

Quantification of 35 benzodiazepines in human plasma by LC-HRAM-MS for clinical research

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Application benefits

- Simple offline sample preparation by protein precipitation
- Increased accuracy of method by implementation of a comprehensive ClinMass[®] kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Analysis of 35 benzodiazepines in a single quantitative method

Goal

Implementation of an analytical method for the quantification of 35 benzodiazepines in human plasma on a Thermo Scientific[™] Orbitrap Exploris[™] 120 mass spectrometer.

Introduction

Benzodiazepines are a group of psychoactive drugs with a broad range of therapeutic effects. These controlled



substances are some of the most frequently prescribed medications worldwide and are frequently prone to abuse by those without valid medical prescriptions.

In this report, a method for quantitative analysis of 35 benzodiazepines in human plasma for clinical research in 7.0 minutes is described. Samples were processed by protein precipitation followed by chromatographic separation on a Thermo Scientific[™] Vanquish[™] Flex Binary UHPLC system. Detection was performed on a high-resolution accurate-mass (HRAM) Orbitrap Exploris 120 hybrid quadrupole mass spectrometer with heated electrospray ionization (HESI) operated in positive ion mode, which allows for an accurate, selective, and sensitive analysis. Method performance was evaluated using the ClinMass[®] LC-MS/MS calibrators, controls, and internal

standards from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response, lower limit of quantitation (LLOQ), carryover, accuracy, and intra- and inter-assay precision for all analytes.

Experimental

Target analytes

The complete list of analytes and corresponding internal standards is reported in Table 1. The retention times obtained and the concentration ranges covered by the calibrators used are reported in Table 2.

