Nota de aplicación

Quantitative Analysis of 21 Benzodiazepine Drugs, Zolpidem and Zopiclone in Serum Using UPLC-MS/MS

Mark Roberts, Michelle Wood

Waters Corporation

Abstract

This application note describes how to develop a UPLC-MS/MS method for the quantitation of 21 benzodiazepines, Zolpidem and Zopiclone in human serum.

Introduction

Benzodiazepines (Figure 1) are the most frequently prescribed drugs in the western world. They are indicated for a variety of disorders including: anxiety; insomnia; agitation; muscle spasms and alcohol withdrawal. They work primarily due to their interaction with the $GABA_A$ receptor.

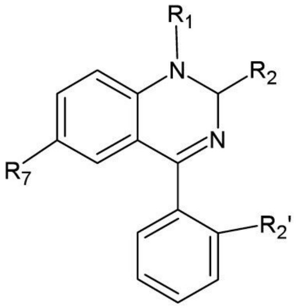

Figure 1. Core Structure of the Benzodiazepines

Many of the benzodiazepines are potentially addictive[1] and longterm use can lead to dependency. Consequently their analysis is of key importance in both clinical and forensic settings. Misuse of these medications by vulnerable populations such as the elderly[2] or the mentally-ill[3] is common. The elderly are at particular risk, as sensitivity to benzodiazepines tends to increase with age thus, these analytes are commonly reported in self-poisonings[4-7]. Recreational use of benzodiazepines has also been reported; they are often used in combination with other narcotics e.g., they can be used to augment the ‘high’ of heroin or cocaine or can be used to reduce the after-effects of LSD or amphetamine use[8]. Drug-facilitated crime often involves benzodiazepines due to their sedative properties and amnesia-producing effects[9].

Traditional techniques used for the quantitation of benzodiazepines include gas chromatography/mass spectrometry (GC-MS) and high performance liquid chromatography/mass spectrometry (HPLC-MS). The former typically requires inclusion of an additional derivatisation step and analysis can be problematic due to the thermo-labile nature of some of the analytes within this class. In contrast, HPLC-MS can separate a wide range of substances without the need for derivatisation and utilises 'softer' ionisation techniques e.g., electrospray ionisation (ESI) which allows the protonated molecular species to remain intact.

Since its introduction in 2004, UltraPerformance LC (UPLC) has repeatedly demonstrated significant advantages compared to HPLC-based methods e.g., enhanced resolution, sensitivity and throughput. Thus our aim was to develop a method utilising this technique.

We describe a quantitative method based on liquid/liquid extraction (LLE) and UPLC with tandem mass spectrometry (MS/MS). The method's performance has been evaluated using authentic samples. Data were compared to results obtained with a validated method based on HPLC-MS/MS[10].

Experimental

Materials

Samples

Twenty-seven authentic serum and plasma samples were received which had previously been analysed by a published method involving conventional SPE and HPLC-MS/MS analysis[10].

Standards, Internal Standards and Blank Serum

Standards (1 mg/mL) and their deuterated internal standards (IS) at 0.1 mg/mL were purchased from LGC Standards (Teddington, UK). Internal standards were made into a mixed stock of 5 µg/mL in water.

Blank serum was obtained from Scipac (Sittingbourne, UK). Serum calibrators (0 to 1000 ng/mL) were prepared by adding mixtures of drug standards to the blank serum.

Sample Preparation

Liquid/Liquid Extraction (LLE)

Spike:
Spike 300 µL sample/calibrator with 10 µL IS, add 150 µL borate buffer*, vortex to mix

Extract:
Add 900 µL extraction mixture**, mix

Centrifuge:
3000 rpm for 5 min

Transfer and Evaporate:
Transfer the supernatant to a clean Eppendorf tube and dry on a 40 °C heating block under nitrogen gas.

Reconstitute and Inject:
Reconstitute in 50 µL 80% water and 20% methanol, (6 x concentration step), mix and inject

**Borate buffer made using saturated solution of disodium tetraborate decahydrate. **Extraction mixture made from dichloromethane/ether/hexane (30/50/20) with 0.5% isoamyl alcohol.*

Column:	ACQUITY UPLC BEH C_{18} Column, 2.1 x 100 mm, 1.7 µm
Column Temp:	50 ˚C
Flow Rate:	400 µL/min.
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in methanol†
Injection Volume:	10 µL
Strong Wash:	Mobile Phase B (500 µL)
Weak Wash:	Mobile Phase A (1500 µL)

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†The organic mobile phase chosen for this UPLC chromatography method was methanol, avoiding the use of acetonitrile which has been more difficult to obtain due to global shortages.

