

Evaluation of an LC-MS/MS Research Method for the Analysis of 33 Benzodiazepines and their Metabolites

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Key Words

TSQ Vantage, Transcend TLX-1, benzodiazepines

Goal

The goal of this work was to evaluate the RECIPE[®] ClinMass[®] LC-MS/MS Complete Kit for research purposes using an online method analysis of 33 benzodiazepines and benzodiazepine metabolites in serum.

Introduction

Benzodiazepines are prescribed for the management of anxiety, sleeping disorders, muscle spasms, and seizures. Benzodiazepines are widely viewed as safe drugs that have relatively few side effects. However, high dosages over prolonged periods can lead to tolerance, which leads to a loss of efficacy and/or physical and psychological dependence, resulting in severe withdrawal symptoms. Benzodiazepines can also be abused in cases of crime, suicide, and drug-facilitated sexual assault. While benzodiazepine intoxication alone is rarely fatal, concurrent use with alcohol or other drugs can be life-threatening. For all of these reasons, additional research into dosage and effects of benzodiazepines is necessary.

Benzodiazepines are active at very low concentrations and have short half lives; therefore, it is of great interest to clinical researchers and forensic toxicologists to simultaneously analyze benzodiazepines and their metabolites in biological samples. However, the analysis is not always easy because of very low blood concentrations and the complexity of detecting multiple drugs at the same time, especially in a biological matrix. Typically, benzodiazepines are quantified in serum.



In this work, the RECIPE kit for benzodiazepines analysis in serum was evaluated for research purposes using a Thermo Scientific[™] Transcend[™] TLX-1 system for performing both the online solid-phase extraction (SPE) and the chromatography. The Transcend system was coupled to a Thermo Scientific[™] TSQ Vantage[™] triple-stage quadrupole mass spectrometer for quantitative analysis. The RECIPE kit allows the analysis of 33 benzodiazepines and their metabolites in 12–15 minutes for research applications, such as those discussed in this application note. It includes the SPE column, analytical column, mobile phases, optimization mixtures, calibrators, quality controls, and an internal standards solution that integrates 20 deuterated internal standards. One kit is sufficient for up to 200 samples.

Experimental

The research method was applied as described in the RECIPE ClinMass LC-MS/MS Complete Kit instructions, with the exception of the loading flow rate, which is described in the HPLC method.

Sample Preparation

As described in the kit instructions, 50 μL of each calibrator and quality control was vortexed for 10 s in a sample preparation tube with 50 μL of internal standards solution. The sample was then centrifuged for 10 min at 10,000 rpm, and 20 μL of the supernatant was injected into the LC-MS/MS system.

HPLC Method

The kit includes an online solid phase extraction (SPE) column and an HPLC separation column that are integrated in a valve system to operate by column switching. A Transcend TLX-1 system was used to perform this column switching. The plumbing diagram is shown in Figure 1.

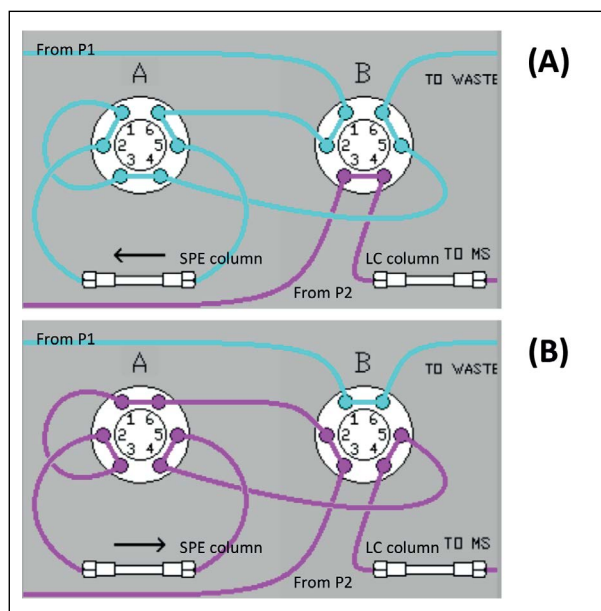


Figure 1. Plumbing of the Transcend system to perform column switching, with load (A) and inject (B) position, P1 is the loading pump and P2 the elution pump

The RECIPE kit was used as described in the instructions for this research purpose; however, the loading flow rate was 2 mL/min instead of the 5 mL/min described in the kit manual. This increased the time of analysis from 11 min to 12–15 min, but it did not impact the quality of the obtained data. In the first step, the valves are in load position (Figure 1A) where the sample is loaded onto the SPE column for the extraction of the analytes from the biological matrix. This step takes 1.9 min. In step two, the valves are switched to the inject position (Figure 1B) where the analytes extracted on the SPE column are eluted to the HPLC column by backflushing with mobile phase for 7.5 min. The analytes are then chromatographically separated with a gradient. For the last step, the valves are switched back to a loading position (Figure 1A) and both columns are re-equilibrated for the next injection. This step lasts 2.75 min.

