlordiazepoxide	223–2881
Clobazam	47–681
Clonazepam	5.25–73.2
Demoxepam	229–3450
Desalkylflurazepam	10.6–155
Desmethylflunitrazepam	4.54–77.7
Diazepam	107–1401
Estazolam	41.9–602
Flunitrazepam	5.48–80.3
Flurazepam	10.9–159
Lorazepam	21.6–298
Lormetazepam	1.84–26.3
Medazepam	76.1–1001
Midazolam	30.8–432
Nitrazepam	22.4–308
Norclobazam	251–4090
Nordiazepam	86.2–1174
Oxazepam	123–1778
Prazepam	89.9–1245
Temazepam	42.2–607
Tetrazepam	44.5–622
Trazodone	166–2370
Triazolam	4.4–55.7
Zaleplon	9.3–136
Zolpidem	38–602
Zopiclone	6.9–108

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as 20 deuterated internal standards for quantification. 50 µL of plasma or serum were protein precipitated using 100 µL of precipitating solution containing the internal standards; precipitated samples were vortex-mixed, centrifuged, and the supernatant was transferred to a clean plate or vial.

Liquid chromatography

Chromatographic separation was achieved using mobile phases and analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. Total run time was 7.5 minutes.

Table 2. Liquid chromatographic method description.

Gradient profile:			
Time (min)	*Flow Rate (mL/min)*	*A (%)*	*B (%)*
0.00	0.6	85	10
0.21	0.6	70	10
0.30	0.6	70	24
3.00	0.6	62	26
6.20	0.6	62	60
6.21	0.6	25	85
6.70	0.6	25	85
7.50	0.6	85	10
Injection volume:	20 µL		
Column temp.:	40 °C		

Mass spectrometry

Analytes and internal standards were detected by SRM on a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. A summary of the MS conditions is reported in Table 3. Two SRM transitions for each analyte are included in the acquisition method for quantification and confirmation, respectively.

Table 3. MS settings.

Source type:	Heated electrospray ionization (HESI)
Vaporizer temperature:	450 °C
Capillary temperature:	350 °C
Spray voltage (positive mode):	1300 V
Sheath gas:	75 AU
Sweep gas:	0 AU
Auxiliary gas:	25 AU
Data acquisition mode:	Selected-reaction monitoring (SRM)
Collision gas pressure:	1.5 mTorr
Cycle time:	0.300 s
Q1 mass resolution (FWMH):	0.7
Q3 mass resolution (FWMH):	0.7

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, accuracy, and intra- and inter-assay precision for each analyte. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at two different levels provided by RECIPE (MS6082 batch #1267) prepared and analyzed in replicates of five on three different days. Intra-assay precision was evaluated for each day on the same set of runs (control samples at two levels, replicates of five each day, three days) in terms of percentage coefficient of variation (%CV). Inter-assay precision was evaluated on the same controls including all the 15 replicates of the three days.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

The method proved to be linear in the calibration ranges covered by the calibrators. Representative chromatograms for the lowest calibrator for 7-aminoflunitrazepam, oxazepam, and their internal standards are reported in Figure 1. Representative calibration curves for the same analytes are reported in Figure 2.

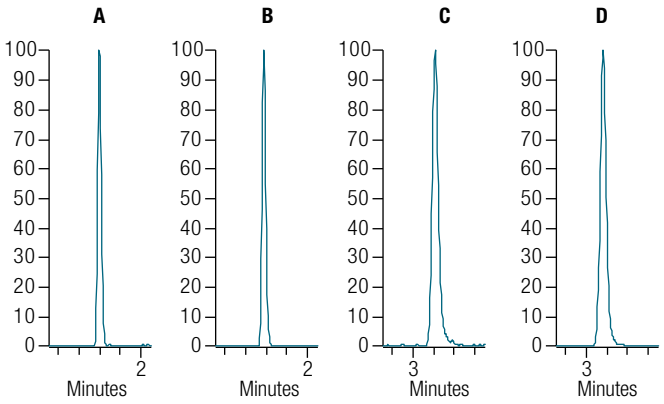

Figure 1. Representative chromatograms for the lowest calibrator for (A) 7-aminoflunitrazepam, (B) d7-7-aminoflunitrazepam, (C) oxazepam, and (D) d5-oxazepam.

