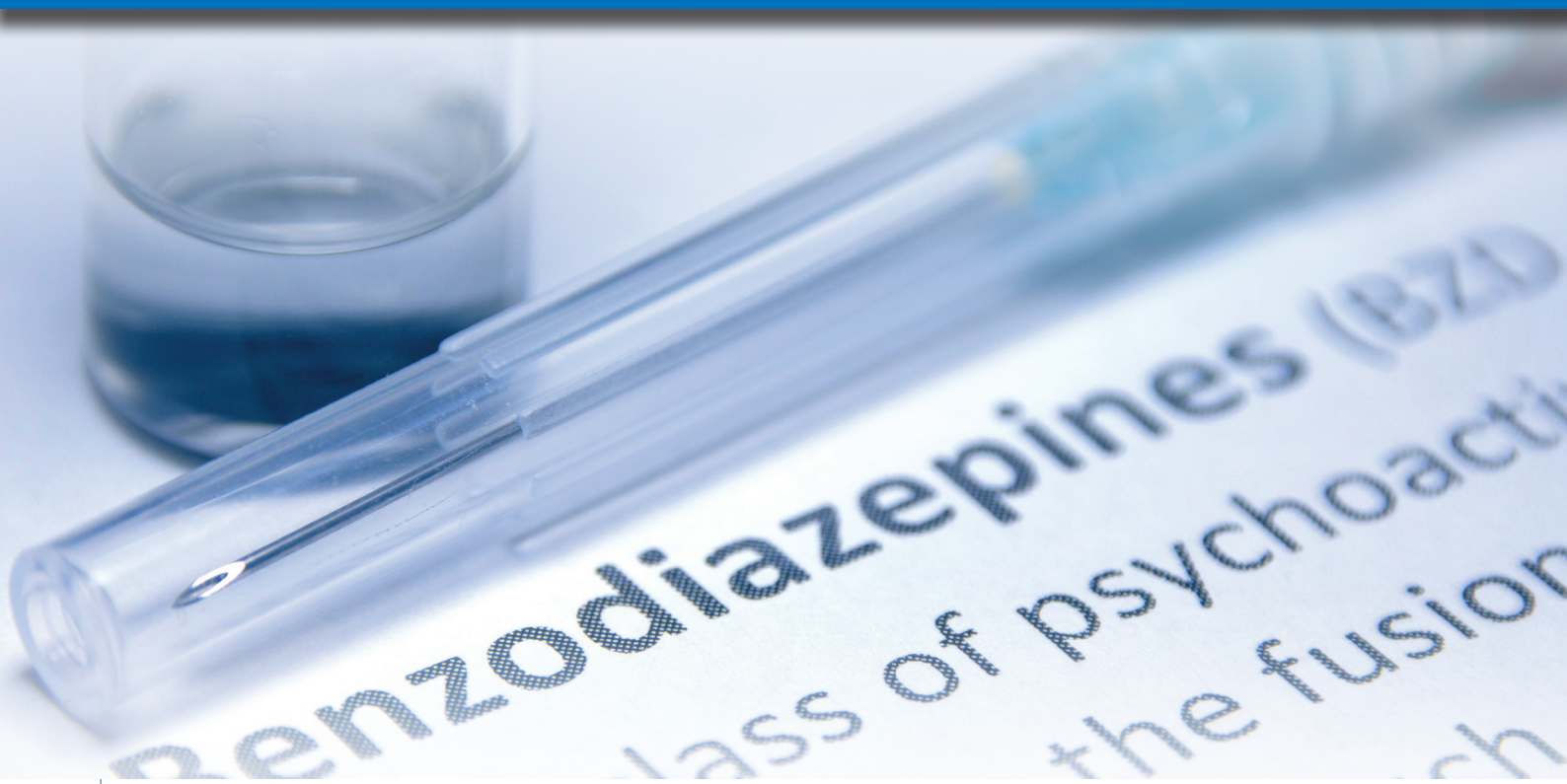

Quantitation of Benzodiazepines in Serum by UHPLC-Triple Quadrupole Mass Spectrometry

This study demonstrates a simple, rapid, and reliable method for the simultaneous detection and quantitation of 35 benzodiazepines in serum using the Bruker Elute UHPLC coupled to the EVOQ LC-TQ Elite MS/MS system. Sample preparation was performed via protein precipitation using the ClinMass TDM kit system.

