

Fast, Sensitive Analysis of Benzodiazepines by LC/MS/MS

Quantify an Order of Magnitude below Typical Methods

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- Achieve full chromatographic separation of compounds with shared precursor ions
- Quantify compounds at 10ng/mL or less in urine.
- Increase accuracy with improved desolvation efficiency from highly organic mobile phase.

Benzodiazepines are widely prescribed drugs used for treating anxiety and sleep disorders. Since addiction and abuse can occur, efficient screening methods are critical to clinical, forensic, and toxicology laboratories. The liquid chromatography tandem mass spectrometry (LC/MS/MS) method presented here offers several advantages over other techniques: minimal sample preparation, fast analysis times, multiple reaction monitoring transitions for quantification and confirmation, and sensitivity down to 0.10-10ng/mL. This method uses the Allure® PFP Propyl stationary phase, which retains compounds long enough to minimize matrix interferences and chromatographically separate compounds that share the same precursor ion.

Procedure

Samples were prepared by adding 100µL of internal standard solution (1µg/mL D5-Diazepam and D3-Dioxepine) to 100µL urine, diluting with 800µL LC grade water, and centrifuging. The samples were then analyzed by LC/MS/MS. Compound separation was achieved using an Allure® PFP Propyl column and a mobile phase gradient program.

A 3200 QTrap® LC/MS/MS system equipped with a Turbo V™ source with electrospray ionization was used to develop and detect the two MRM transitions (Table 1). For each compound, MRM 1 was used to quantify, and the ratio to MRM 2 was used to confirm.

Clivid™ Drug Screen & Quant Software was used to process data and generate automatic reporting relevant to forensic guidelines. Limits of quantification were determined and the automated reporting allowed for positive confirmation based on the detected MRM ratios.

Results

By diluting the urine samples ten-fold, matrix effects are reduced (reducing ion suppression) and LOQs between 0.10ng/mL and 10ng/mL can be achieved (Table 1). Ion suppression is further reduced by using a retentive column which 1) elutes matrix interferences before the compounds of interest, and 2) allows for better desolvation efficiency due to the ability to use 90% organic in the mobile phase composition. The Allure® PFP Propyl is such a column; it has high retention and selectivity for basic drug compounds, such as benzodiazepines (Figure 1).

Figure 1 MRM transitions of 27 benzodiazepines, 3 nonbenzodiazepine hypnotics, and two internal standards on the Allure® PFP Propyl column.