MS Method

Mass spectrometric analysis was performed using a TSQ Vantage triple-stage quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. Source parameters are summarized in Table 1. MS analysis was performed in positive-ion selected-reaction monitoring (SRM) mode. The optimized SRM parameters for all the analytes and internal standards are presented in Table 2. The cycle time was set to 600 ms with a data acquisition window of 4 min for each analyte.

Table 1. Optimized source parameters

Ion Source	APCI, Positive
Resolution Q1 and Q3	0.7 amu
Discharge Current	5.0 μA
Vaporizer Temperature	450 $^{\circ}\text{C}$
Sheath Gas Pressure	30 au
Aux Gas Pressure	10 au
Capillary Temp	250 $^{\circ}\text{C}$
Collision Pressure	1.5 mTorr

Results and Discussion

Calibration curves were plotted for each analyte with the three calibrators provided in the kit. The regression model for all the analytes was linear with different weighting according to the analyte. The limits of quantification (LOQ) were obtained by diluting the first calibrator with blank serum either two times or five times (the blank serum is the 0 calibrator solution). The LOQ were then determined as the lowest concentration for which the %RSD for 5 injections was less than 20% and the bias was less than 20%. The weighting, internal standards, correlation factor, and LOQ of the analytes are presented in Table 3. Examples of chromatograms obtained at the LOQ for some of the analyzed compounds are presented in Figure 2. As can be seen in Table 3, good linearity was obtained for all of the analytes in the concentration ranges of the kit calibrators. A blank sample injected after the upper limit of quantification (ULQ) was used to evaluate carryover. The carryover was less than 10% of the signal obtained for the LOQ for all the analytes tested with this kit.

Table 2. SRM parameters used for the analysis

Compound	Retention Time (minutes)	Precursor Ion	Product Ion	S-Lens	Collision Energy
7-Aminoclonazepam	4.56	286.1	222.2	114	24
7-Aminoflunitrazepam	4.70	284.1	135.1	101	26
7-Aminonitrazepam	4.56	252.1	121.1	96	26
α -OH-Alprazolam	6.20	325.1	297.1	102	24
α -OH-Midazolam	7.24	342.1	324.1	102	19
α -OH-Triazolam	5.95	359.1	331.0	101	23
Alprazolam	6.66	309.1	205.1	112	38
Bromazepam	5.73	316.0	182.1	100	31
Chlordiazepoxide	7.64	300.1	227.1	95	24
Clobazam	6.35	301.1	259.1	96	19
Clonazepam	6.10	316.1	270.1	114	23
Demoxepam	5.67	287.1	178.0	91	21
Desalkylflurazepam	7.02	289.1	140.1	123	28
Desmethylflunitrazepam	5.80	300.1	254.1	95	23
Diazepam	8.47	285.1	193.1	91	28
Estazolam	6.31	295.1	267.1	119	22
Flunitrazepam	6.20	314.1	268.1	89	25
Flurazepam	9.16	388.2	315.1	117	20
Lorazepam	6.67	323.0	277.1	100	21
Lormetazepam	7.32	335.1	289.1	88	21
Medazepam	10.57	271.1	207.1	86	26
Midazolam	8.41	326.1	291.1	129	25
Nitrazepam	6.20	282.1	236.1	95	23
Norclobazam	5.96	287.1	245.1	81	19
Nordiazepam	7.98	271.1	140.1	99	27
Oxazepam	6.76	287.1	241.1	106	21
Prazepam	10.03	325.1	271.1	100	21
Temazepam	7.12	301.1	255.1	96	22
Tetrazepam	9.96	289.1	225.2	117	28
Trazodone	9.34	372.2	148.1	115	32
Triazolam	6.49	343.1	308.1	146	25
Zaleplone	5.43	306.1	236.1	100	26
Zolpidem	7.10	308.2	235.2	116	33