Table 1. List of analytes and internal standards

Analyte	Chemical formula	Expected mass (m/z)	Internal standard name	Chemical formula	Expected mass (m/z)
3-Hydroxybromomazepam	C ₁₄ H ₁₀ BrN ₃ O ₂	332.0029	d ₇ -7-Aminoflunitrazepam	C ₁₆ H ₇ D ₇ FN ₃ O	291.1633
7-Aminoclonazepam	C ₁₅ H ₁₂ ClN ₃ O	286.0742	d ₄ -7-Aminoclonazepam	C ₁₅ H ₈ D ₄ ClN ₃ O	290.0993
7-Aminoflunitrazepam	C ₁₆ H ₁₄ FN ₃ O	284.1194	d ₇ -7-Aminoflunitrazepam	C ₁₆ H ₇ D ₇ FN ₃ O	291.1633
7-Aminonitrazepam	C ₁₅ H ₁₃ N ₃ O	252.1131	d ₇ -7-Aminoflunitrazepam	C ₁₆ H ₇ D ₇ FN ₃ O	291.1633
alpha-Hydroxyalprazolam	C ₁₇ H ₁₃ ClN ₄ O	325.0851	d ₅ -alpha-Hydroxyalprazolam	C ₁₇ H ₈ D ₅ ClN ₄ O	330.1165
alpha-Hydroxymidazolam	C ₁₈ H ₁₃ ClFN ₃ O	342.0804	d ₄ -alpha-Hydroxymidazolam	C ₁₈ H ₉ D ₄ ClFN ₃ O	346.1055
alpha-Hydroxytriazolam	C ₁₇ H ₁₂ Cl ₂ N ₄ O	359.0461	d ₄ -alpha-Hydroxytriazolam	C ₁₇ H ₈ D ₄ Cl ₂ N ₄ O	363.0712
Alprazolam	C ₁₇ H ₁₃ ClN ₄	309.0902	d ₅ -Alprazolam	C ₁₇ H ₈ D ₅ ClN ₄	314.1215
Bromazepam	C ₁₄ H ₁₀ BrN ₃ O	316.0080	d ₅ -alpha-Hydroxyalprazolam	C ₁₇ H ₈ D ₅ ClN ₄ O	330.1165
Chlordiazepoxide	C ₁₆ H ₁₄ ClN ₃ O	300.0898	d ₅ -Chlordiazepoxide	C ₁₆ H ₉ D ₅ ClN ₃ O	305.1212
Clobazam	C ₁₅ H ₁₀ ClN ₃ O ₃	301.0738	d ₅ -Nordiazepam	C ₁₅ H ₆ D ₅ ClN ₂ O	276.0947
Clonazepam	C ₁₆ H ₁₃ ClN ₂ O ₂	316.0484	d ₄ -Clonazepam	C ₁₅ H ₆ D ₄ ClN ₃ O ₃	320.0735
Demoxepam	C ₁₅ H ₁₁ ClN ₂ O ₂	287.0582	d ₇ -7-Aminoflunitrazepam	C ₁₆ H ₇ D ₇ FN ₃ O	291.1633
Desalkylflurazepam	C ₁₅ H ₁₀ ClFN ₂ O	289.0539	d ₆ -Zopiclone	C ₁₉ H ₁₅ D ₆ N ₃ O	314.2134
Desmethylflunitrazepam	C ₁₅ H ₁₀ FN ₃ O ₃	300.0779	d ₅ -Oxazepam	C ₁₅ H ₆ D ₅ ClN ₂ O ₂	292.0896
Diazepam	C ₁₆ H ₁₃ ClN ₂ O	285.0789	d ₅ -Diazepam	C ₁₆ H ₈ D ₅ ClN ₂ O	290.1103
Estazolam	C ₁₆ H ₁₁ ClN ₄	295.0745	d ₅ -Estazolam	C ₁₆ H ₆ D ₅ ClN ₄	300.1059
Flunitrazepam	C ₁₆ H ₁₂ FN ₃ O ₃	314.0936	d ₇ -Flunitrazepam	C ₁₆ H ₅ D ₇ FN ₃ O ₃	321.1375
Flurazepam	C ₂₁ H ₂₃ ClFN ₃ O	388.1586	d ₄ -Midazolam	C ₁₈ H ₉ D ₄ ClFN ₃	330.1106
Lorazepam	C ₁₅ H ₁₀ Cl ₂ N ₂ O ₂	321.0192	d ₄ -Lorazepam	C ₁₅ H ₆ D ₄ Cl ₂ N ₂ O ₂	325.0443
Lormetazepam	C ₁₆ H ₁₂ Cl ₂ N ₂ O ₂	335.0349	d ₄ -Triazolam	C ₁₇ H ₈ D ₄ Cl ₂ N ₄	347.0763
Medazepam	C ₁₆ H ₁₅ ClN ₂	271.0997	d ₅ -Przepam	C ₁₉ H ₁₂ D ₅ ClN ₂ O	330.1416
Midazolam	C ₁₈ H ₁₃ ClFN ₃	326.0855	d ₄ -Midazolam	C ₁₈ H ₉ D ₄ ClFN ₃	330.1106
Nitrazepam	C ₁₅ H ₁₁ N ₃ O ₃	282.0873	d ₅ -Nitrazepam	C ₁₅ H ₆ D ₅ N ₃ O ₃	287.1187
Norclobazam	C ₁₅ H ₁₁ ClN ₂ O ₂	287.0582	d ₅ -Estazolam	C ₁₆ H ₆ D ₅ ClN ₄	300.1059
Nordiazepam	C ₁₅ H ₁₁ ClN ₂ O	271.0633	d ₅ -Nordiazepam	C ₁₅ H ₆ D ₅ ClN ₂ O	276.0947
Oxazepam	C ₁₅ H ₁₁ ClN ₂ O ₂	287.0582	d ₅ -Oxazepam	C ₁₅ H ₆ D ₅ ClN ₂ O ₂	292.0896
Przepam	C ₁₉ H ₁₇ ClN ₂ O	325.1102	d ₅ -Przepam	C ₁₉ H ₁₂ D ₅ ClN ₂ O	330.1416
Temazepam	C ₁₆ H ₁₃ ClN ₂ O ₂	301.0738	d ₅ -Temazepam	C ₁₆ H ₈ D ₅ ClN ₂ O ₂	306.1052
Tetrazepam	C ₁₆ H ₁₇ ClN ₂ O	289.1102	d ₅ -Przepam	C ₁₉ H ₁₂ D ₅ ClN ₂ O	330.1416
Trazodone	C ₁₉ H ₂₂ ClN ₅ O	372.1586	d ₄ -Midazolam	C ₁₈ H ₉ D ₄ ClFN ₃	330.1106
Triazolam	C ₁₇ H ₁₂ Cl ₂ N ₄	343.0512	d ₄ -Triazolam	C ₁₇ H ₈ D ₄ Cl ₂ N ₄	347.0763
Zaleplone	C ₁₇ H ₁₅ N ₅ O	306.1349	d ₇ -7-Aminoflunitrazepam	C ₁₇ H ₈ D ₄ Cl ₂ N ₄ O	291.1633
Zolpidem	C ₁₉ H ₂₁ N ₃ O	308.1757	d ₆ -Zolpidem	C ₁₉ H ₁₅ D ₆ N ₃ O	314.2134
Zopiclone	C ₁₇ H ₁₇ ClN ₆ O ₃	389.1123	d ₅ -Chlordiazepoxide	C ₁₆ H ₉ D ₅ ClN ₃ O	305.1212

