

Quantification of 34 benzodiazepines in human plasma by LC-HRAM-MS for clinical research

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Application benefits

- Simple offline sample preparation by protein precipitation
- Fast acquisition time allows for increased productivity of the assay
- Robust, sensitive hardware enables increased confidence in data
- Analysis of 34 benzodiazepines in a single quantitative method

Goal

Implementation of an analytical method for the quantification of thirty-four 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 34 benzodiazepines in human plasma for clinical research in 6.5 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 an Orbitrap Exploris 120 hybrid quadrupole mass spectrometer with

heated electrospray ionization (HESI) operated in positive ion mode. 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

Compound name	Formula	Expected mass (m/z)	Internal standard name	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 ₄ -7-Aminoclonazepam	C ₁₅ H ₈ D ₄ ClN ₃ O	290.0993
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 ₂ 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 ₈ 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	300.10588
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 -Prazepam	C ₁₉ H ₁₂ D ₅ ClN ₂ O	330.1416
Midazolam	C ₁₈ H ₁₃ ClFN ₃	326.0855	d ₅ -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
Prazepam	C ₁₉ H ₁₇ ClN ₂ O	325.1102	d ₅ -Prazepam	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 -Prazepam	C ₁₉ H ₁₂ D ₅ ClN ₂ O	330.1416
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. Retention times and concentration ranges (MS6013 batch #1069)

Analyte	Retention time (min)	Concentration range (µg/L)
3-Hydroxybromomazepam	3.1	16.6–258
7-Aminoclonazepam	2.8	5.02–70.3
7-Aminoflunitrazepam	3.1	5.36–76.5
7-Aminonitrazepam	2.3	21.6–304
alpha-Hydroxyalprazolam	3.6	5.74–81.1
alpha-Hydroxymidazolam	3.2	10.6–135
alpha-Hydroxytriazolam	3.6	3.92–63.5
Alprazolam	3.9	5.14–76.4
Bromazepam	3.3	21.3–319
Chlordiazepoxide	3.0	260–3512
Clobazam	4.0	27.7–460
Clonazepam	3.7	4.94–72.3
Demoxepam	3.4	231–3524
Desalkylflunitrazepam	3.8	17.1–274
Desmethyflunitrazepam	3.7	4.78–84.3
Diazepam	4.1	128–1733
Estazolam	3.8	44.6–679
Flunitrazepam	4.0	5.25–76.6
Flurazepam	3.2	10.4–155
Lorazepam	3.6	19.2–276
Lormetazepam	3.9	1.97–27.2
Medazepam	3.3	75.3–385
Midazolam	3.2	20.5–104
Nitrazepam	3.6	18.9–102
Norclobazam	3.7	240–4281
Nordiazepam	3.6	116–1653
Oxazepam	3.6	117–587
Prazepam	4.7	89.4–1259
Temazepam	3.9	115–1587
Tetrazepam	3.6	45.0–640
Triazolam	3.9	3.46–43.8
Zaleplone	3.7	17.3–253
Zolpidem	3.0	36.6–603
Zopiclone	2.8	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 acetonitrile containing

the internal standards. Precipitated samples were vortex-mixed and centrifuged for 10 minutes. Fifty microliters of the supernatant were transferred to a clean vial and diluted with 50 µL of water.

Liquid chromatography

The processed sample was injected onto a Vanquish Flex Binary UHPLC system connected to an Orbitrap Exploris 120 mass spectrometer. Chromatographic separation was achieved by gradient elution on a Thermo Scientific™ Biphenyl 100 x 2.1 mm (2.6 µm) column (P/N #17826-102130) kept at 40 °C.

Mobile phases composition was the following:

- Mobile phase A: Water + 0.1% formic acid
- Mobile phase B: Acetonitrile + 0.1% formic acid

Injection volume: 2 µL.

The LC method is described in detail in Table 3. Total runtime was 6.5 minutes.

Table 3. LC gradient profile

Time (min)	Flow (mL/min)	B (%)
0	0.5	5
1.00	0.5	5
3.00	0.5	50
4.00	0.5	50
4.01	0.5	100
5.00	0.5	100
5.10	0.5	5
6.50	0.5	5

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 included in the acquisition method for confirmation based on ion ratio.