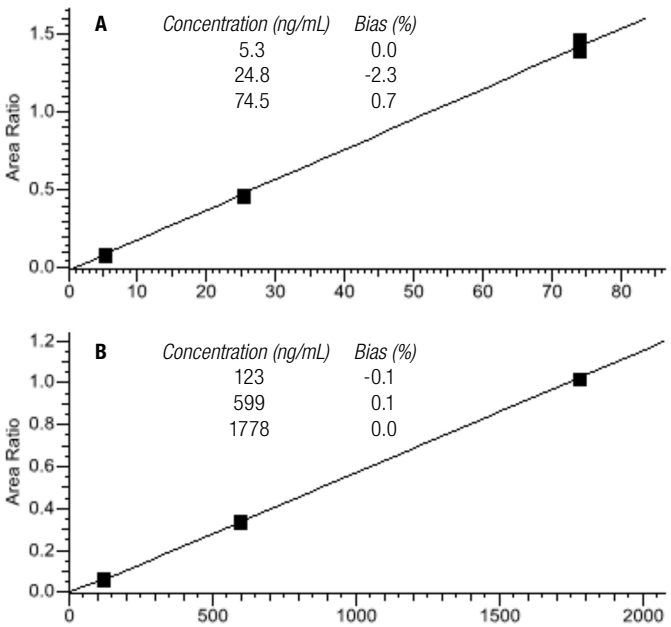

Figure 2. Representative calibration curves for (A) 7-aminoflunitrazepam and (B) oxazepam.

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -9.3% and 10.3%. Results are reported in Table 4.

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 -9.3% and 10.3%. Results are reported in Table 4.

Table 4. Analytical accuracy results for control MS6082 batch #1267.

Analyte	Control 1			Control 2		
	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	Bias (%)	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	Bias (%)
3-Hydroxybromazepam	42.4	44.9	5.9	144	154	6.8
7-Aminoclonazepam	14.3	14.0	-1.9	48.5	45.4	-6.3
7-Aminoflunitrazepam	15.0	14.8	-1.4	49.5	48.3	-2.5
7-Aminonitrazepam	63.5	62.7	-1.3	211	201	-4.6
alpha-Hydroxyalprazolam	16.2	16.6	2.7	54.1	54.8	1.2
alpha-Hydroxymidazolam	55.0	55.0	0.0	177	174	-1.5
alpha-Hydroxytriazolam	15.7	16.0	1.8	51.9	53.6	3.4
Alprazolam	16.9	15.7	-7.3	57.2	52.2	-8.7
Bromazepam	93.3	96.0	2.9	305	314	3.0
Chlordiazepoxide	631	572	-9.3	2053	1878	-8.5
Clobazam	89.5	85.5	-4.4	292	296	1.5
Clonazepam	8.13	8.0	-2.2	61.3	57.0	-7.0
Demoxepam	646	623	-3.5	2189	2051	-6.3
Desalkylflurazepam	29.9	29.2	-2.3	101.0	95.4	-5.5
Desmethylflunitrazepam	14.6	14.5	-0.5	50.9	48.4	-4.8
Diazepam	290	282	-2.8	939	918	-2.3
Estazolam	127	123	-3.5	425	407	-4.3
Flunitrazepam	16.2	15.4	-4.9	54.2	49.8	-8.1
Flurazepam	62.3	62.5	0.4	199	206	3.4
Lorazepam	65.6	62.7	-4.4	203	196	-3.4
Lormetazepam	5.65	5.41	-4.2	18.3	17.4	-5.0
Medazepam	274	257	-6.1	838	814	-2.8
Midazolam	30.2	30.5	0.9	78.9	76.2	-3.4
Nitrazepam	45.3	43.5	-4.0	148	136	-8.1
Norclobazam	771	776	0.6	2733	2657	-2.8
Nordiazepam	244	227	-7.1	782	729	-6.8
Oxazepam	360	344	-4.4	1205	1137	-5.7
Prazepam	271	256	-5.4	866	817	-5.6
Temazepam	202	191	-5.3	562	533	-5.2
Tetrazepam	126	124	-1.9	418	423	1.1
Trazodone	509	506	-0.7	1664	1540	-7.4
Triazolam	7.55	7.53	-0.3	24.1	23.5	-2.3
Zaleplon	24.7	25.5	3.2	83.2	83.2	0.0
Zolpidem	128	119	-7.4	426	398	-6.5
Zopiclone	18.9	20.2	6.8	66.2	73.0	10.3

The %CV for intra-assay precision was always below 14.8% for all the analytes; results are reported in Table 5.