Gradient:

Time/min	%A	%B	Curve
0	70	30	-
2.5	35	65	6
3.25	30	70	6
4.5	23	77	6
4.55	5	95	6
5.55	5	95	6

Time/min	%A	%B	Curve
5.6	70	30	6
7.5	70	30	6

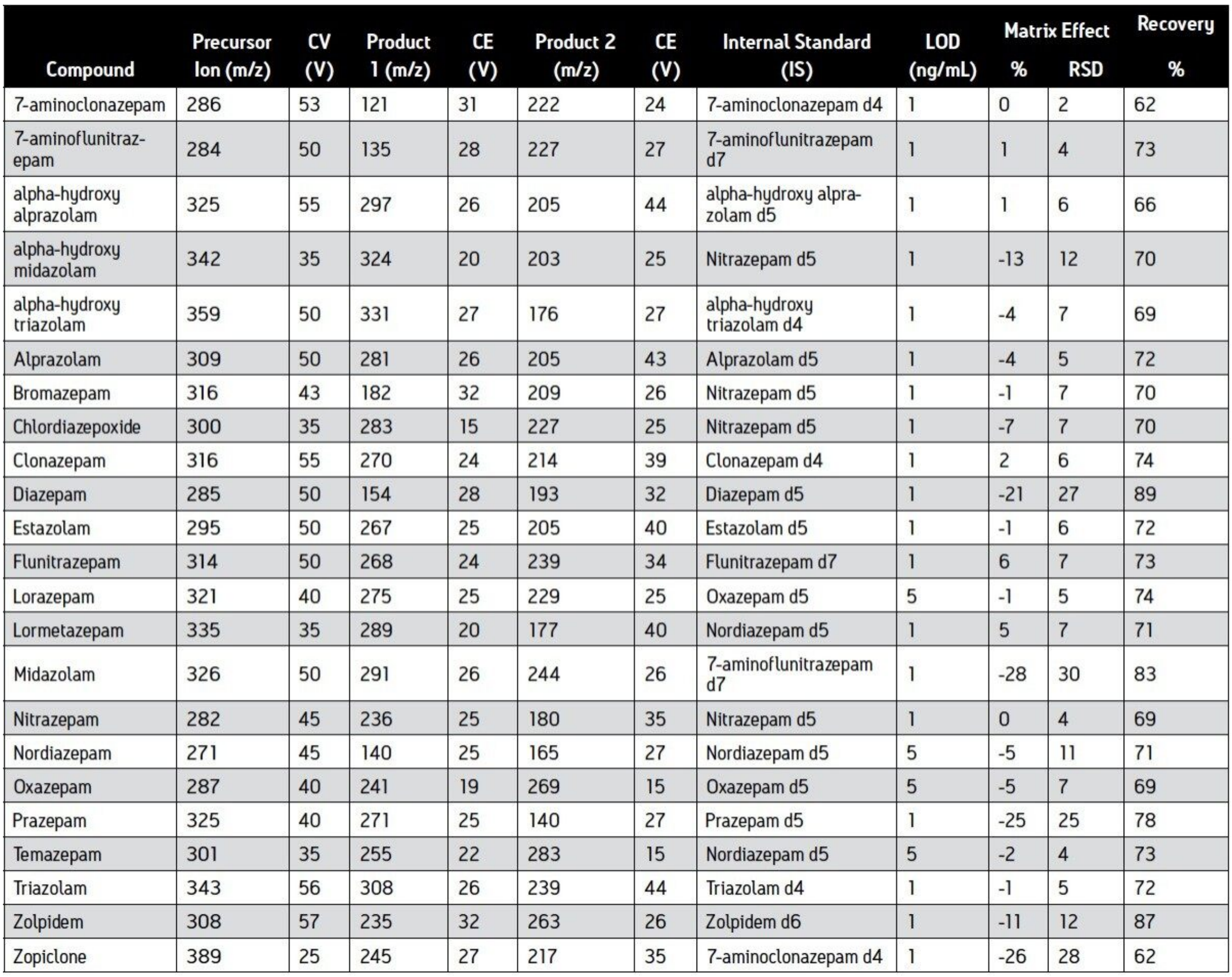
MS Conditions

MS System:	Waters TQ Detector (TQD)
Ionization Mode:	ESI Positive
Capillary Voltage:	3 kV
Desolvation Temp:	400 ˚C
Desolvation Gas:	800 L/Hr
Source Temp:	120 ˚C
Acquisition Mode:	Multiple Reaction Monitoring (MRM)

Results and Discussion

Method Validation

The MRM transitions and optimised conditions for all of the drugs and metabolites are shown in Table 1. Each analyte was monitored using two transitions i.e., a quantifier and qualifier. Internal standards were monitored using a single transition.