Introduction

Benzodiazepines and the so-called z-drugs are among the most prescribed drugs worldwide as they have sedative, hypnotic, anxiolytic, anticonvulsant and muscle relaxant properties; however, they also bear a risk of dependence and have a very high rate of abuse. Therefore, they are not suitable for permanent treatment except as an antiepileptic agent. During benzodiazepine therapy, the ability of patients to drive is strongly impaired as reaction times are negatively affected, making this group of analytes of interest for forensic toxicology as well.

The half-life of benzodiazepines varies from a few hours to some days. Often the metabolites are also pharmacologically effective, and those with a long half-life can accumulate in the body.

Keywords: Benzodiazepines, serum, quantitation, therapeutic drug monitoring

Authors: Rafaela Martin, Zoltan Czentnar; Bruker Daltonik GmbH, Bremen, Germany

Benzodiazepines all have a similar chemical backbone consisting of a benzene ring combined with a diazepine ring with different substituents.

This manuscript describes a UHPLC-triple quadrupole mass spectrometry method to rapidly and reliably detect and quantify 35 benzodiazepines and z-drugs in human serum.

Experimental

The analysis was performed on an EVOQ LC-TQ Elite MS/MS system coupled to an Elute UHPLC using the ClinMass® TDM Platform (RECIPE Chemicals + Instruments GmbH, Munich, Germany) which included the mobile phases, autosampler washing solution, precipitation reagent, and the HPLC column with prefilter. Serum calibrators and quality control serum samples were from the ClinMass Add-On Set for 35 Benzodiazepines (MS9500), also provided by RECIPE.

Sample Preparation

Following the ClinMass kit protocol, 100 µL of precipitation reagent containing isotopically labelled internal standards were added to 50 µL serum samples and vortexed for 30 seconds. After centrifugation for 5 minutes at 10,000 x g, the supernatants were transferred to HPLC vials for analysis by UHPLC-MS/MS.

Table 1: Mass Spectrometry Method Conditions

Liquid Chromatography	
Instrument	Bruker Elute UHPLC
Column	ClinMass® TDM MS9030 with Prefilter MS9032 (RECIPE)
Mobile Phase A	Included within the ClinMass MS9000 Kit (RECIPE)
Mobile Phase B	Included within the ClinMass MS9000 Kit (RECIPE)
Gradient	0.00 – 0.21 min 10% B 0.21 – 0.30 min to 24% B 0.30 – 3.00 min to 26% B 3.00 – 6.20 min to 60% B 6.20 – 6.21 min to 85% B 6.21 – 6.40 min 85% B 6.40 – 6.41 min to 10% B 6.41 – 6.80 min 10% B
Flow Rate	600 µL/min
Injection Volume	3 µL
Column Oven	40°C
Mass Spectrometry	
Instrument	EVOQ LC-TQ Elite MS/MS system
Ion Source	VIP H-ESI positive, 4500 V
Probe Gas	50 units at 350°C
Cone Gas	25 units at 350°C
Nebulizing Gas	50 units
Active Exhaust	on
Collision Gas	Argon, 1.5 mTorr
MRM Transitions	see Table 2

Results and Discussion

Following simple and rapid sample preparation using only 50 µl of serum, the chromatographic separation of the 35 benzodiazepines was performed in 6.8 minutes using the Elute UHPLC system. Overlaid MRM traces for all target drugs are shown in Figure 1. The quantitation of the analytes was performed using 20 internal standards. Calibration curves included three calibrator levels and provided excellent linearity with R^2 values from 0.9931 – 1.0000 (Table 2). The calibration curve of clonazepam is shown in Figure 2 as an example. Quality controls (QC) in serum with a low (QC I) and a high (QC II) concentration were measured four times. The RSD of < 9% for all analytes underscores the very good precision of the instrumentation used. The experiments also showed a high accuracy with a bias within ±10% for most of the analytes (highest value -16.3%). Detailed results are presented in Table 3. The values for precision and accuracy are well within the range required by common guidelines for quantitative results.

