Determination of Benzodiazepines in Urine by CE-MS/MS

Application Note

Forensic Toxicology

Authors

Claudimir Lucio do Lago
Department of Fundamental Chemistry,
Institute of Chemistry
University of São Paulo, Brazil

Daniela Daniel
Agilent Technologies, Inc.

Abstract

Using capillary electrophoresis combined with tandem mass spectrometry, we have developed a method to simultaneously detect lorazepam, clonazepam, temazepam, oxazepam, diazepam, and nitrazepam in urine. The urine samples were submitted to a modified QuEChERS extraction procedure, followed by electrophoretic separation in 0.1 M formic acid electrolyte (pH 2.4) using a polyvinyl alcohol (PVA)-coated capillary. The correlation coefficients of the calibration curves, using matrix-matched benzodiazepines standard solutions at six different concentrations from 10 to 500 ng/mL, were up to 0.998. The limits of detection ranged from 0.9 to 1.4 ng/mL. We verified precision and accuracy through recovery for spiked urine blank samples at five concentration levels (10, 20, 50, 100, and 200 ng/mL) in triplicate. The recovery values ranged from 89 to 121 %, with a relative standard deviation lower than 6.0 %.

Agilent Technologies

Introduction

Benzodiazepines, shown in Figure 1, are frequently used to treat anxiety and sleep disturbance, and are widely prescribed throughout the world[1]. However, they are commonly involved in cases of drug intoxication, or traffic accidents, and criminals use them to incapacitate their victims. Therefore, a fast and accurate method to detect these drugs in body fluids is important for forensic toxicologists[2].
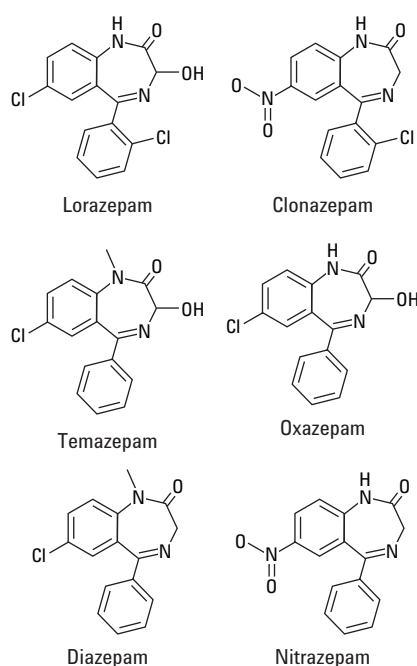

Figure 1. Chemical structures of benzodiazepines.

In forensic analysis, the results generated must prove the nature of the substance. Seized substances are commonly analyzed using multiple uncorrelated techniques[3]. Various analytical methods for the measurement of benzodiazepines have been documented[1,4], but capillary electrophoresis (CE), especially when coupled with tandem mass spectrometry (MS/MS) is getting more attention in forensic laboratories. CE-MS/MS combines the advantages of both quantitative and migration time information with molecular masses or fragmentation patterns in one analysis. This approach presents a high probability of elucidating the chemical compound and its concentration using either an analytical curve or standard addition methods.

This Application Note describes a CE-MS/MS method for the detection and quantitation of lorazepam, clonazepam, temazepam, oxazepam, diazepam, and nitrazepam in urine. This sensitive, fast method requires low sample volumes (nL) and produces low levels of chemical waste.

All separations were performed at 25 °C using a 0.1 M formic acid, pH 2.4, as background electrolyte (BGE). The PVA-coated capillary was preconditioned by flushing with Milli-Q water for 3 minutes followed by BGE for 5 minutes. An additional post-conditioning step by flushing with BGE for 60 seconds was included between the runs. Samples were introduced hydrodynamically in 12 seconds at 50 mbar, and analyzed with an applied voltage of 28 kV. The mass spectrometer was operated in positive ionization mode, using a multiple reaction monitoring (MRM) mode for two specific transitions for each benzodiazepine. The most intense transition was used for quantification, and the other was used as a qualifying ion. Table 1 lists the monitored ions and other MS/MS acquisition parameters.

Table 1. Migration time (t_M) and MS/MS acquisition parameters used for the identification and quantification of benzodiazepines in urine.

Compound	t_M (min)	Q1[a] (m/z)	Q3[b] (m/z)	CE[c] (V)	FE[d] (V)
Diazepam	5.18	285.1	193.1*	32	166
			154.1	24	
Nitrazepam	5.57	282.1	236.1*	24	204
			180.1	40	
Oxazepam	8.43	287.1	269.1*	12	131
			241.1	20	
Clonazepam	8.54	316.1	270.1*	24	214
			214.0	40	
Temazepam	8.79	301.1	283.1*	8	123
			255.1	16	
Lorazepam	9.59	321.0	303.0*	12	108
			275.0	20	

[a] Precursor ion (Q1), [b] Fragment ions (Q3), [c] Collision energy, [d] Fragmentor energy. * Transition used for quantification.