Compound	Precursor Ion (m/z)	CV (V)	Product 1 (m/z)	CE (V)	Product 2 (m/z)	CE (V)	Internal Standard (IS)	LOD (ng/mL)	Matrix Effect		Recovery
									%	RSD	%
7-aminoclonazepam	286	53	121	31	222	24	7-aminoclonazepam d4	1	0	2	62
7-aminoflunitrazepam	284	50	135	28	227	27	7-aminoflunitrazepam d7	1	1	4	73
alpha-hydroxy alprazolam	325	55	297	26	205	44	alpha-hydroxy alprazolam d5	1	1	6	66
alpha-hydroxy midazolam	342	35	324	20	203	25	Nitrazepam d5	1	-13	12	70
alpha-hydroxy triazolam	359	50	331	27	176	27	alpha-hydroxy triazolam d4	1	-4	7	69
Alprazolam	309	50	281	26	205	43	Alprazolam d5	1	-4	5	72
Bromazepam	316	43	182	32	209	26	Nitrazepam d5	1	-1	7	70
Chlordiazepoxide	300	35	283	15	227	25	Nitrazepam d5	1	-7	7	70
Clonazepam	316	55	270	24	214	39	Clonazepam d4	1	2	6	74
Diazepam	285	50	154	28	193	32	Diazepam d5	1	-21	27	89
Estazolam	295	50	267	25	205	40	Estazolam d5	1	-1	6	72
Flunitrazepam	314	50	268	24	239	34	Flunitrazepam d7	1	6	7	73
Lorazepam	321	40	275	25	229	25	Oxazepam d5	5	-1	5	74
Lormetazepam	335	35	289	20	177	40	Nordiazepam d5	1	5	7	71
Midazolam	326	50	291	26	244	26	7-aminoflunitrazepam d7	1	-28	30	83
Nitrazepam	282	45	236	25	180	35	Nitrazepam d5	1	0	4	69
Nordiazepam	271	45	140	25	165	27	Nordiazepam d5	5	-5	11	71
Oxazepam	287	40	241	19	269	15	Oxazepam d5	5	-5	7	69
Prazepam	325	40	271	25	140	27	Prazepam d5	1	-25	25	78
Temazepam	301	35	255	22	283	15	Nordiazepam d5	5	-2	4	73
Triazolam	343	56	308	26	239	44	Triazolam d4	1	-1	5	72
Zolpidem	308	57	235	32	263	26	Zolpidem d6	1	-11	12	87
Zopiclone	389	25	245	27	217	35	7-aminoclonazepam d4	1	-26	28	62

Table 1. MRM transitions with cone voltages (CV) and collision energies (CE) for 23 analytes, product 1 is the quantifier ion and product 2 is the qualifier ion.

To investigate linearity for all of the analytes, spiked serum calibrators were prepared in triplicate at 0, 1, 5, 10, 100, and 1,000 ng/mL, and extracted using LLE as previously described. Following analysis, calibration curves were plotted with a 1/x weighting. Average r^2 values were all >0.995 except for alphahydroxy triazolam which was 0.975 for 1-100 ng/mL.

Quantitation was performed by integrating the area under the peak for each analyte MRM trace and referencing to the appropriate deuterated internal standard peak area. Figure 2 shows the quantifier ion traces for all analytes at 1 ng/mL. The limit of detection (LOD) was defined as the concentration which gave a signal to noise (S:N) ratio >7:1 (for both qualifier and quantifier). The data is summarised in Table 1.

