

Quantitative Analysis of Fourteen Benzodiazepines Listed by the Society of Forensic Toxicology Using Liquid Chromatography - Mass Spectrometry

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Overview

Purpose: To develop a quantitative method for 14 benzodiazepines that satisfies the requirements of the Drug-Facilitated Sexual Assault Committee of Forensic Toxicologists

Methods: A TSQ Quantum Access™ triple quadrupole mass spectrometer and 30-minute LC method divided into time segments

Results: A robust and reliable LC-MS/MS method was developed for the analysis of benzodiazepines in urine at the recommended detection limit of 5 or 10 ng/mL

Introduction

Benzodiazepines include a wide range of drugs used as hypnotics, tranquilizers, muscle relaxants and anticonvulsants. Misuse of these drugs is often implicated in crimes such as murder, suicide and traffic accidents. This class of compounds is frequently encountered in clinical and forensic cases. Most forensic laboratories set up benzodiazepine assays in urine, blood or hair to document drug exposure. In this method, 14 benzodiazepines and their deuterated internal standards were analyzed, with 3 SRM transitions per compound. Prezepam-D5 was used in place of deuterated Chlordiazepoxide, due to problems with the availability of the compound.

Methods

Sample Preparation: Glucuronide Hydrolysis followed by Solid Phase Extraction

LC Method: Surveyor MS Pump
Solvent A: Water, 0.1%Formic Acid
Solvent B: Acetonitrile, 0.1%Formic Acid
Column: 150 x 2.1 mm id packed with 5 µm, BDS-C8 stationary phase

MS Conditions: SPE prepped samples were analyzed on a TSQ Quantum Access equipped with an ESI+ Ion Max source in SRM data acquisition mode.

Results

Table 1 is the list of benzodiazepines supported in this method, as well as their recommended detection limits in urine.

TABLE 1. Benzodiazepines, Metabolites and Recommended Detection Limits in Urine Specified by the Society of Forensic Toxicologists

Target Analyte	Recommended Maximum Detection Limit
Alprazolam	10 ng/mL
a-hydroxy-alprazolam	10 ng/mL
Chlordiazepoxide	10 ng/mL
Clonazepam	5 ng/mL
7-amino-clonazepam	5 ng/mL
Diazepam	10 ng/mL
Flunitrazepam	5 ng/mL
7-amino-flunitrazepam	5 ng/mL
Lorazepam	10 ng/mL
Nordiazepam	10 ng/mL
Oxazepam	10 ng/mL
Temazepam	10 ng/mL
Triazolam	10 ng/mL
a-hydroxy-triazolam	5 ng/mL

FIGURE 2. Workflow

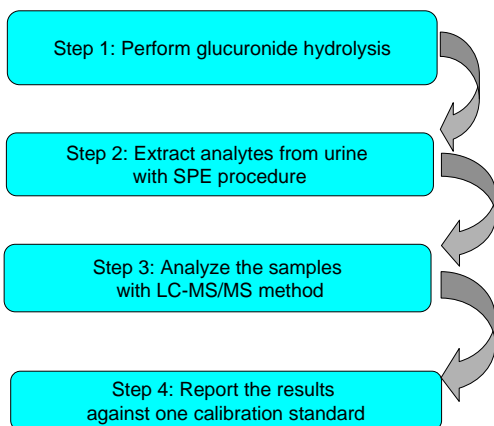


Figure 2 shows an overall workflow for the method. Initially, urine spiked samples undergo glucuronide hydrolysis. Next, analytes are extracted with SPE. Samples are then analyzed using an SRM method with values that were optimized for each analyte. This data is compared to one standard to check whether the concentration of the analyte is above or below the specified cutoff.

FIGURE 2. MS/MS Transitions of 14 Analytes

Analyte	Retention Time (min)	Parent Ion/Tube Lens Voltage	Quantifying Ion	Confirming Ion #1	Confirming Ion #2
7-amino-clonazepam	7.12	286/90	222@22	121@32	195@25
7-amino-flunitrazepam	7.83	284/97	135@25	226@31	240@40
a-hydroxy-triazolam	8.69	359/105	175.7@23	277@32	331.1@29
a-hydroxy-alprazolam	8.76	325/103	243@31	216@42	205@44
Oxazepam	9.16	287/79	241@21	163@41	104@31
Lorazepam	9.43	321/92	275@22	229@38	194@41
Clonazepam	9.63	316/96	270@24	214@41	241@30
Triazolam	9.70	343/103	308@26	239@36	315@24
Alprazolam	9.66	309/103	205@39	281@27	274@26
Flunitrazepam	10.23	314/92	268@27	239@38	183@47
Temazepam	10.26	301/84	255@24	177@44	193@40
Nordiazepam	10.94	271/95	208@26	140@27	165@24
Diazepam	12.17	285/92	193@32	222@30	154@29
Chlordiazepoxide	14.47	300/83	227@23	165@45	241@21