Experimental

CE Conditions

Parameter	Value
Instrument	Agilent 7100 CE system
Background electrolyte	0.1 M formic acid, pH 2.4
Applied voltage	28 kV
Capillary	PVA-coated silica capillary 50 µm id with 58 cm total length (p/n G1600-67219, 125 cm length, cut to 58 cm)
Injection	12 seconds at 50 mbar
Temperature	25 °C

MS Conditions

Parameter	Value
Instrument	Agilent 6430 MS
Ion mode	ESI, positive ionization
Sheath liquid	0.01 M Formic acid/methanol (50:50 v/v)
Flow rate	5.0 µL/min
Capillary voltage	1,000 V
Drying gas flow (N_2)	5 L/min
Drying gas temperature	150 °C
Nebulizer pressure	6 psi

Sample preparation

Samples of blank urine were stored at –20 °C before analysis. Extraction of the benzodiazepines from urine was performed using a modified QuEChERS method. This method involved placing a 10-mL aliquot of the sample into a 50-mL PP tube followed by extraction, using 10.0 mL of acetonitrile (containing 10 mg of NaOH, apparent pH 12.4). A partition step was performed by adding 4 g of anhydrous magnesium sulfate ($MgSO_4$) and 1 g of anhydrous sodium chloride (NaCl) using Agilent original QuEChERS method (nonbuffered) extraction tubes (p/n 5982-5550) followed by shaking for 1 minute, and centrifugation for 5 minutes at 5,000 rpm. Next, a 2-mL aliquot of the supernatant was filtered through a 0.2-µm PVDF and PP membrane (Agilent Captiva filter cartridges, p/n A5300002), and analyzed. The dSPE cleanup step was unnecessary.

The recovery tests were carried out by spiking the samples before the shaking step with a known amount of the analytes. This spiking resulted in five different levels of benzodiazepines (10, 20, 50, 100, and 200 ng/mL) in the blank urine samples. The recovery was calculated for each analyte as the average peak area of benzodiazepines found in the spiked blank sample with the response of the same analytes from post-extracted samples at the equivalent concentrations, and was expressed as a percentage.

Results and Discussion

The BGE and sheath liquid composition, applied potential, and hydrodynamic injection were optimized for separation efficiency and sensitivity. A PVA-coated capillary (p/n G1600-67219) was used to achieve a good compromise between analysis time and peak resolution by reducing the electro-osmotic flow (EOF). The PVA coating minimized the interaction between highly polar compounds and the surface of the capillary to avoid excessive peak tailing. Figure 2 shows a representative electropherogram of a standard solution of benzodiazepines at 200 ng/mL in BGE, using a PVA-coated capillary.
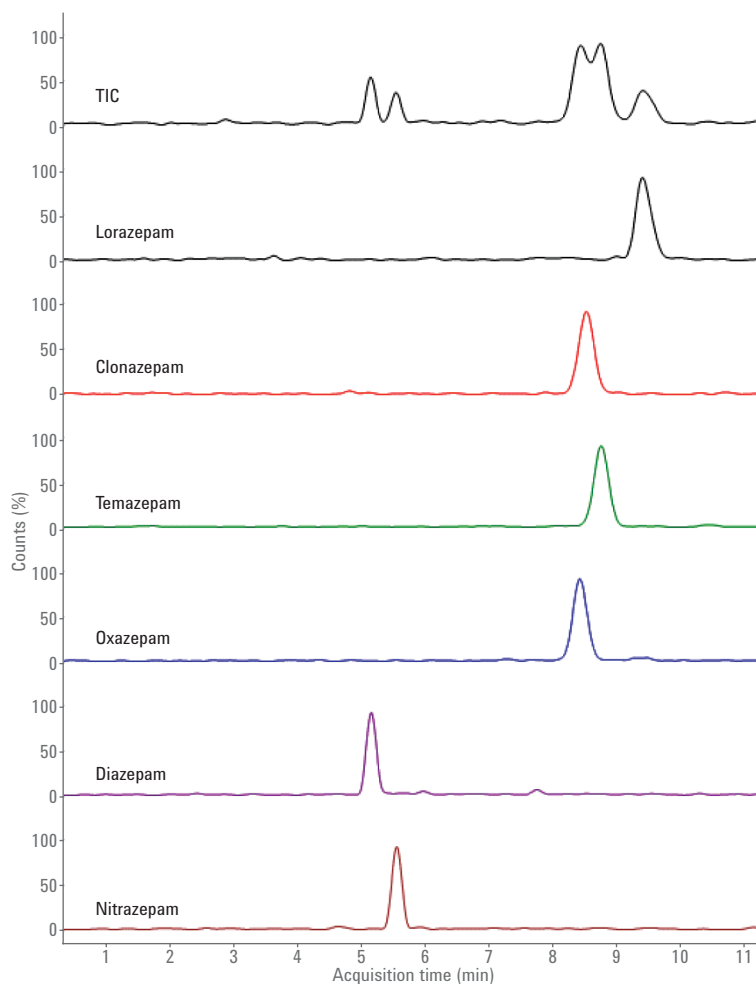

Figure 2. MRM electropherogram of a mixture of the benzodiazepines at 200 ng/mL each in BGE using a PVA-coated capillary. Total ion electropherogram (TIC), lorazepam, clonazepam, temazepam, oxazepam, diazepam, and nitrazepam.