Table 4. MS parameters

Ion source parameters	
Source type	Heated Electrospray Source Ionization (HESI)
Spray voltage - positive (V)	3,500
Sheath gas (Arb)	60
Aux gas (Arb)	10
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	320
Settings	
Mild trapping	No
Internal mass calibration	RunStart Easy-IC™
Data acquisition mode	Full Scan - ddMS
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 (%)	100
AGC target	Standard (1e6)
Polarity	Positive
Data-dependent MS ² scan properties	
Isolation window (<i>m/z</i>)	2
Collision energy type	Normalized
HCD collision energy (%)	30
Resolution (at <i>m/z</i> 200)	15,000
Scan range mode	Auto

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 just 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 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 ±16% 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.

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

The data demonstrated accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -16% and +11% (Table 5). The %CV for intra-assay precision was always below 7.6% for all the analytes. The maximum %CV for inter-assay precision including all the analytes was 14%. Results for intra- and inter-assay precision are reported in Table 6.

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

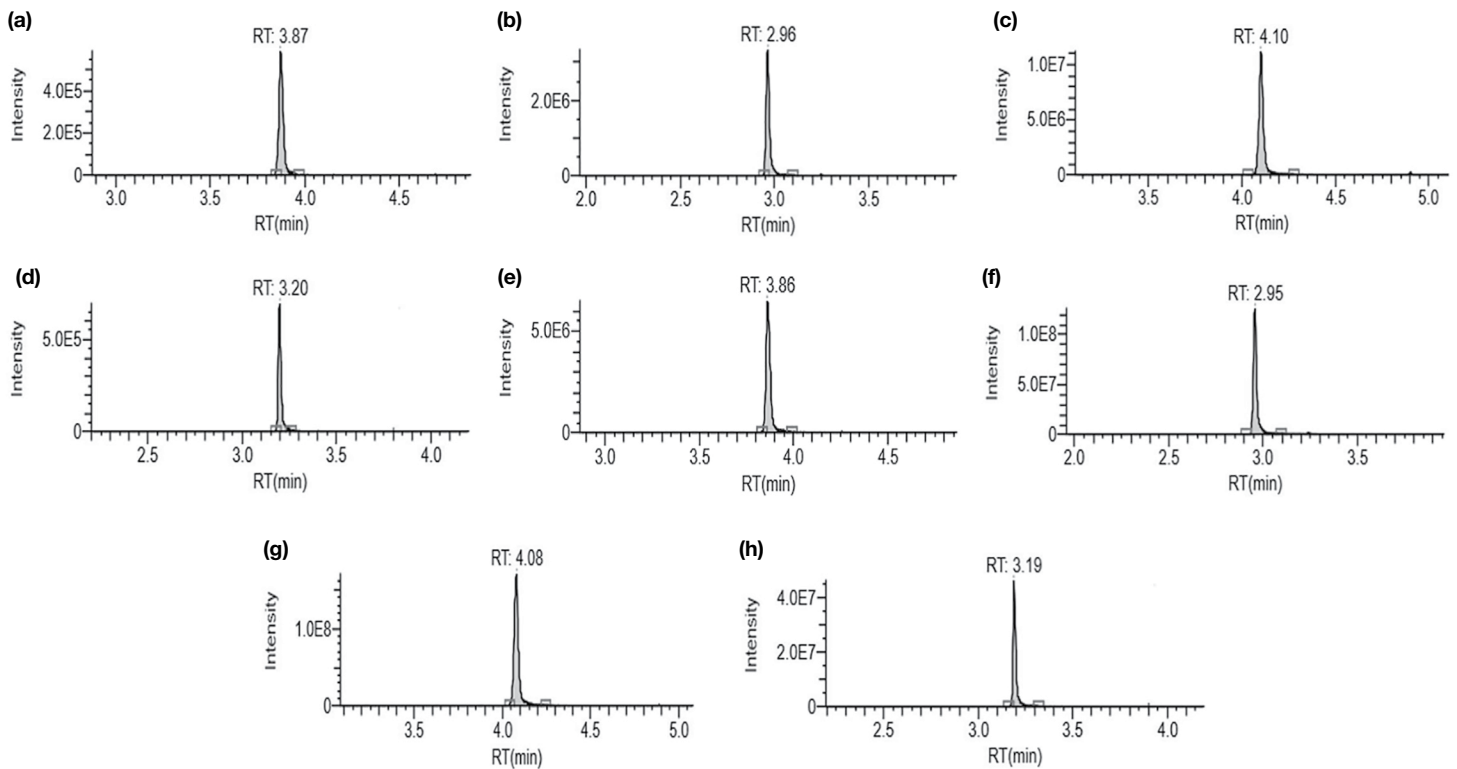


Figure 1. Representative chromatograms of the lower limits of quantification for (a) alprazolam, (b) chlordiazepoxide, (c) diazepam, (d) midazolam, (e) d₅-alprazolam, (f) d₅-chlordiazepoxide, (g) d₅-diazepam, (h) d₄-midazolam