Table 2. Concentration ranges covered by the calibrators (MS6013 batch #1069) and retention times

Analyte	Retention time (min)	Concentration range (µg/L)
3-Hydroxybromomazepam	1.4	16.6–258
7-Aminoclonazepam	1.2	5.02–70.3
7-Aminoflunitrazepam	1.4	5.36–76.5
7-Aminonitrazepam	1.1	21.6–304
alpha-Hydroxyalprazolam	2.7	5.74–81.1
alpha-Hydroxymidazolam	3.9	10.6–135
alpha-Hydroxytriazolam	2.6	3.92–63.5
Alprazolam	3.8	5.14–76.4
Bromazepam	1.9	21.3–319
Chlordiazepoxide	3.5	260–3512
Clobazam	4.6	27.7–460
Clonazepam	3.7	4.94–72.3
Demoxepam	2.1	231–3524
Desalkylflurazepam	4.2	17.1–274
Desmethylflunitrazepam	3.0	4.78–84.3
Diazepam	5.1	128–1733
Estazolam	3.1	44.6–679
Flunitrazepam	4.2	5.25–76.6
Flurazepam	4.8	10.4–155
Lorazepam	3.4	19.2–276
Lormetazepam	4.6	1.97–27.2
Medazepam	6.4	75.3–385
Midazolam	5.0	20.5–104
Nitrazepam	3.1	18.9–102
Norclobazam	3.4	240–4281
Nordiazepam	4.4	116–1653
Oxazepam	3.0	117–587
Prazepam	6.2	89.4–1259
Temazepam	4.3	115–1587
Tetrazepam	5.9	45.0–640
Trazodone	4.6	180–2396
Triazolam	4.0	3.46–43.8
Zaleplone	2.4	17.3–253
Zolpidem	3.2	36.6–603
Zopiclone	2.1	16.5–279

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as an internal standard mix for quantitation. Samples of 50 µL of plasma were protein precipitated using 100 µL of precipitating solution containing the internal standard (MS9021 and MS9512). Precipitated samples were vortex-mixed and centrifuged for 5 minutes. 50 µL of the supernatant were transferred to a clean vial.

Liquid chromatography

The supernatant was injected onto a Vanquish Flex Binary UHPLC system connected to an Orbitrap Exploris 120 mass spectrometer. Chromatographic separation was achieved using the mobile phases and analytical column provided by RECIPE.

Details of the analytical method are reported in Table 3. Total runtime was 7.0 minutes.

Table 3. LC method

Time (min)	Flow rate (mL/min)	B (%)
0.00	0.6	10
0.21	0.6	10
0.30	0.6	24
3.00	0.6	26
6.20	0.6	60
6.21	0.6	85
6.40	0.6	85
6.41	0.6	10
7.00	0.6	10
Phase A		MS9007
Phase B		MS9008
Column temperature (°C)		40
Injection volume (µL)		2

Mass spectrometry

Analytes and internal standards were detected by Full-Scan – data-dependent MS² acquisition mode on an Orbitrap Exploris 120 mass spectrometer using HESI operated in positive ionization mode. A summary of the MS conditions is reported in Table 4. Two fragments for each analyte were used for confirmation based on the average ion ratio of all samples.

Table 4. MS parameters

Ion source parameters	
Source type	Heated Electrospray Ionization (HESI)
Spray voltage – Positive (V)	3,500
Sheath gas (Arb)	60
Aux gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	350
Vaporizer temp. (°C)	400
Settings	
Mild trapping	No
Internal mass calibration	RunStart EASY-IC™
Data acquisition mode	Full scan – ddMS ²
Full scan parameters	
Resolution (at <i>m/z</i> 200)	60,000
Scan range (<i>m/z</i>)	70–450
Expected peak width (s)	6
RF lens (%)	90
AGC target	Standard (1e6)
Polarity	Positive

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, LLOQ, carryover, accuracy, and intra- and inter-assay precision for all analytes.

Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it.

The LLOQ was evaluated by diluting the lowest calibrator 20-fold using blank matrix. LLOQs were determined as the lowest concentration with inaccuracy and precision below 20%. 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).

Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five, prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A linear interpolation with 1/x weighting was used for all analytes. The percentage bias between nominal and back-calculated concentration was always within ±15% for all the calibrators in all the runs. Chromatograms of representative analytes and their internal standards at their respective lowest limit of quantitation are reported in Figure 1. Representative calibration curves are reported in Figure 2.

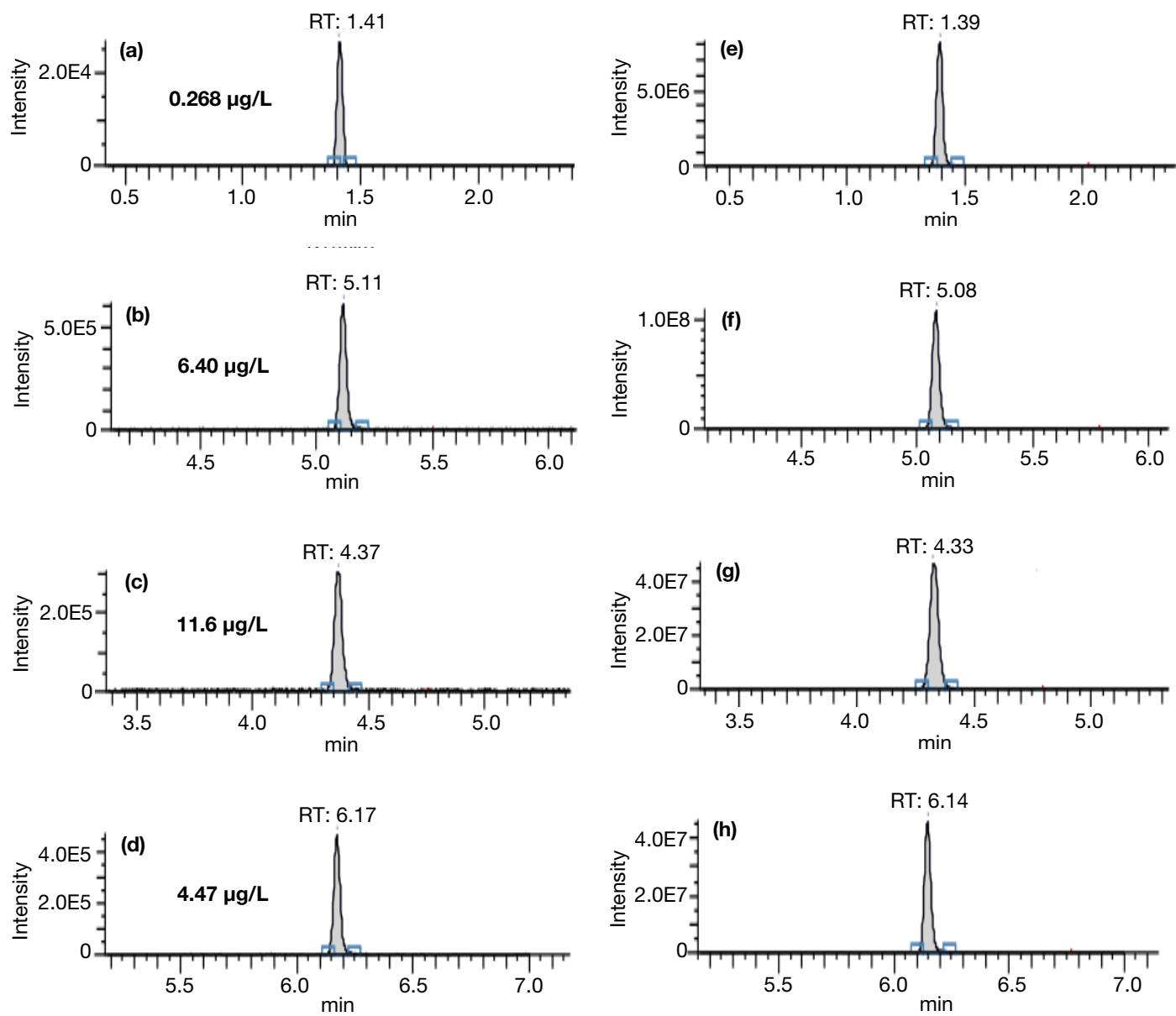


Figure 1. Representative chromatograms of the lower limits of quantification for (a) 7-aminoflunitrazepam, (b) diazepam, (c) nordiazepam, (d) prazepam, (e) d₇-7-aminonitrazepam, (f) d₅-diazepam, (g) d₅-nordiazepam, (h) d₅-prazepam