TABLE 2. SPE Extraction Efficiencies

Compound	%Recovery
7-amino-clonazepam	41
7-amino-flunitrazepam	29
a-hydroxyalprazolam	87
a-hydroxytriazolam	109
Oxazepam	128
Lorazepam	108
Clonazepam	86
Triazolam	92
Alprazolam	86
Flunitrazepam	111
Temazepam	99
Nordiazepam	96
Diazepam	95
Chlordiazepoxide	75

FIGURE 3. TIC Chromatogram of 14 Benzodiazepines and Internal Standard in Processed Urine Samples

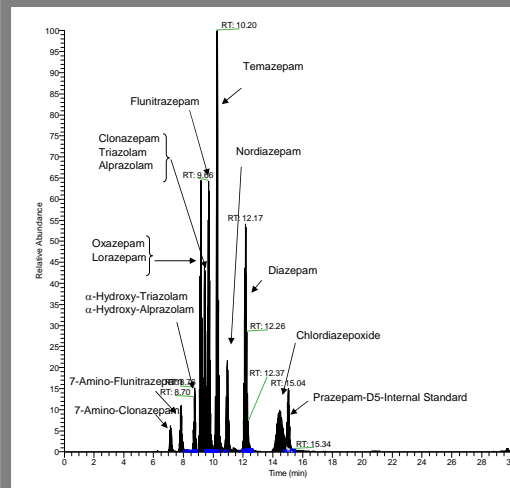


FIGURE 3. Time Segments

Segment Number	Time (min)	Analyte	Precursor Ion [M+H] ⁺
1	0 - 8.3	7-amino-clonazepam	286.1
		7-amino-flunitrazepam	284.2
2	8.3 - 9.2	a-hydroxy-alprazolam	325.0
		a-hydroxy-triazolam	359.1
		Oxazepam	287.1
3	9.2 - 10.3	Oxazepam	287.1
		Lorazepam	321.0
		Clonazepam	316.0
		Alprazolam	309.2
		Triazolam	343.0
		Temazepam	301.0
4	10.3 - 11.4	Flunitrazepam	314.0
		Temazepam	301.0
		Nordiazepam	271.0
5	11.4 - 14.1	Diazepam	285.0
		Chlordiazepoxide	300.1
6	14.1 - 20.0	Chlordiazepoxide	300.1
		Prazepam - Internal Standard	325.1

FIGURE 4. Chromatograms From Time Segment 3

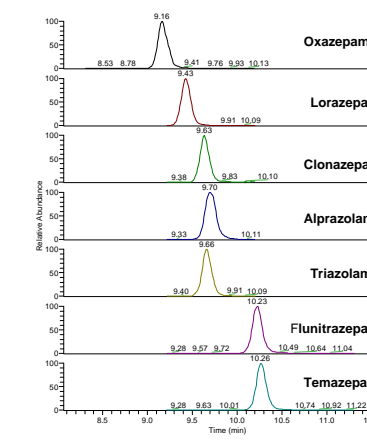


FIGURE 5. Method Validation

Compound	QC1		QC2	
	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay
7-Amino-Clonazepam	5.6	6.9	8.1	1.2
7-Amino-Flunitrazepam	6.0	9.7	4.6	9.7
Hydroxy-Alprazolam	8.9	3.8	9.6	5.1
Hydroxy-Triazolam	13.0	8.6	10.8	12.1
Oxazepam	5.3	7.6	5.9	3.5
Lorazepam	6.0	1.4	3.0	2.7
Clonazepam	8.3	4.8	5.7	7.3
Triazolam	6.3	4.3	5.0	2.7
Alprazolam	4.7	2.4	3.5	2.3
Flunitrazepam	5.0	3.1	4.5	1.4
Temazepam	2.2	1.9	2.5	1.5
Nordiazepam	5.1	3.2	5.8	3.1
Diazepam	1.4	2.7	1.8	0.9
Chlordiazepoxide	15.0	2.4	8.8	9.9

Five QC1 samples (15% below standard concentration) and five QC2 samples (15% above standard concentration) were processed and analyzed in three batches to obtain intra and inter assay variability.

FIGURE 6. Method Variability

Compound	%RSD
7-amino-clonazepam	1.2
7-amino-flunitrazepam	1.5
a-hydroxyalprazolam	4.0
a-hydroxytriazolam	3.4
Oxazepam	3.6
Lorazepam	5.9
Clonazepam	4.7
Triazolam	4.7
Alprazolam	3.2
Flunitrazepam	3.5
Temazepam	3.9
Nordiazepam	3.5
Diazepam	1.8
Chlordiazepoxide	4.6

LC-MS method variability was evaluated by analysis of 10 replicate injections, one processed urine sample spiked with all analytes at cut off value concentrations.

Conclusions

A robust and reliable LC-MS/MS method has been developed for the analysis of benzodiazepines in urine at the recommended detection limit of 5-10 ng/mL. Intra Assay and Inter Assay variabilities were 15% or lower. To obtain a success rate of 99% for QC samples prepared in concentrations of +/-15% of cut off concentration, it is recommended that samples are analyzed in triplicate and mean values reported. A method using fast LC chromatography (<5 min) is currently being validated.

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