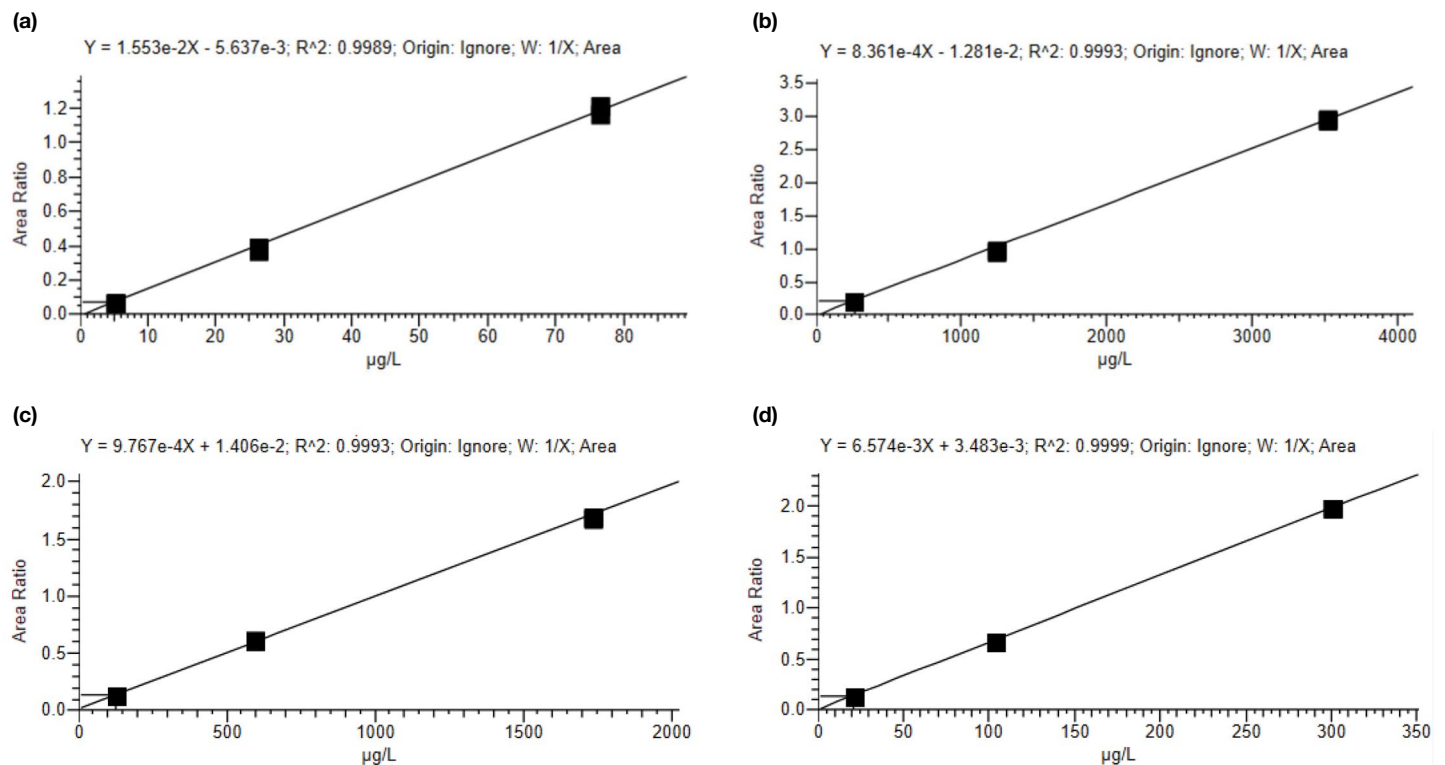


Figure 2. Representative calibration curves for (a) alprazolam, (b) chlordiazepoxide, (c) diazepam, (d) midazolam

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	41.1	-3.2
	Level II	144	159	11.0
7-Aminoclonazepam	Level I	14.3	14.2	-0.7
	Level II	48.5	47.1	-2.8
7-Aminoflunitrazepam	Level I	15.0	16.2	8.3
	Level II	49.5	53.2	7.5
7-Aminonitrazepam	Level I	63.5	68.6	8.1
	Level II	211	217	2.7
alpha-Hydroxyalprazolam	Level I	16.2	15.7	-3.0
	Level II	54.1	53.6	-0.9
alpha-Hydroxymidazolam	Level I	55.0	50.6	-8.0
	Level II	177	172	-2.7
alpha-Hydroxytriazolam	Level I	15.7	14.9	-5.1
	Level II	51.9	52.3	0.8
Alprazolam	Level I	15.2	15.0	-1.0
	Level II	52.9	51.3	-3.1
Bromazepam	Level I	93.3	93.8	0.5
	Level II	305	311	2.0
Chlordiazepoxide	Level I	631	607	-3.9
	Level II	2053	1982	-3.5
Clobazam	Level I	89.5	76.9	-14.0
	Level II	292	273	-6.6
Clonazepam	Level I	8.13	7.66	-5.8
	Level II	61.3	58.6	-4.4
Demoxepam	Level I	646	676	4.7
	Level II	2189	2227	1.7
Desalkylflurazepam	Level I	29.9	30.1	0.5
	Level II	101	102	1.0
Desmethylflunitrazepam	Level I	14.6	15.3	4.8
	Level II	50.9	52.8	3.7