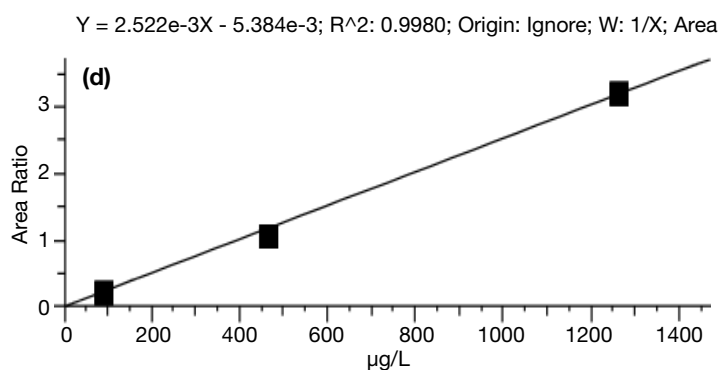
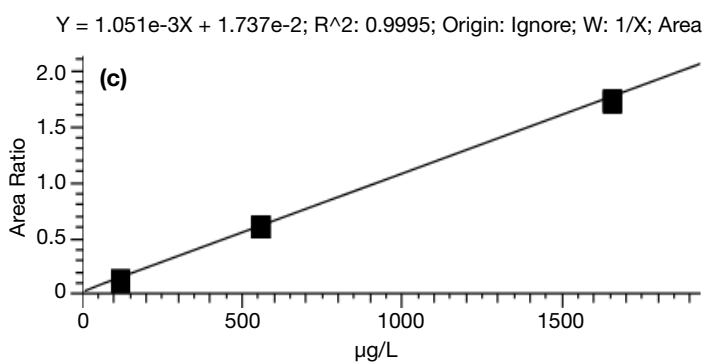
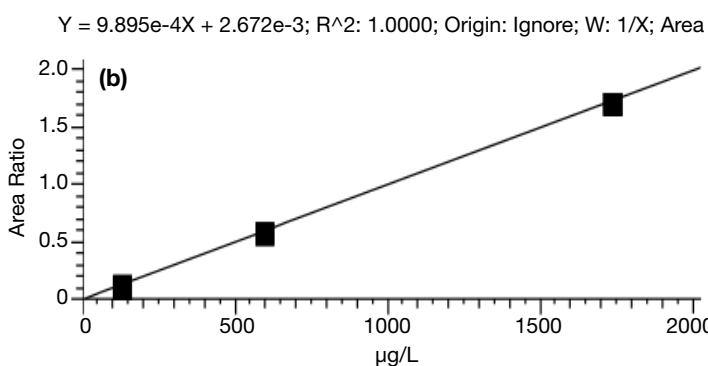
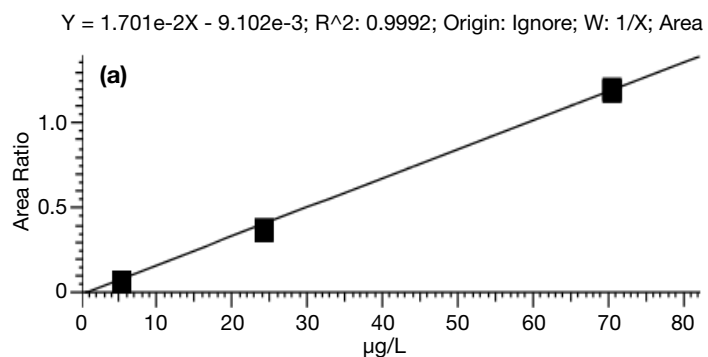


Figure 2. Representative calibration curves for (a) 7-aminoflunitrazepam, (b) diazepam, (c) nordiazepam, (d) prazepam

No significant carryover was observed for any of the analytes, with no signal detected in the blank injected immediately after the highest calibrator.