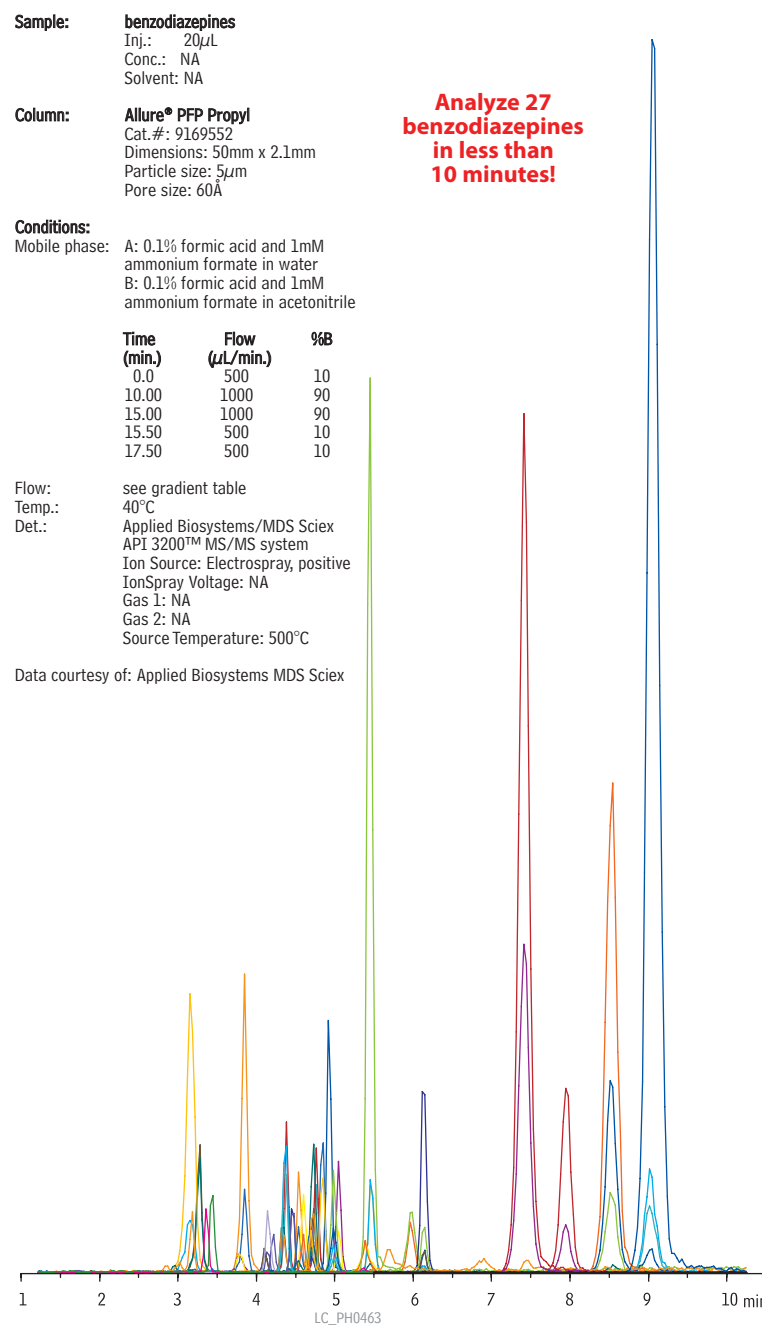


Table I MRM Transitions, retention times, and LOQ values.

Compound Name	Retention Time (min.)	Precursor Ion (amu)	MRM 1 (amu)	MRM 2 (amu)	DP	CE (MRM 1)	CE (MRM 2)	LOQ (ng/mL)
7-aminonitrazepam	3.2	252.1	121.1	94.0	51	35	53	1.0
7-aminoclonazepam	3.3	286.1	121.0	222.2	46	41	35	0.5
7-aminoflunitrazepam	3.8	284.1	135.1	226.0	51	39	49	0.5
Bromazepam	3.8	316.0/318.0	182.1	182.1	51	45	45	5.0
α -hydroxyalprazolam	4.1	325.1	297.2	204.9	51	31	59	2.0
α -hydroxytriazolam	4.1	359.0	239.2	176.0	61	63	37	5.0
Oxazepam	4.2	287.0	241.1	268.9	41	27	19	10.0
Lorazepam	4.3	321.0/323.1	275.0	277.0	41	31	27	5.0
Estazolam	4.4	295.0	205.0	267.1	51	53	31	2.0
Zaleplon	4.4	306.2	236.3	264.2	56	35	27	0.5
2-hydroxyethylflurazepam	4.5	333.1	211.2	109.0	56	51	41	1.0
Desmethyflunitrazepam	4.5	300.1	254.2	198.2	56	35	51	2.0
Nitrazepam	4.6	282.0	236.1	180.2	71	35	51	2.0
Clonazepam	4.7	316.0	270.2	214.0	56	41	51	2.0
Desalkylflurazepam	4.7	289.1	140.1	226.1	71	41	39	2.0
Temazepam	4.7	301.1/303.1	255.1	257.2	35	30	30	5.0
Triazolam	4.7	343.0	238.9	314.9	61	53	37	1.0
Alprazolam	4.8	309.1	205.1	281.1	56	53	35	1.0
Lormetazepam	4.8	335.0/337.1	289.0	291.1	41	29	29	2.0
Clobazam	4.9	301.1	259.1	224.3	46	29	47	1.0
Flunitrazepam	5.0	314.0	268.1	239.1	56	35	49	1.0
Nordiazepam	5.0	271.1	140.2	164.9	46	37	35	2.0
Zolpiclone	5.4	389.1	244.8	217.0	16	25	41	1.0
D5-Diazepam	5.4	290.1	198.2	-	55	41	-	-
Diazepam	5.5	285.0	193.2	154.1	55	41	37	1.0
Chlordiazepoxide	6.0	300.1	227.1	283.2	36	31	21	5.0
Prazepam	6.1	325.1	271.1	140.0	81	31	53	2.0
Zolpidem	7.4	308.1	235.1	236.1	56	39	35	0.2
Midazolam	7.9	326.1	291.3	222.0	56	33	63	0.5
Flurazepam	8.5	388.2	315.1	317.1	36	27	27	0.1
Medazepam	9.0	271.0	91.1	207.3	46	41	39	2.0
D3-Doxepine	9.1	283.0	107.1	-	41	35	-	-