Table 3. Calibration parameters and LOQ

Compound	Internal Standard	Weighting	R ²	LOQ (µg/L)
7-Aminoclonazepam	7-Aminoclonazepam-D4	1/X	0.999	2.6
7-Aminoflunitrazepam	7-Aminoflunitrazepam-D7	1/X ²	0.995	2.6
7-Aminonitrazepam	7-Aminoclonazepam-D4	1/X	0.995	22.1
α-OH-Alprazolam	α-OH-Alprazolam-D5	1/X	0.993	2.7
α-OH-Midazolam	α-OH-Midazolam-D4	1/X	0.995	10.8
α-OH-Triazolam	α-OH-Triazolam-D4	1/X	0.997	4.6
Alprazolam	Alprazolam-D5	1/X	0.996	1.1
Bromazepam	7-Aminoflunitrazepam-D7	1/X	0.997	15.8
Chlordiazepoxide	Chlordiazepoxide-D5	1/X ²	0.993	111.5
Clobazam	Triazolam-D4	1/X ²	0.992	9.5
Clonazepam	Clonazepam-D4	1/X ²	0.994	2.5
Demoxepam	Clonazepam-D4	1/X	0.997	216.0
Desalkylflurazepam	Temazepam-D5	1/X ²	0.993	9.7
Desmethylflunitrazepam	Clonazepam-D4	1/X ²	0.990	4.5
Diazepam	Diazepam-D5	1/X ²	0.993	20.4
Estazolam	Estazolam-D5	1/X	0.996	21.4
Flunitrazepam	Flunitrazepam-D7	1/X	0.990	5.2
Flurazepam	Prazepam-D5	Equal	0.996	4.3
Lorazepam	Lorazepam-D4	1/X ²	0.996	20.6
Lormetazepam	Nordiazepam-D5	1/X ²	0.989	1.8
Medazepam	Nordiazepam-D5	Equal	0.997	8.6
Midazolam	Midazolam-D4	Equal	0.995	15.5
Nitrazepam	Nitrazepam-D5	1/X ²	0.997	10.5
Norclobazam	α-OH-Triazolam-D4	1/X	0.997	133.0
Nordiazepam	Nordiazepam-D5	1/X	0.997	16.7
Oxazepam	Oxazepam-D5	1/X	0.995	62.5
Prazepam	Prazepam-D5	1/X	0.999	41.7
Temazepam	Temazepam-D5	1/X ²	0.996	21.7
Tetrazepam	Prazepam-D5	Equal	0.999	8.2
Trazodone	Prazepam-D5	1/X	0.997	82.5
Triazolam	Triazolam-D4	1/X	0.988	4.0
Zaleplone	7-Aminoflunitrazepam-D7	1/X	0.999	4.2
Zolpidem	Zolpidem-D6	Equal	0.998	21.2

The intraday and interday analytical accuracies and variability are presented in Tables 4 and 5, respectively. For each analyte, intraday variability and accuracy were determined by analyzing five samples at each QC level. For interday variability and accuracy, the process performed for intraday data was repeated on three different days. For the intraday study, the accuracy obtained was between 94% and 112% with a %RSD less than 12%. In the case of interday assays, the accuracy was between 93% and 111% with a %RSD less than 13%. The obtained results show a low variability for the two QC levels as well as good accuracy.