The data demonstrated accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -13.5% and +8.8% (Table 5). The %CV for intra-assay precision was always below 7.1% for all the analytes. The maximum %CV for inter-assay precision including all the analytes was 9.7%. Results for intra- and inter-assay precision are reported in Table 6.

LLOQs of all compounds were determined and reported in Table 7.

Table 5. Analytical accuracy results for control MS6082 batch #1267

Analyte	Control	Nominal conc. (µg/L)	Average calculated conc. (µg/L)	Bias (%)
3-Hydroxybromomazepam	Level I	42.4	45.3	6.8
	Level II	144	157	8.8
7-Aminoclonazepam	Level I	14.3	14.0	-2.2
	Level II	48.5	46.3	-4.5
7-Aminoflunitrazepam	Level I	15.0	15.1	0.8
	Level II	49.5	50.7	2.4
7-Aminonitrazepam	Level I	63.5	64.4	1.5
	Level II	211	210	-0.6
alpha-Hydroxyalprazolam	Level I	16.2	16.1	-0.7
	Level II	54.1	53.3	-1.4
alpha-Hydroxymidazolam	Level I	55.0	51.7	-5.9
	Level II	177	174	-2.0
alpha-Hydroxytriazolam	Level I	15.7	15.3	-2.8
	Level II	51.9	51.2	-1.5
Alprazolam	Level I	15.2	14.9	-2.3
	Level II	52.9	50.3	-4.8
Bromazepam	Level I	93.3	91.8	-1.6
	Level II	305	304	-0.2
Chlordiazepoxide	Level I	631	615	-2.5
	Level II	2053	2011	-2.1
Clobazam	Level I	89.5	81.8	-8.6
	Level II	292	286	-2.2
Clonazepam	Level I	8.13	7.62	-6.3
	Level II	61.3	57.4	-6.3
Demoxepam	Level I	646	642	-0.6
	Level II	2189	2258	3.1

Table 5 (continued). Analytical accuracy results for control MS6082 batch #1267

Analyte	Control	Nominal conc. (µg/L)	Average calculated conc. (µg/L)	Bias (%)	Analyte	Control	Nominal conc. (µg/L)	Average calculated conc. (µg/L)	Bias (%)
Desalkylflurazepam	Level I	29.9	28.7	-4.0	Norclobazam	Level I	771	764	-0.9
	Level II	101	100	-0.7		Level II	2733	2741	0.3
Desmethylflunitrazepam	Level I	14.6	14.9	2.2	Nordiazepam	Level I	219	215	-1.9
	Level II	50.9	52.8	3.7		Level II	704	730	3.7
Diazepam	Level I	290	297	2.4	Oxazepam	Level I	360	348	-3.4
	Level II	939.0	971.3	3.4		Level II	1205	1185	-1.7
Estazolam	Level I	127	127	-0.3	Prazepam	Level I	271	253	-6.6
	Level II	425	435	2.5		Level II	866	825	-4.8
Flunitrazepam	Level I	16.2	15.0	-7.6	Temazepam	Level I	186	178	-4.1
	Level II	54.2	50.3	-7.3		Level II	520	486	-6.6
Flurazepam	Level I	62.30	57.04	-8.4	Tetraazepam	Level I	126	129	2.0
	Level II	199	194	-2.4		Level II	418	427	2.2
Lorazepam	Level I	60.7	54.9	-9.6	Trazodone	Level I	509	490	-3.7
	Level II	193	185	-4.3		Level II	1581	1549	-2.0
Lormetazepam	Level I	5.65	5.46	-3.3	Triazolam	Level I	7.55	7.79	3.2
	Level II	18.3	17.8	-2.8		Level II	24.1	24.9	3.5
Medazepam	Level I	274	255	-6.8	Zaleplone	Level I	24.7	25.8	4.6
	Level II	838	830	-1.0		Level II	83.2	87.8	5.5
Midazolam	Level I	30.2	29.1	-3.5	Zolpidem	Level I	128	116	-9.2
	Level II	78.9	79.1	0.2		Level II	426	403	-5.3
Nitrazepam	Level I	41.9	39.4	-6.1	Zopiclone	Level I	18.9	16.3	-13.5
	Level II	133	137	2.7		Level II	66.2	60.8	-8.1