Diazepam	Level I	290	301	3.9
	Level II	939	969	3.2
Estazolam	Level I	127	128	1.0
	Level II	425	434	2.2

Analyte	Control	Nominal conc. (µg/L)	Average calculated conc. (µg/L)	Bias (%)
Flunitrazepam	Level I	16.2	14.8	-8.5
	Level II	54.2	50.7	-6.5
Flurazepam	Level I	62.3	52.9	-15.1
	Level II	199	185	-7.1
Lorazepam	Level I	60.7	55.7	-8.2
	Level II	193	188	-2.7
Lormetazepam	Level I	5.65	5.36	-5.1
	Level II	18.3	17.9	-2.4
Medazepam	Level I	274	282	3.0
	Level II	838	892	6.5
Midazolam	Level I	30.2	29.5	-2.2
	Level II	78.9	80.4	1.9
Nitrazepam	Level I	41.9	40.1	-4.4
	Level II	133	137	2.7
Norclobazam	Level I	771	788	2.2
	Level II	2733	2792	2.1
Nordiazepam	Level I	219	215	-1.8
	Level II	704	715	1.6
Oxazepam	Level I	360	349	-3.1
	Level II	1205	1191	-1.1
Prazepam	Level I	271	247	-8.8
	Level II	866	822	-5.1
Temazepam	Level I	186	179	-3.7
	Level II	520	490	-5.7
Tetrazepam	Level I	126	139	10.0
	Level II	418	431	3.1
Triazolam	Level I	7.55	7.83	3.7
	Level II	24.1	25.2	4.4
Zaleplone	Level I	24.7	24.6	-0.5
	Level II	83.2	85.6	2.9
Zolpidem	Level I	128	120	-6.5
	Level II	426	410	-3.8
Zopiclone	Level I	18.9	15.8	-16.0
	Level II	66.2	59.5	-10.0