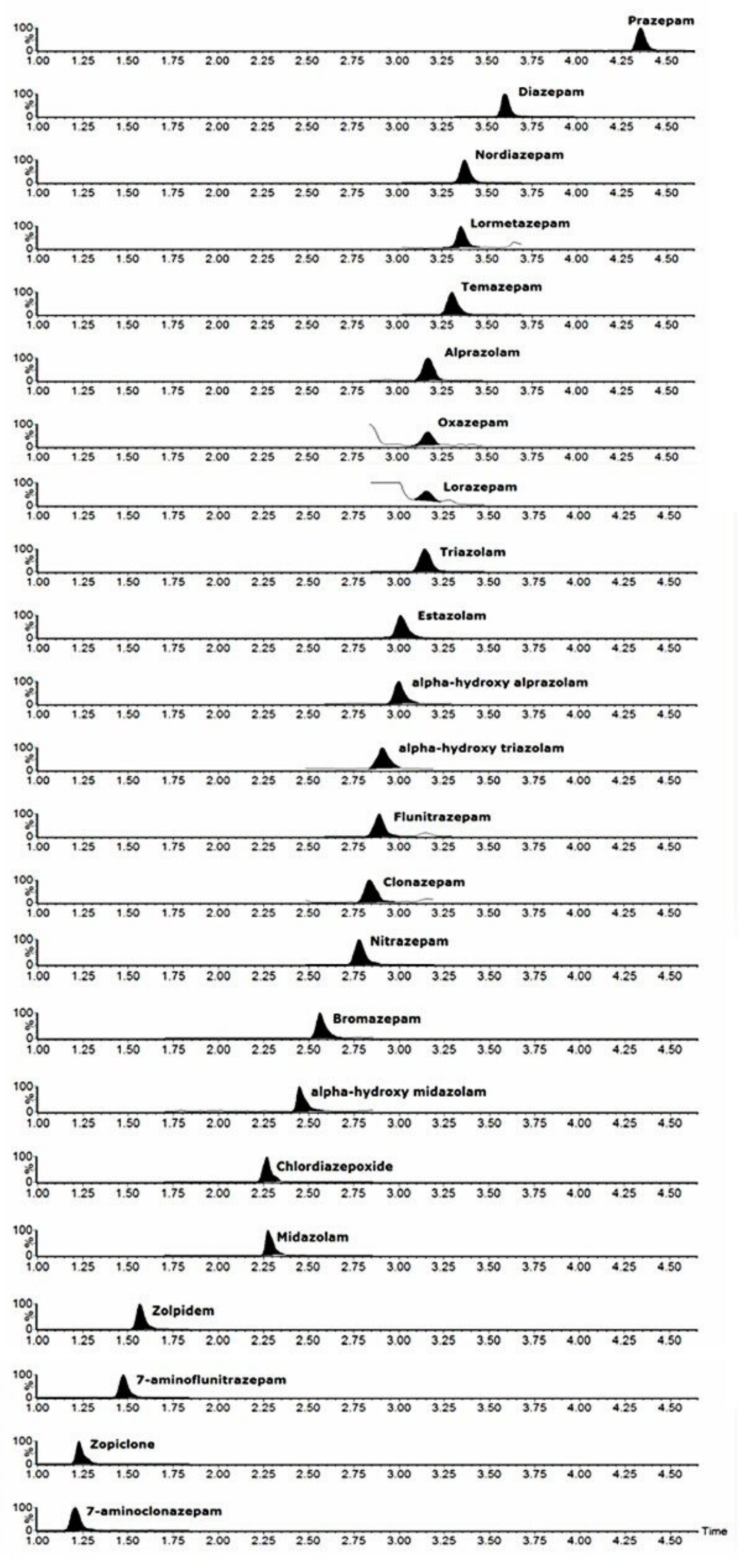


Figure 2. Quantifier ion traces for all analytes for the extracted 1 ng/mL

Recoveries and matrix effects were assessed using six different sources of blank sera (Table 1). Recoveries

Matrix effects were determined by comparing the responses for analytes spiked into extracted blank sera to

Extracted sample stability was assessed using 16 blank serum samples which were spiked at 50 ng/mL and values above 0.98) between the newly-developed method and the published method. An example of a positive authentic sample is shown in Figure 5; a negative control is also included for comparison.