Table 2: Retention times, MRM transitions, calibration ranges, and coefficient of determination R^2

Analyte	Retention time (min)	Precursor Ion	Product Ion 1	CE 1 (V)	Product Ion 2	CE 2 (V)	Calibration Range [µg/L]	R^2
3-OH-Bromazepam	1.47	332.1	314.8	12	286.8	18	15.0 – 246	0.9968
7-Aminoclonazepam	1.28	286.1	121.2	29	222.1	23	4.98 – 69.9	0.9973
7-Aminoflunitrazepam	1.50	284.0	135.1	26	225.9	28	5.26 – 74.0	0.9975
7-Aminonitrazepam	1.21	252.0	121.1	24	94.2	36	21.7 – 306	0.9989
α-Hydroxyalprazolam	2.76	325.0	297.0	23	216.0	38	5.67 – 80.6	0.9939
α-Hydroxymidazolam	3.94	342.0	324.0	18	203.0	24	20.1 – 277	0.9980
α-Hydroxytriazolam	2.75	359.0	330.8	27	175.9	27	4.40 – 63.0	0.9931
Alprazolam	3.74	309.0	280.9	24	204.9	43	5.28 – 81.0	0.9988
Bromazepam	2.03	316.0	209.0	28	182.1	34	34.2 – 465	0.9989
Chlordiazepoxide	3.59	300.1	282.1	16	227.0	23	223 – 2881	0.9984
Clobazam	4.46	301.0	258.9	17	224.0	33	47.0 – 681	0.9980
Clonazepam	3.46	316.0	270.1	27	214.0	40	5.25 – 73.2	0.9998
Demoxepam	2.18	287.0	180.0	20	241.0	31	229 – 3450	0.9991
Desalkylflurazepam	4.12	289.0	140.0	27	226.0	26	10.6 – 155	1.0000
Desmethylflunitrazepam	2.89	300.0	198.0	34	254.0	20	4.54 – 77.7	0.9970
Diazepam	5.09	285.0	154.0	26	193.0	32	107 – 1401	0.9993
Estazolam	3.15	295.0	266.9	22	204.8	41	41.9 – 602	0.9988
Flunitrazepam	4.13	314.0	267.9	25	239.0	34	5.48 – 80.3	0.9990
Flurazepam	4.75	388.2	315.0	21	288.0	24	10.9 – 159	0.9998
Lorazepam	3.38	321.0	274.9	21	302.9	14	21.6 – 298	1.0000
Lormetazepam	4.59	335.0	288.9	19	177.0	40	1.84 – 26.3	0.9981
Medazepam	6.40	271.0	91.2	28	207.0	27	76.1 – 1001	0.9986
Midazolam	4.95	326.0	290.9	25	223.0	41	30.8 – 432	0.9980
Nitrazepam	3.07	282.0	235.9	23	180.0	39	22.4 – 308	0.9976
Norclobazam	3.25	287.1	244.9	16	210.0	30	251 – 4090	1.0000
Nordazepam	4.35	271.0	140.0	27	208.0	28	86.2 – 1174	0.9986
Oxazepam	2.99	287.0	240.9	28	268.9	9	123 – 1778	0.9984
Prazepam	6.16	325.0	270.9	21	140.0	34	89.9 – 1245	0.9985
Temazepam	4.23	301.0	255.0	20	283.0	10	42.2 – 607	0.9982
Tetrazepam	5.82	289.0	225.1	24	197.1	32	44.5 – 622	1.0000
Trazodone	4.71	372.0	176.0	22	148.0	31	166 – 2370	0.9999
Triazolam	3.96	343.0	307.9	25	238.8	43	4.40 – 55.7	0.9969
Zaleplon	2.53	306.1	236.1	25	264.1	20	9.30 – 136	0.9989
Zolpidem	3.58	308.0	235.0	30	263.0	23	38.0 – 602	0.9994
Zopiclone	2.23	389.0	244.9	12	216.9	31	6.90 – 108	0.9996

Table 3: Quantitative results, bias, and relative standard deviation of fourfold measurement of Quality Controls I and II