Table 6 (part 1). 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	43.2	2.2	40.9	2.6	39.0	3.9	41.1	5.1
	Level II	155	2.1	173	7.6	149	1.9	159	7.9
7-Aminoclonazepam	Level I	14.1	0.9	14.2	0.8	14.3	1.0	14.2	0.8
	Level II	45.0	0.4	48.6	0.9	47.8	0.4	47.1	4.0
7-Aminoflunitrazepam	Level I	16.1	1.5	16.7	1.2	15.9	3.2	16.2	2.5
	Level II	49.0	2.0	58.0	2.1	52.7	1.7	53.2	8.4
7-Aminonitrazepam	Level I	66.6	1.2	70.3	1.1	69.0	1.5	68.7	2.8
	Level II	211	0.7	225	1.4	215	0.5	217	3.5
alpha-Hydroxyalprazolam	Level I	15.9	2.4	15.7	2.8	15.6	1.6	15.7	1.1
	Level II	51.8	1.3	55.1	2.2	53.9	1.8	53.6	3.1
alpha-Hydroxymidazolam	Level I	52.1	0.9	49.1	1.0	50.7	1.4	50.6	3.0
	Level II	174	2.1	171	1.5	172	0.6	172	0.8
alpha-Hydroxytriazolam	Level I	15.1	3.0	14.9	2.2	14.7	3.0	14.9	1.3
	Level II	50.4	0.4	53.5	1.8	53.0	1.5	52.3	3.1
Alprazolam	Level I	15.1	1.5	14.9	0.7	15.1	1.3	15.0	1.0
	Level II	49.6	1.0	52.1	1.8	52.2	1.3	51.3	2.9
Bromazepam	Level I	94.3	0.9	93.8	1.1	93.2	2.2	93.8	0.6
	Level II	300	1.2	324	1.3	309	2.8	311	4.0
Chlordiazepoxide	Level I	616	0.6	595	0.9	609	1.0	607	1.8
	Level II	1918	0.5	2024	1.7	2004	1.9	1982	2.8
Clobazam	Level I	78.7	1.2	75.8	1.6	76.3	1.7	76.9	2.0
	Level II	266	1.4	286	7.6	266	2.9	273	4.2
Clonazepam	Level I	8.04	2.1	7.44	2.0	7.50	3.2	7.66	4.3
	Level II	56.1	1.6	60.3	2.5	59.5	1.8	58.6	3.9
Demoxepam	Level I	673	1.8	677	0.6	679	2.2	676	0.4
	Level II	2117	1.3	2253	5.2	2310	3.3	2227	4.4
Desalkylflurazepam	Level I	29.8	1.8	30.8	1.8	29.6	7.5	30.1	2.2
	Level II	96.9	2.2	105	3.7	104	2.3	102	4.4
Desmethylflunitrazepam	Level I	15.5	1.6	14.9	1.5	15.6	1.9	15.3	2.5
	Level II	51.7	1.5	54.4	2.2	52.3	1.7	52.8	2.7
Diazepam	Level I	306	0.5	298	0.5	301	1.5	301	1.3
	Level II	937	0.5	980	1.8	991	0.9	969	2.9
Estazolam	Level I	130	0.9	128	1.7	127	1.6	128	0.9
	Level II	422	0.8	439	1.1	443	2.4	434	2.6