The maximum %CV for inter-assay precision including all the analytes was 11.3%; results are reported in Table 6.

Table 5. Intra-assay precision results for control MS6082 batch #1267.

Analyte	Control 1						Control 2					
	Day 1		Day 2		Day 3		Day 1		Day 2		Day 3	
	Average Calculated Concentration (ng/mL)	CV (%)	Average Calculated Concentration (ng/mL)	CV (%)	Average Calculated Concentration (ng/mL)	CV (%)	Average Calculated Concentration (ng/mL)	CV (%)	Average Calculated Concentration (ng/mL)	CV (%)	Average Calculated Concentration (ng/mL)	CV (%)
3-Hydroxybromazepam	43.3	7.1	45.7	7.7	45.6	5.7	161	2.4	157	5.0	143	7.6
7-Aminoclonazepam	13.9	4.6	13.9	5.0	14.2	4.4	49.2	4.2	45.7	3.0	41.4	4.7
7-Aminoflunitrazepam	14.4	3.5	14.9	3.6	15.1	1.7	50.4	3.9	49.1	2.1	45.4	5.8
7-Aminonitrazepam	62.5	4.0	62.6	3.3	63.0	4.8	213	3.7	209	2.3	182	4.6
alpha-Hydroxyalprazolam	15.7	14.8	18.0	4.6	16.5	5.7	58.5	5.8	53.3	6.7	52.5	10.9
alpha-Hydroxymidazolam	52.8	4.7	54.7	3.6	57.5	5.3	182	4.3	183	2.9	158	9.1
alpha-Hydroxytriazolam	14.4	13.4	16.6	7.5	17.0	5.7	49.1	10.9	57.0	4.8	54.7	8.8
Alprazolam	15.1	5.3	16.8	9.0	15.1	5.2	55.1	4.4	52.5	4.8	49.1	4.7
Bromazepam	97.3	6.3	97.0	6.0	93.6	5.7	330	4.4	307	5.1	305	9.8
Chlordiazepoxide	555	3.8	577	2.9	585	3.0	1895	5.4	2009	3.1	1729	5.7
Clobazam	86.2	2.9	85.7	2.6	84.4	5.1	292	4.4	319.0	8.4	278	5.0
Clonazepam	7.85	14.0	7.74	10.1	8.34	8.2	59.2	7.7	57.7	3.9	54.2	10.5
Demoxepam	604	4.1	615	3.6	650	3.8	2128	4.3	2087	1.9	1939	8.3
Desalkylflurazepam	28.3	6.6	29.5	3.3	29.9	7.0	104.2	4.3	93.7	7.1	88.4	6.6
Desmethylflunitrazepam	14.0	10.5	14.2	9.8	15.4	9.6	52.3	6.0	50.6	3.4	42.4	2.9
Diazepam	273	9.1	283	2.3	289	1.5	959	4.0	938	1.7	856	6.2
Estazolam	120	4.7	126	2.9	122	3.6	421	3.2	424	2.6	376	8.7
Flunitrazepam	14.7	6.1	15.5	8.7	16.0	10.0	49.2	3.5	51.0	4.3	49.2	7.3
Flurazepam	59.5	4.7	66.4	3.7	61.6	11.5	219	6.0	214	2.9	185	8.4
Lorazepam	61.3	11.6	61.8	7.8	65.0	5.7	200	9.9	204	6.7	185	4.2
Lormetazepam	5.48	7.6	5.41	11.6	5.34	12.3	18.4	6.6	17.2	3.0	16.6	8.7
Medazepam	248	1.9	272	3.3	252	4.5	877	4.5	857	2.0	709	6.5
Midazolam	29.9	5.3	29.4	4.2	32.1	3.3	79.9	3.3	78.1	2.2	70.7	8.5
Nitrazepam	42.4	5.6	42.8	7.3	45.4	3.0	141	4.9	134	2.2	133	7.5
Norclobazam	744	6.0	816	4.1	768	1.3	2748	1.8	2756	2.7	2467	6.1
Nordiazepam	229	2.2	226	3.7	233	2.9	784	3.4	699	3.5	698	6.3
Oxazepam	333	5.1	344	3.7	355	3.2	1186	4.2	1189	3.3	1035	6.4
Prazepam	250	3.7	261	2.3	259	1.8	856	3.2	843	1.5	753	6.2
Temazepam	192	3.6	188	6.0	194	4.7	579	4.8	528	8.7	491	8.0
Tetrazepam	123	4.3	124	4.1	124	8.3	430	2.1	436	3.9	401	5.3
Trazodone	497	2.9	483	7.2	546	6.7	1701	5.4	1449	3.6	1470	8.9
Triazolam	8.31	7.4	7.38	4.4	7.1	5.3	25.8	2.2	23.0	3.5	21.8	6.7
Zaleplon	24.3	4.5	25.4	5.5	26.79	3.6	85.0	2.6	84.7	1.8	79.9	6.4
Zolpidem	115	5.0	121	5.2	120	4.0	420	4.4	408	6.7	367	7.6
Zopiclone	18.2	7.6	21.1	2.7	21.4	1.8	67.3	7.2	75.8	2.9	76.0	1.4