Sample	QC I				QC II			
Analyte	**Specified Value [µg/L]**	**Actual Value [µg/L]**	**Bias [%]**	**RSD [%]**	**Specified Value [µg/L]**	**Actual Value [µg/L]**	**Bias [%]**	**RSD [%]**
3-OH-Bromazepam	46.7	53.0	13.6	3.9	165	176	6.7	5.6
7-Aminoclonazepam	14.1	14.9	5.6	3.7	48.2	45.7	-5.1	4.3
7-Aminoflunitrazepam	15.5	16.9	8.8	1.1	52.9	49.5	-6.5	2.5
7-Aminonitrazepam	74.5	73.4	-1.5	1.4	252	234	-7.2	4.6
α-Hydroxyalprazolam	16.5	15.5	-5.8	5.2	54.9	54.3	-1.1	5.5
α-Hydroxymidazolam	69.0	67.7	-1.9	4.6	227	214	-5.8	2.1
α-hydroxytriazolam	13.6	13.5	-1.1	8.8	41.8	44.0	5.4	6.0
Alprazolam	15.5	15.4	-0.9	2.9	51.0	49.3	-3.3	3.1
Bromazepam	92.8	86.2	-7.1	1.8	305	278	-8.8	4.1
Chlordiazepoxide	608	660	8.6	1.3	1984	1944	-2.0	2.9
Clobazam	147	149	1.4	2.5	474	464	-2.1	2.1
Clonazepam	14.8	14.8	0.0	5.1	48.1	46.6	-3.2	2.7
Demoxepam	655	683	4.3	3.5	2204	2117	-3.9	5.8
Desalkylflurazepam	31.9	31.1	-2.6	2.4	104	103	-1.0	4.5
Desmethylflunitrazepam	15.1	15.4	2.3	2.1	49.9	51.7	3.7	2.2
Diazepam	292	301	3.1	0.4	895	908	1.4	1.3
Estazolam	129	132	2.4	2.3	444	413	-6.9	2.0
Flunitrazepam	15.7	16.1	2.6	2.3	51.8	49.6	-4.2	1.2
Flurazepam	25.2	24.5	-2.8	3.4	84.9	75.0	-11.7	5.2
Lorazepam	67.1	68.5	2.1	6.6	208	199	-4.4	8.5
Lormetazepam	6.92	7.2	4.3	2.8	22.7	22.4	-1.2	6.2
Medazepam	118	105	-11.4	5.6	393	329	-16.3	2.6
Midazolam	82.6	86.2	4.4	1.3	282	271	-3.8	3.2
Nitrazepam	63.6	67.7	6.5	1.7	214	203	-5.4	3.7
Norclobazam	850	821	-3.4	1.2	2763	2539	-8.1	4.2
Nordazepam	248	245	-1.1	1.3	797	746	-6.5	2.9
Oxazepam	372	350	-5.9	2.2	1227	1107	-9.8	1.0
Prazepam	265	260	-1.9	2.3	869	790	-9.1	1.6
Temazepam	130	128	-1.4	2.0	430	395	-8.3	2.3
Tetrazepam	126	137	8.3	2.2	414	404	-2.5	3.9
Trazodone	525	538	2.4	4.0	1708	1543	-9.7	3.3
Triazolam	12.1	12.5	3.7	7.6	38.3	38.2	-0.3	1.0
Zaleplon	26.2	27.6	5.4	1.6	86.6	88.0	1.6	4.2
Zolpidem	122	129	5.8	2.7	417	418	0.3	5.3
Zopiclone	16.5	18.6	12.9	2.2	58.6	60.6	3.4	3.7

