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Application Data Sheet

LC-MS
Liquid Chromatograph Mass Spectrometer

Quantitation of 33 benzodiazepines by LCMS-8030 from human serum using RECIPE ClinMass® LC MS/MS Complete Kit MS6000

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Introduction

Benzodiazepines belong to a group of psychotropic drugs that are used for the treatment of anxiety and restlessness as well as for epileptic seizures. Diazepam, one of the most commonly prescribed benzodiazepines, is used to treat a number of conditions by the pharmacological action of enhancing the neurotransmitter GABA by binding to the $GABA_A$ receptor, causing CNS depression. Benzodiazepines comprise a heterocyclic unsaturated diazepine ring to which a benzene ring is attached. Most benzodiazepines also have a second six-membered ring with additional side chain groups which give different benzodiazepines their unique properties. Although considered safe, dose duration of 4 weeks is not normally exceeded because of the risk of dependence and other side effects. In situations of long-term administration the drug levels are monitored to optimize the administered drug dose.
Due to the heterogeneity of the compound class the convenience of the pre-optimized kit accelerates method development by combining many compounds and respective internal standards which would be costly to source individually. In these experiments the
RECIPE ClinMass® LC MS/MS Complete Kit for Benzodiazepines in Serum was evaluated and optimized on the LCMS-8030.

Materials and methods

The LCMS-8030 triple quadrupole mass spectrometer was coupled to a Nexera UHPLC system equipped with a 6-port 2-way switching valve (load/inject). The LC system was configured with pumps A and B performing analytical separation. Pump C was configured for sample loading to the on-line solid phase extraction (SPE) column (0.02-0.75 min) and SPE column re-equilibration (8.25-9.19 min).
Chemical standards, control samples, column and mobile phase solvents were taken from the commercially available test kit RECIPE ClinMass®
LC MS/MS Complete Kit for Benzodiazepines in Serum, MS6000 (RECIPE Chemicals + Instruments GmbH, Dessauerstraße 3, 80992 München, Germany).
MRM optimization was performed on five different optimization mixes. For all compounds the $[M+H]^+$ ion was measured and used as the precursor ion. Positive atmospheric chemical ionization (APCI) was used as ionization mode.

LC Conditions

UHPLC:	Nexera UHPLC
Mobile phase:	RECIPE kit MS6000.
Injection volume:	20 µL
Column temperature:	30° C

LCMS Conditions

Mass spectrometer:	LCMS-8030
Source conditions:	Interface: 425 ° C
Desolvation Line:	200 ° C
Heat Block:	300 ° C
Nebulizer Gas:	3 L/min
Drying Gas:	5 L/min
Interface voltage:	4.5 kV
Ionization:	APCI, positive mode
Scan Type:	MRM
Dwell time:	20 msec
Pause time:	3 msec

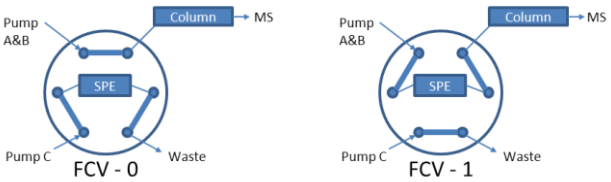
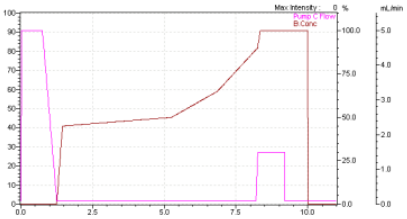


Fig. 1 LC time program; The FCV-12AH 6 port valve was used to switch between sample loading (pump C) and separation (pumps A & B).



FCV-12AH position	Time
0	0.01
1	0.75
0	8.25

Pump	Time	%B	Flow (mL/min)
A&B	0.00	0	0.7
A&B	1.25	0	0.7
A&B	1.45	45	0.7
A&B	5.25	50	0.7
A&B	6.85	65	0.7
A&B	8.25	90	0.7
A&B	8.35	100	0.7
A&B	10.00	100	0.7
A&B	10.01	0	0.7
A&B	11.00	0	0.7

Pump	Time	Flow (mL/min)
C	0.01	0.1
C	0.02	5
C	0.75	5
C	1.25	0.1
C	8.20	0.1
C	8.25	1.5
C	9.19	1.5
C	9.20	0.1
C	11.0	0.1

Table 1 Benzodiazepine compound transitions and retention times (RT).