Table 6. Analytical intra- and inter-assay precision results for control MS6082 batch #1267

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3		Average calculated concentration (µg/L)	CV (%)
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)		
3-Hydroxybromomazepam	Level I	42.9	3.9	46.0	5.8	46.9	3.2	45.3	4.6
	Level II	152	3.5	162	4.1	156	1.7	157	3.4
7-Aminoclonazepam	Level I	13.7	4.8	13.3	5.0	15.0	2.1	14.0	6.2
	Level II	45.7	3.5	45.7	5.1	47.6	1.9	46.3	2.4
7-Aminoflunitrazepam	Level I	14.8	4.9	14.4	4.1	16.2	3.2	15.1	6.5
	Level II	50.1	3.1	50.2	5.0	51.8	1.7	50.7	1.9
7-Aminonitrazepam	Level I	62.6	5.1	62.6	4.8	68.1	2.5	64.4	5.0
	Level II	208	2.6	211	4.3	211	2.1	210	0.8
alpha-Hydroxyalprazolam	Level I	15.8	7.1	15.0	5.3	17.5	1.8	16.1	7.8
	Level II	53.3	1.8	51.9	5.0	54.9	2.6	53.3	2.8
alpha-Hydroxymidazolam	Level I	51.5	5.1	49.2	4.4	54.6	3.0	51.7	5.2
	Level II	174	2.8	174	4.1	173	3.2	174	0.4

Table 6 (continued). Analytical intra- and inter-assay precision results for control MS6082 batch #1267

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3		Average calculated concentration (µg/L)	CV (%)
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)		
alpha-Hydroxytriazolam	Level I	15.4	4.0	14.3	4.3	16.1	3.3	15.3	5.9
	Level II	50.8	3.4	50.0	5.6	52.7	2.5	51.2	2.7
Alprazolam	Level I	14.6	5.0	14.1	5.0	15.9	2.9	14.9	6.3
	Level II	49.8	2.8	49.5	4.5	51.8	1.8	50.3	2.4
Bromazepam	Level I	92.8	5.3	88.3	4.8	94.3	5.3	91.8	3.4
	Level II	313	2.3	300	1.9	300	3.7	304	2.5
Chlordiazepoxide	Level I	598	5.2	583	4.4	664	2.6	615	7.0
	Level II	1973	2.9	1982	4.2	2077	2.4	2011	2.8
Clobazam	Level I	82.5	4.5	75.9	4.6	87.2	3.6	81.8	6.9
	Level II	284	1.9	277	4.2	295	1.6	286	3.2
Clonazepam	Level I	7.35	4.6	7.29	4.7	8.21	2.8	7.62	6.7
	Level II	56.8	3.9	56.4	4.7	59.1	2.0	57.4	2.6
Demoxepam	Level I	614	5.5	631	6.9	681	3.3	642	5.5
	Level II	2176	3.3	2311	4.5	2286	2.1	2258	3.2
Desalkylflurazepam	Level I	27.8	6.1	27.2	5.2	31.1	2.5	28.7	7.3
	Level II	99.0	3.2	100	4.3	102	2.2	100	1.4
Desmethylflunitrazepam	Level I	14.7	6.8	14.0	5.2	16.0	3.2	14.9	6.9
	Level II	52.2	2.9	51.4	5.6	54.7	1.6	52.8	3.2
Diazepam	Level I	287	5.5	281	5.0	322	2.5	297	7.6
	Level II	954	3.2	965	4.4	995	1.7	971	2.2
Estazolam	Level I	124	5.1	120	4.7	136	3.0	127	6.5
	Level II	430	3.0	430	5.2	447	2.1	435	2.2
Flunitrazepam	Level I	14.9	4.9	14.3	4.3	15.7	2.5	15.0	4.8
	Level II	50.1	2.6	49.4	4.9	51.3	0.9	50.3	1.9
Flurazepam	Level I	56.5	5.3	54.4	4.5	60.2	3.1	57.0	5.1
	Level II	193	2.8	194	4.8	196	1.6	194	0.9
Lorazepam	Level I	53.8	5.4	51.7	6.0	59.2	3.1	54.9	7.0
	Level II	182	1.8	182	5.0	190	2.5	185	2.5
Lormetazepam	Level I	5.19	5.1	5.27	3.5	5.93	2.1	5.46	7.4
	Level II	17.5	2.9	17.5	4.8	18.4	1.9	17.8	3.0
Medazepam	Level I	256	5.4	237	4.5	273	1.9	255	7.2
	Level II	836	3.2	807	5.3	847	2.2	830	2.5
Midazolam	Level I	28.2	5.7	27.5	4.8	31.8	2.7	29.1	7.9
	Level II	77.9	2.6	78.0	4.9	81.4	1.7	79.1	2.6
Nitrazepam	Level I	38.7	4.5	37.2	5.0	42.3	3.2	39.4	6.7
	Level II	135	3.0	134	4.7	141	1.7	137	2.8
Norclobazam	Level I	749	6.1	699	4.7	844	4.0	764	9.7
	Level II	2691	3.1	2630	4.5	2903	3.4	2741	5.2
Nordiazepam	Level I	211	5.9	202	4.5	232	2.6	215	7.2
	Level II	718	2.3	721	3.9	752	1.6	730	2.6