Bar color indicates shared precursor ions. Note compounds with shared precursor ions are baseline resolved on the Allure® PFP Propyl column, as shown by retention time comparison. Data courtesy of Applied Biosystems MDS Sciex.

The Allure® PFP Propyl stationary phase provides baseline resolution for compounds sharing the same precursor ion, such as nordiazepam and medazepam. The ability to chromatographically separate compounds with similar spectra allows this method to be adapted for single stage MS, however, the LOQ values would be affected. Tandem MS is advantageous since two MRM transitions are collected, allowing quantification and confirmation to be accomplished in a single run, without loss of sensitivity.

Conclusion

The method presented here provides significant advantages over other techniques for benzodiazepine analysis: simple sample preparation, fast analysis time (less than 10 minutes), LOQs of 0.10-10ng/mL in matrix, and quantification and confirmation in a single run. Further, using the retentive Allure® PFP Propyl column eliminates coelution of matrix peaks with target compounds and assures full chromatographic resolution of analytes with shared precursor ions.

Acknowledgement

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References

Schreiber, Andre PhD, El Arbi, Houssain and Gibbons, John. 2007. A Fast and Sensitive LC/MS/MS Method for the Quantitation and Confirmation of 30 Benzodiazepines and Nonbenzodiazepine Hypnotics in Forensic Urine Samples. Applied Biosystems MDS Sciex.

Trident Direct Guard Cartridge System

Description	qty.	cat.#
High-pressure filter	ea.	25082
10mm guard cartridge holder without filter	ea.	25083
10mm guard cartridge holder with filter	ea.	25084
20mm guard cartridge holder without filter	ea.	25085
20mm guard cartridge holder with filter	ea.	25086

*The standard PEEK™ tip in Trident Direct systems is compatible with Parker®, Upchurch Scientific®, Valco™, and other CPI-style fittings. To use Trident Direct systems with Waters-style end fittings, replace the tip with cat.# 25088.

Allure® PFP Propyl Columns (USP L43) Excellent Columns for LC/MS and ELSD

Physical Characteristics:

particle size: 3 μ m or 5 μ m, spherical
pore size: 60Å
carbon load: 17%

endcap: fully endcapped
pH range: 2.5 to 7.5
temperature limit: 80°C

5 μ m Column, 2.1mm	cat. #
50mm	9169552
50mm (with Trident Inlet Fitting)	9169552-700

Allure® PFP Propyl Guard Cartridges	qty.	cat. #
10 x 2.1mm	3-pk.	916950212
10 x 4.0mm	3-pk.	916950210
20 x 2.1mm	2-pk.	916950222
20 x 4.0mm	2-pk.	916950220

Exempted Drug of Abuse Reference

Materials: Benzodiazepines

Concentration is μ g/mL. Volume is 1mL/ampul.

Compound	CAS#	Solvent		
		Code	Conc.	cat.#
alprazolam	28981-97-7	PTM	1,000	34042
bromazepam	1812-30-2	PTM	1,000	34043
chlordiazepoxide	438-41-5	PTM	1,000	34044
clobazam	22316-47-8	PTM	1,000	34045
clonazepam	1622-61-3	PTM	1,000	34046
diazepam	439-14-5	PTM	1,000	34047
flunitrazepam	1622-62-4	PTM	1,000	34049
flurazepam	1172-18-5	PTM	1,000	34050
lorazepam	846-49-1	PTM	1,000	34051
nitrazepam	146-22-5	PTM	1,000	34053
oxazepam	604-75-1	PTM	1,000	34054
prazepam	2955-38-6	PTM	1,000	34055
temazepam	896-50-4	PTM	1,000	34056
triazolam	28911-01-5	PTM	1,000	34057

PTM=purge & trap grade methanol