Compound	MRM1	MRM2	RT
7-Aminoclonazepam	286.10>222.10	-	2.90
7-Aminonitrazepam	252.15>121.00	252.15>94.10	2.91
7-Aminoflunitrazepam	284.15>135.00	284.15>226.95	3.03
Zaleplon	306.00>236.10	-	3.78
Demoxepam	287.10>179.90	287.10>268.90	4.09
Desmethylflunitrazepam	300.15>254.00	300.15>198.00	4.23
a-OH-Triazolam	359.10>176.00	-	4.34
Norclobazam	287.10>244.85	287.10>209.90	4.37
Bromazepam	316.00>182.15	316.00>209.00	4.47
Clonazepam	316.10>270.00	316.10>213.90	4.56
Flunitrazepam	314.05>268.00	314.05>238.95	4.64
Nitrazepam	282.15>236.15	282.15>179.95	4.66
a-OH-Alprazolam	324.95>297.00	324.95>216.10	4.68
Estazolam	294.85>267.10	294.85>204.90	4.74
Clobazam	301.05>258.90	301.05>224.10	4.80
Triazolam	343.10>308.00	343.10>239.10	4.93
Alprazolam	308.95>204.90	-	5.11
Lorazepam	323.00>276.90	320.90>228.90	5.11
Oxazepam	287.10>240.90	287.10>269.00	5.23
Zolpidem	308.20>235.00	308.20>236.00	5.44
Desalkylflurazepam	289.10>140.10	289.10>226.00	5.50
Temazepam	301.05>255.00	-	5.60
a-OH-Midazolam	342.15>324.10	342.15>203.00	5.67
Lormetazepam	335.00>288.85	337.10>290.90	5.79
Chlordiazepoxid	300.15>227.00	-	6.17
Nordiazepam	271.05>140.05	271.05>164.95	6.52
Midazolam	325.95>291.10	325.95>249.00	6.84
Diazepam	285.10>154.00	285.10>193.10	6.99
Flurazepam	388.20>314.90	390.20>316.90	7.35
Trazodon	372.05>176.00	372.05>148.00	7.82
Tetrazepam	289.15>197.00	289.15>167.10	8.38
Prazepam	325.15>271.05	-	8.44
Medazepam	271.15>207.10	271.15>91.00	9.01

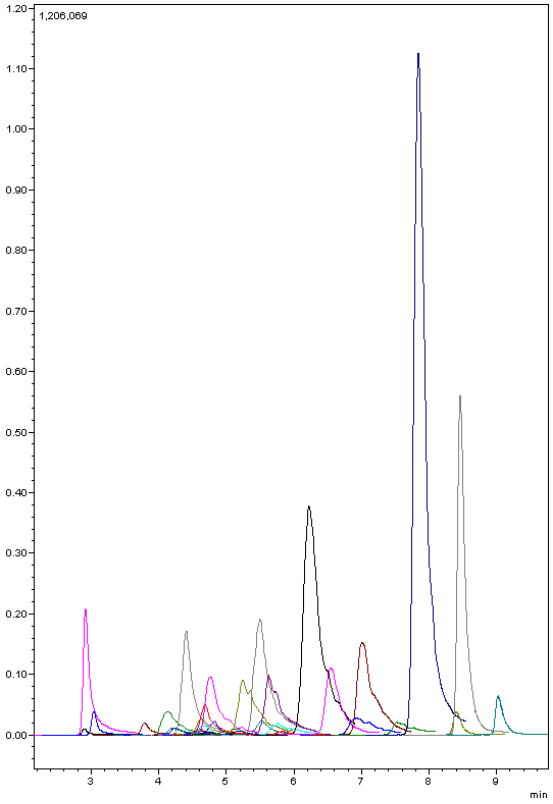


Fig. 2 Separation of 33 benzodiazepine compounds separated over 10 min using on-line SPE extraction from human serum.

Table 2 Benzodiazepine internal standard transitions and retention times (RT).