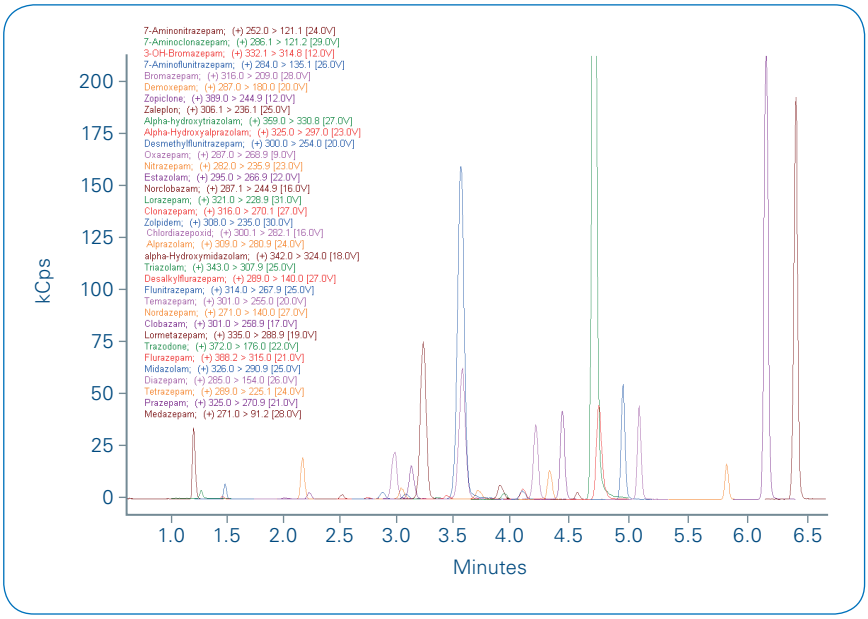

Figure 1: Overlaid MRM traces of all analytes (lowest calibrator level)

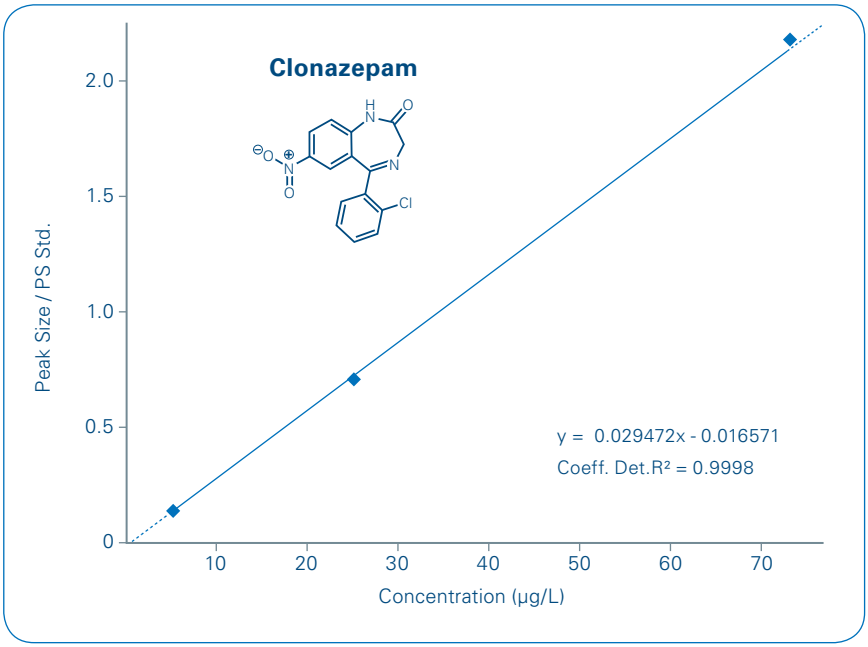

Figure 2: Calibration curve of clonazepam. Calibration range 5.25 – 73.2 µg/L

Acknowledgement

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Further Reading

To learn more about the EVOQ LC-TQ Elite MS/MS and Elute LC systems, please see:
https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/evoq/overview.html
https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/liquid-chromatography/elute-lc-series/overview.html

Please see Application Notes LCMS-138, LCMS-139, LCMS-145, and LCMS-147 for additional examples of quantitation of clinically relevant drug panels using the ClinMass TDM platform.

Conclusion

- The Bruker Elute UHPLC coupled to the EVOQ LC-TQ Elite MS/MS system in conjunction with the ClinMass TDM kit provide a quick and reliable method to easily detect and quantitate 35 benzodiazepines in serum.
- Low sample requirements (50 µl serum), easy preparation, and short run time (6.8 minutes) support high sample throughput. Linearity of calibration, precision, and accuracy were outstanding, supporting the use of this combined system in clinical research and toxicological workflows.

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Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com