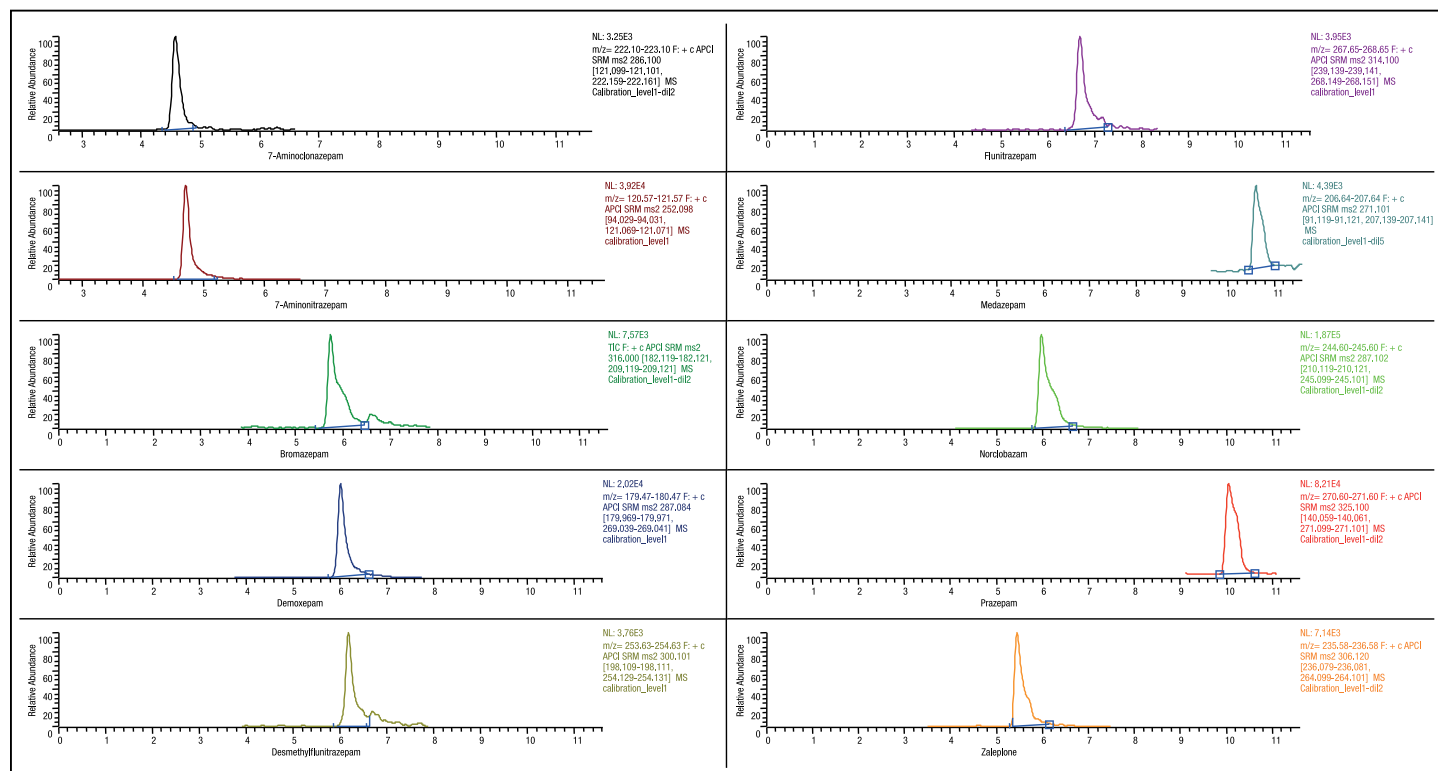


Figure 2. Chromatograms of the SRM transitions of some commonly found benzodiazepines at the LOQ

Table 4. Intraday repeatability and accuracy for QC1 and QC2

Compound	QC 1				QC 2			
	Specified Concentration	Measured Concentration	%RSD	%Accuracy	Specified Concentration	Measured Concentration	%RSD	%Accuracy
7-Aminoclonazepam	16.0	15.5	3.8	97	52.4	54.6	5.2	104
7-Aminoflunitrazepam	16.6	16.8	8.6	101	54.5	55.9	3.0	103
7-Aminonitrazepam	66.4	68.5	3.9	103	221	237	5.1	107
α -OH-Alprazolam	16.7	17.4	7.1	104	57	59.4	3.9	104
α -OH-Midazolam	64.6	65.4	3.8	101	203	217	4.1	107
α -OH-Triazolam	13.6	13.7	8.8	101	43.3	45.7	2.9	105
Alprazolam	15.4	15.7	9.2	102	55.5	55.2	5.8	99
Bromazepam	94.3	97.0	6.9	103	296	320	2.3	108
Chlordiazepoxide	621	602	3.1	97	2040	2150	3.3	105
Clobazam	143	144	7.7	101	491	489	4.4	100
Clonazepam	14.6	14.6	5.2	100	49.7	54.7	7.5	110
Demoxepam	682	673	5.5	99	2270	2350	4.4	103
Desalkylflurazepam	31.4	29.3	9.6	94	102	107	5.0	94
Desmethylflunitrazepam	15.2	14.4	4.6	95	51.1	54.2	3.8	106
Diazepam	292	297	3.4	102	949	941	5.2	99
Estazolam	132	127	2.4	96	441	447	6.6	101
Flunitrazepam	15.9	16.8	12.1	106	53.5	54.7	8.2	102
Flurazepam	25.9	26.9	5.4	104	86.2	93.0	4.7	108
Lorazepam	62.1	67.3	5.6	108	205	210	4.2	103
Lormetazepam	5.46	5.70	9.0	104	18.2	18.9	9.3	104
Medazepam	127	126	5.1	99	426	437	6.1	102
Midazolam	93.5	91.1	4.7	97	308	316	4.1	103
Nitrazepam	62.6	66.5	4.6	106	206	215	3.0	104
Norclobazam	835	811	3.2	97	2670	2770	3.8	104
Nordiazepam	239	245	4.2	103	821	804	4.3	98
Oxazepam	377	383	7.6	101	1240	1240	6.9	100
Prazepam	262	256	1.7	98	843	862	1.6	102
Temazepam	128	135	3.7	105	409	459	1.8	112
Tetrazepam	123	124	1.1	101	409	422	1.6	103
Trazodone	516	503	4.3	98	1630	1810	9.0	111
Triazolam	12.1	11.6	8.4	96	40.2	40.0	11.1	100
Zaleplone	26.8	27.3	8.5	102	88.6	94.1	5.6	106
Zolpidem	139	131	2.3	94	468	463	3.4	99