Table 6 (continued). Analytical intra- and inter-assay precision results for control MS6082 batch #1267

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3		Average calculated concentration (µg/L)	CV (%)
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)		
Oxazepam	Level I	342	4.9	327	4.8	374	2.5	348	6.8
	Level II	1175	2.7	1164	4.8	1215	3.3	1185	2.3
Prazepam	Level I	249	5.2	240	4.9	270	2.3	253	6.1
	Level II	823	2.9	817	4.6	835	1.9	825	1.1
Temazepam	Level I	173	5.2	170	5.2	192	2.1	178	6.7
	Level II	477	2.8	480	4.8	500	2.1	486	2.6
Tetrazeepam	Level I	128	5.1	121	5.0	137	2.1	129	6.4
	Level II	426	2.3	423	4.7	433	1.8	427	1.1
Trazodone	Level I	484	5.0	461	3.7	526	2.4	490	6.7
	Level II	1545	2.9	1517	4.9	1587	1.7	1550	2.3
Triazolam	Level I	7.77	5.2	7.29	3.2	8.30	3.7	7.79	6.5
	Level II	24.7	2.9	24.5	5.1	25.6	1.2	24.9	2.4
Zaleplone	Level I	24.0	5.4	26.2	6.0	27.4	3.4	25.8	6.6
	Level II	83.8	2.9	90.5	4.0	89.1	1.9	87.8	4.0
Zolpidem	Level I	114	5.3	110	5.0	125	2.4	116	6.8
	Level II	400	3.3	396	4.5	414	1.8	403	2.2
Zopiclone	Level I	16.7	6.1	16.0	5.2	16.4	2.6	16.3	2.4
	Level II	61.3	4.3	59.0	5.6	62.1	2.6	60.8	2.6

Table 7. LLOQs for all compounds

Analyte	LLOQ (µg/L)	Analyte	LLOQ (µg/L)
3-Hydroxybromomazepam	1.66	Flurazepam	0.52
7-Aminoclonazepam	0.251	Lorazepam	1.92
7-Aminoflunitrazepam	0.268	Lormetazepam	1.97
7-Aminonitrazepam	2.16	Medazepam	3.77
alpha-Hydroxyalprazolam	1.15	Midazolam	1.03
alpha-Hydroxymidazolam	0.53	Nitrazepam	0.945
alpha-Hydroxytriazolam	3.92	Norclobazam	24.0
Alprazolam	0.257	Nordiazepam	11.6
Bromazepam	21.3	Oxazepam	11.7
Chlordiazepoxide	13.0	Prazepam	4.47
Clobazam	2.77	Temazepam	5.75
Clonazepam	0.988	Tetrazeepam	2.25
Demoxepam	23.1	Trazodone	18.0
Desalkylflurazepam	1.71	Triazolam	0.173
Desmethylflunitrazepam	0.956	Zaleplone	1.73
Diazepam	6.40	Zolpidem	3.66
Estazolam	2.23	Zopiclone	16.5
Flunitrazepam	0.263		

Conclusions

A robust, reproducible, and sensitive seven-minute liquid chromatography-HRAM mass spectrometry method for clinical research for quantification of 35 benzodiazepines in human plasma was developed. The ClinMass TDM Platform with the ClinMass Add-On Set for Benzodiazepines from RECIPE was used.

The method was analytically implemented and validated on an Vanquish Flex UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. The method described here 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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