Table 6. Inter-assay precision results for control MS6082 batch #1267.

Analyte	Control 1		Control 2	
	Average Calculated Concentration (ng/mL)	CV (%)	Average Calculated Concentration (ng/mL)	CV (%)
3-Hydroxybromazepam	44.9	6.8	154	7.1
7-Aminoclonazepam	14.0	4.4	45.4	8.2
7-Aminoflunitrazepam	14.8	3.6	48.3	6.0
7-Aminonitrazepam	62.7	3.8	201	7.7
alpha-Hydroxyalprazolam	16.6	10.4	54.8	8.9
alpha-Hydroxymidazolam	55.0	5.6	174.3	8.6
alpha-Hydroxytriazolam	16.0	11.3	53.6	10.0
Alprazolam	15.7	8.3	52.2	6.5
Bromazepam	96.0	5.9	314	7.3
Chlordiazepoxide	572	3.7	1878	7.7
Clobazam	85.5	3.4	296	8.4
Clonazepam	7.95	10.8	57.0	8.1
Demoxepam	623	4.8	2051	6.4
Desalkylflurazepam	29.2	6.0	95.4	9.0
Desmethylflunitrazepam	14.5	10.2	48.4	10.1
Diazepam	282	5.5	918	6.4
Estazolam	123	4.2	407	7.4
Flunitrazepam	15.4	8.8	49.8	5.2
Flurazepam	62.5	8.1	206	9.4
Lorazepam	62.7	8.4	196	8.1
Lormetazepam	5.41	9.7	17.4	7.5
Medazepam	257	5.3	814	10.4
Midazolam	30.5	5.6	76.2	7.2
Nitrazepam	43.5	6.0	136	5.6
Norclobazam	776	5.6	2657	6.3
Nordiazepam	229	3.0	729	7.2
Oxazepam	344	4.6	1137	7.9
Prazepam	256	3.2	817	6.8
Temazepam	191	4.7	533	9.7
Tetrazepam	124	5.5	423	5.2
Trazodone	506	7.6	1540	9.6
Triazolam	7.53	8.9	23.5	8.4
Zaleplon	25.5	5.9	83.2	4.7
Zolpidem	119	4.9	398	8.2
Zopiclone	20.2	8.7	73.0	7.0

Conclusions

A liquid chromatography-tandem mass spectrometry method for clinical research for the quantification of 35 different benzodiazepines in human plasma or serum using the ClinMass TDM Platform with the ClinMass Add-On Set for Benzodiazepines from RECIPE was implemented and analytically evaluated on a Vanquish Flex Binary system connected to a TSQ Quantis triple quadrupole mass spectrometer. The method offers quick and simple offline protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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TN65135-EN 0118S