Table 5. Interday repeatability and accuracy for QC1 and QC2

Compound	QC 1				QC 2			
	Specified Concentration	Measured Concentration	%RSD	%Accuracy	Specified Concentration	Measured Concentration	%RSD	%Accuracy
7-Aminoclonazepam	16.0	16.7	6.3	104	52.4	53.9	5.3	103
7-Aminoflunitrazepam	16.6	17.4	6.6	105	54.5	53.2	6.5	98
7-Aminonitrazepam	66.4	70.7	5.8	107	221	224	6.1	101
α -OH-Alprazolam	16.7	17.8	7.4	107	57.0	56.8	5.7	100
α -OH-Midazolam	64.6	68.0	9.2	105	203	212	8.0	104
α -OH-Triazolam	13.6	13.4	6.3	98	43.3	43.5	5.3	100
Alprazolam	15.4	16.0	10.3	104	55.5	52.5	6.9	95
Bromazepam	94.3	91.9	12.9	97	296	310	7.9	105
Chlordiazepoxide	621	607	8.8	98	2040	2090	8.2	103
Clobazam	143	146	6.8	102	491	473	5.9	96
Clonazepam	14.6	15.0	5.6	103	49.7	52.7	5.9	106
Demoxepam	682	711	7.5	104	2270	2280	4.3	100
Desalkylflurazepam	31.4	29.5	10.1	94	102	102	7.3	100
Desmethylflunitrazepam	15.2	15.3	7.3	101	51.1	51.8	6.3	101
Diazepam	292	309	8.8	106	949	945	9.5	100
Estazolam	132	129	3.7	98	441	437	7.1	99
Flunitrazepam	15.9	17.4	9.5	109	53.5	52.6	8.7	98
Flurazepam	25.9	27.3	13.4	106	86.2	89.5	8.1	104
Lorazepam	62.1	66.0	6.1	106	205	200	5.7	97
Lormetazepam	5.46	6.00	11.0	111	18.2	18.8	8.8	103
Medazepam	127	131	6.9	103	426	437	12.5	102
Midazolam	93.5	89.0	4.4	95	308	307	4.3	100
Nitrazepam	62.6	69.3	6.9	111	206	203	6.7	99
Norclobazam	835	835	6.3	100	2670	2640	5.6	99
Nordiazepam	239	262	11.6	110	821	802	8.1	98
Oxazepam	377	387	9.3	103	1240	1250	6.5	101
Prazepam	262	265	3.7	101	843	845	4.1	100
Temazepam	128	134	5.8	105	409	437	6.2	107
Tetrazepam	123	127	4.4	103	409	416	7.5	102
Trazodone	516	501	6.6	97	1630	1750	11.6	107
Triazolam	12.1	11.7	6.1	97	40.2	39.7	6.8	99
Zaleplone	26.8	27.1	9.8	101	88.6	85.2	11.2	96
Zolpidem	139	130	7.8	93	468	450	8.0	96

Conclusion

The analysis of benzodiazepines for research purposes presents a challenge due to their low concentrations, their short half life, and their diversity. In this work, the RECIPE ClinMass® complete kit for the quantification of 33 benzodiazepines and metabolites in serum was evaluated for research use with a TSQ Vantage triple quadrupole. The online SPE configuration was achieved by using a Transcend TLX-1 system. HPLC coupled to tandem mass spectrometry may be a suitable tool for the quantification of benzodiazepines in research applications since it can deal with the differences in polarities of the benzodiazepines and their metabolites. The use of SRM detection increases the analytical specificity needed to attain the low quantification ranges for these types of molecules. Finally, the use of LC-MS/MS simplifies sample preparation since the matrix clean-up is performed by online SPE.

This research method showed good results in terms of variability, precision, and dynamic range requirements for a clinical research method.

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