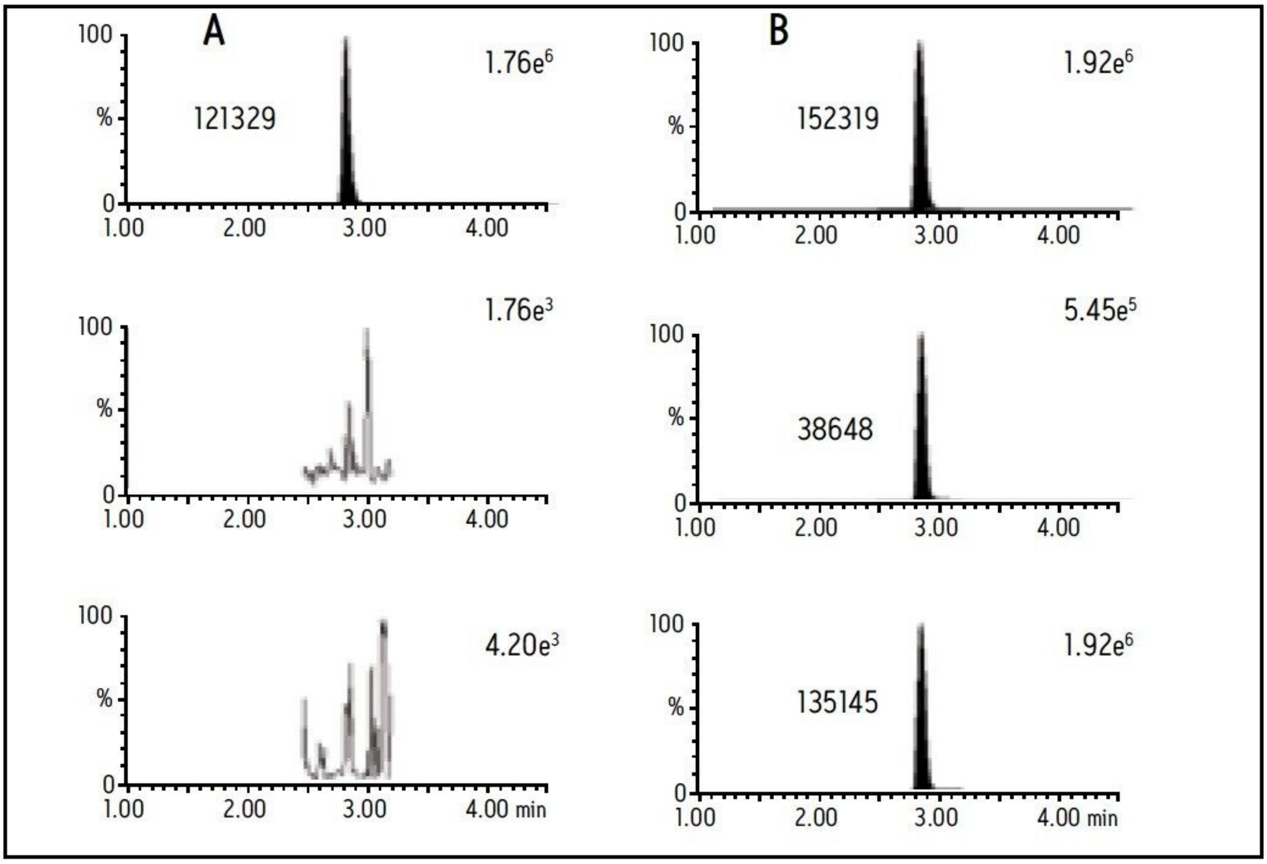


Figure 5. A positive result for clonazepam at 40 ng/mL. A is the zero serum calibrator and B is the authentic sample. The top trace is the internal standard, middle is 316>214 (qualifier) and bottom is 316>270 (quantifier). The numbers above the peaks show peak area and to the right of the peak is the peak intensity.

Conclusion

Benzodiazepines need to be monitored in both clinical and forensic laboratories and so an accurate, reliable and robust method is needed to quantitate these drugs in biological samples. To this end we have developed a fast, sensitive method for an extensive panel of commonly-prescribed benzodiazepines using UPLC-MS/MS.

Unlike GC-MS, this technique requires no derivatisation and due to softer ionisation it is possible to monitor specific fragmentations from the protonated molecular species of the analytes. In comparison to HPLC-based methods, those based on UPLC offer superior chromatographic resolution, enhanced sensitivity and shorter analytical run times.

The results for 27 authentic serum and plasma samples analysed using the described LLE-UPLC-MS/MS method were compared to those obtained previously with a published, validated method; the data showed excellent agreement.

This application is an example of an assay that can be performed using Waters systems. Complete method validation by the end user is required.

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Acknowledgements

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Featured Products

ACQUITY UPLC System <https://www.waters.com/514207>

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