Compound	MRM1	MRM2	RT
7-Aminoclonazepam-D4	290.15>226.10	-	2.91
7-Aminoflunitrazepam-D7	291.20>138.00	-	3.03
a-OH-Triazolam-D4	363.05>243.20	-	4.33
Clonazepam-D4	320.05>274.00	-	4.53
Flunitrazepam-D7	321.20>246.20	-	4.60
Nitrazepam-D5	287.15>185.10	-	4.62
a-OH-Alprazolam-D5	330.15>301.90	-	4.65
Estazolam-D5	300.10>272.00	-	4.71
Triazolam-D4	347.05>312.00	-	4.86
Alprazolam-D5	314.10>286.00	-	5.06
Lorazepam-D4	325.05>279.10	-	5.12
Oxazepam-D5	292.10>245.90	-	5.19
Zolpidem-D6	314.00>235.30	-	5.41
Temazepam-D5	306.10>260.00	-	5.56
a-OH-Midazolam-D5	346.10>168.20	-	5.65
Chlordiazepoxid-D5	305.10>285.90	-	6.14
Nordiazepam-D5	276.05>139.90	-	6.47
Midazolam-D4	330.10>295.10	-	6.79
Diazepam-D5	290.15>154.00	-	6.93
Prazepam-D5	330.10>276.00	-	8.44

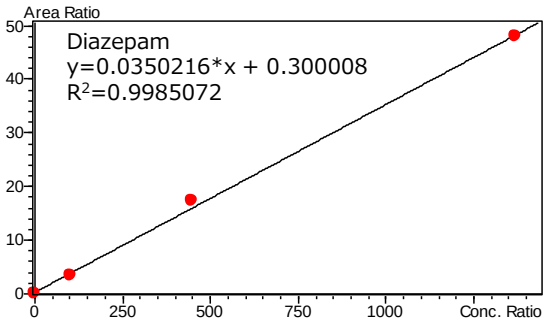


Fig. 3 Calibration curve for diazepam; a typical calibration curve for the 33 benzodiazepine compounds analyzed.

Results

The successful application of the RECIPE ClinMass® Kit for benzodiazepines demonstrated good sensitivity with calibration curves showing excellent R^2 values (Table 3). Typically serum diazepam concentrations are in the range of 0.1-1.0 mg/L in patients receiving the drug therapeutically, therefore these experiments have shown measurement in clinically relevant concentration ranges. Likewise the concentration ranges measured for other benzodiazepine compounds and metabolites were measured in clinically relevant concentration ranges.

Two control samples at high and low concentration were analyzed in duplicate to measure analytical reproducibility. The percentage relative standard deviation was typically lower than 5% from these measurements.

Table 3 Concentration range measured for 33 benzodiazepine compounds (RT = retention times).

Compound	RT	Concentration range (µg/L)	R^2
7-Aminoclonazepam	2.90	5.17-52.4	0.9992
7-Aminonitrazepam	2.91	22.1-221	0.9971
7-Aminoflunitrazepam	3.03	5.24-54.5	0.9999
Zaleplon	3.78	8.37-88.6	0.9998
Demoxepam	4.09	216-2270	0.9998
Desmethylflunitrazepam	4.23	4.54-51.1	0.9842
a-OH-Triazolam	4.34	4.62-43.3	0.9992
Norclobazam	4.37	266-2670	0.9999
Bromazepam	4.47	31.5-296	0.9952
Clonazepam	4.56	5.06-49.7	0.9973
Flunitrazepam	4.64	5.23-53.5	0.9916
Nitrazepam	4.66	21-206	0.9986
a-OH-Alprazolam	4.68	5.34-57	0.9917
Estazolam	4.74	42.8-441	0.9997
Clobazam	4.80	47.7-491	0.9925
Triazolam	4.93	4.02-40.2	0.9995
Alprazolam	5.11	5.25-55.5	0.9998
Lorazepam	5.11	20.6-205	0.9973
Oxazepam	5.23	125-1240	0.9994
Zolpidem	5.44	42.4-468	0.9930
Desalkylflurazepam	5.50	9.67-102	0.9998
Temazepam	5.60	43.3-409	0.9973
a-OH-Midazolam	5.67	21.5-203	0.9997
Lormetazepam	5.79	1.82-18.2	0.9970
Chlordiazepoxid	6.17	223-2040	0.9984
Nordiazepam	6.52	83.3-821	0.9998
Midazolam	6.84	31-308	0.9927
Diazepam	6.99	102-949	0.9985
Flurazepam	7.35	8.54-86.2	0.9994
Trazodon	7.82	165-1630	0.9970
Tetrazepam	8.38	40.9-409	0.9970
Prazepam	8.44	83.3-843	0.9996
Medazepam	9.01	42.8-426	0.9998

Conclusion

The clinical application of the benzodiazepine kit enables reliable and simple measurement of benzodiazepines from human serum.

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