Table 6 (part 2). 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 (%)		
Flunitrazepam	Level I	14.8	0.7	14.8	0.3	14.8	2.2	14.8	0.1
	Level II	48.2	0.7	51.7	2.2	52.2	1.4	50.7	4.3
Flurazepam	Level I	52.9	1.6	51.8	1.8	53.9	2.6	52.9	2.0
	Level II	183	2.1	188	4.2	184	2.6	185	1.3
Lorazepam	Level I	55.7	1.3	54.8	0.9	56.7	2.2	55.7	1.7
	Level II	179	1.2	195	1.2	189	1.3	188	4.2
Lormetazepam	Level I	5.31	1.4	5.26	2.2	5.51	3.3	5.36	2.5
	Level II	16.8	1.6	18.6	1.2	18.2	2.9	17.9	5.1
Medazepam	Level I	274	2.0	292	0.7	280	1.1	282	3.2
	Level II	787	3.3	950	2.0	939	2.9	892	10.2
Midazolam	Level I	30.6	1.5	29.1	1.0	28.9	1.4	29.5	3.3
	Level II	79.4	1.1	81.5	1.7	80.2	1.6	80.4	1.3
Nitrazepam	Level I	40.7	2.0	40.8	1.5	38.7	1.3	40.1	3.0
	Level II	132	0.7	143	1.6	135	2.6	137	4.2
Norclobazam	Level I	798	2.5	810	1.2	756	4.1	788	3.6
	Level II	2606	0.6	2900	4.0	2870	4.3	2792	5.8
Nordiazepam	Level I	216	0.9	216	1.1	214	1.5	215	0.5
	Level II	684	1.2	740	1.2	722	1.8	715	4.0
Oxazepam	Level I	349	0.6	349	0.7	349	1.2	349	0.1
	Level II	1155	0.8	1208	0.5	1211	1.7	1191	2.6
Prazepam	Level I	251	1.6	245	0.3	245	1.1	247	1.4
	Level II	794	0.9	835	0.9	836	0.9	822	2.9
Temazepam	Level I	186	0.5	178	0.6	174	1.3	179	3.2
	Level II	474	1.1	498	2.2	499	0.9	490	3.0
Tetraazepam	Level I	139	2.1	139	2.0	140	2.6	139	0.5
	Level II	408	1.1	427	2.5	458	0.8	431	5.8
Triazolam	Level I	7.91	1.3	7.78	1.1	7.80	0.9	7.83	0.9
	Level II	24.1	0.6	25.9	1.2	25.5	0.7	25.2	3.6
Zaleplone	Level I	25.2	1.2	23.5	2.2	25.1	3.7	24.6	3.8
	Level II	82.5	1.4	88.4	3.6	86.0	3.0	85.6	3.4
Zolpidem	Level I	120	1.5	123	0.6	116	2.8	120	2.6
	Level II	393	1.3	424	1.2	413	1.7	410	3.8
Zopiclone	Level I	18.3	1.8	15.3	4.1	14.0	2.7	15.8	13.9
	Level II	62.1	1.5	60.4	0.6	55.9	3.4	59.5	5.4

Table 7. LLOQs for all compounds

Compound Name	LLOQ (µg/L)	Compound Name	LLOQ (µg/L)
3-Hydroxybromazepam	1.66	Flunitrazepam	1.50
7-Aminoclonazepam	0.502	Flurazepam	2.08
7-Aminoflunitrazepam	2.16	Lorazepam	3.18
7-Aminonitrazepam	2.16	Lormetazepam	1.97
alpha-Hydroxyalprazolam	1.15	Medazepam	75.3
alpha-Hydroxymidazolam	2.12	Midazolam	2.05
alpha-Hydroxytriazolam	5.30	Nitrazepam	1.89
Alprazolam	5.14	Norclobazam	42.0
Bromazepam	4.20	Nordiazepam	11.6
Chlordiazepoxide	26.0	Oxazepam	11.7
Clobazam	2.77	Prazepam	8.94
Clonazepam	0.988	Temazepam	23.0
Demoxepam	23.1	Tetrazepam	9.00
Desalkylflurazepam	3.40	Triazolam	3.46
Desmethylflunitrazepam	4.78	Zaleplone	3.40
Diazepam	64.0	Zolpidem	7.10
Estazolam	4.46	Zopiclone	3.30

Conclusions

A robust, reproducible, and sensitive liquid chromatography-HRAM mass spectrometry method for clinical research for quantification of 34 benzodiazepines in human plasma was developed and implemented. The method was analytically validated on an Vanquish Flex

Binary 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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