The linearity of the analytical curve was studied in BGE at seven different concentration levels ranging from 5 to 500.0 ng/mL. Figure 3 shows an example of the response for lorazepam, using Agilent MassHunter quantitative software. Calibration curves also were constructed using matrix-matched benzodiazepines standard solutions at six different concentration levels ranging from 10 to 500 ng/mL. The response function was found to be linear, with coefficient of determination (R^2) values higher than 0.998 for all calibration curves. The limits of detection (LOD) and limits of quantification (LOQ) were determined considering the corresponding concentration to produce a signal 3 and 10 times the baseline noise, in a close region to the migration time of each benzodiazepine, respectively. The proposed method enabled us to detect benzodiazepines at levels between 0.9 and 1.4 ng/mL. Table 2 shows the regression equations and other characteristic parameters for the developed method.
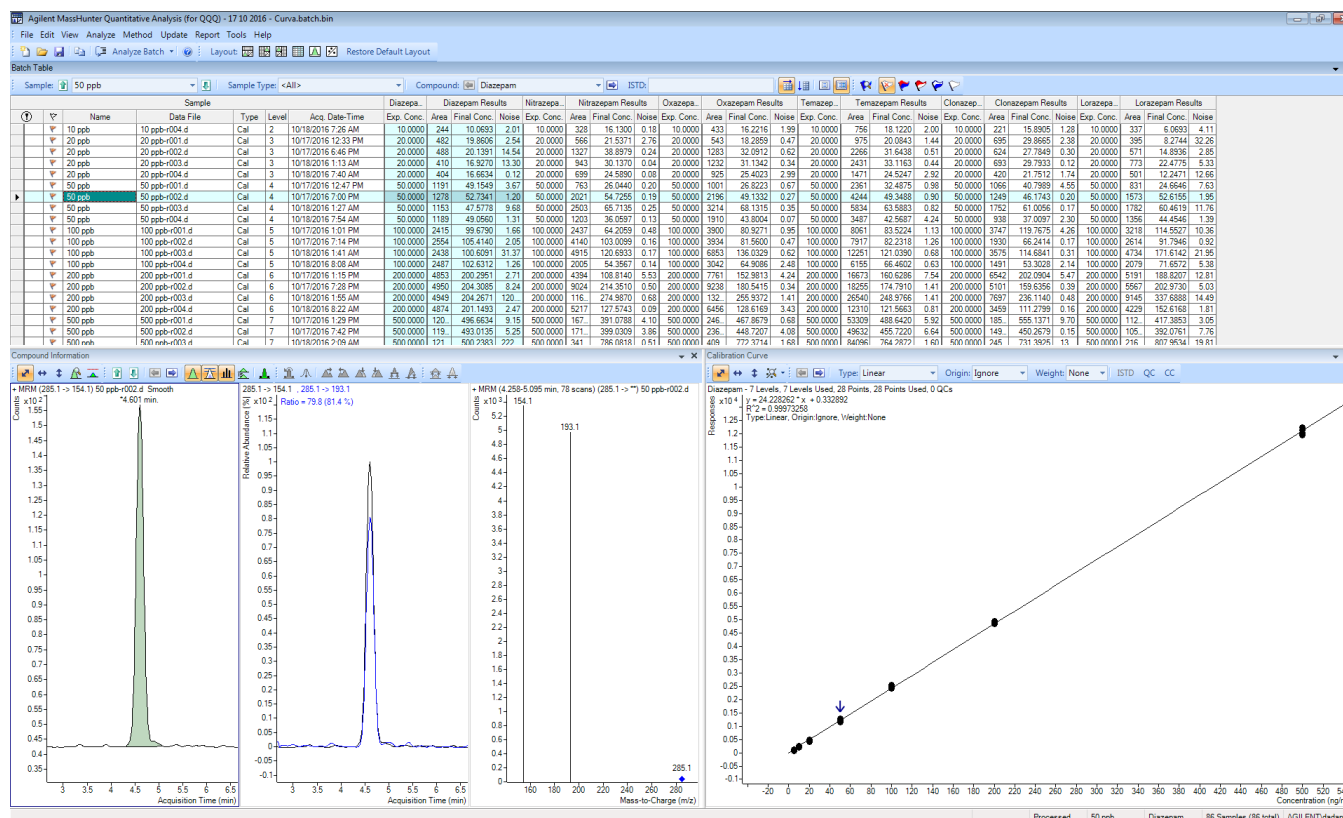

Figure 3. Calibration curve of benzodiazepines using Agilent MassHunter quantitative software.

Table 2. Analysis of results from the proposed method for the determination of benzodiazepines in urine by CE-MS/MS.

Compound	Slope	Intercept	R^2	LOD (ng/mL)	LOQ (ng/mL)
Diazepam	10.1	160.8	0.999	1.3	4.4
Nitrazepam	15.8	223.4	0.999	0.9	3.1
Oxazepam	31.5	567.6	0.998	0.9	2.9
Clonazepam	20.4	46.4	0.999	1.4	4.6
Temazepam	68.3	16.1	0.999	1.1	3.5
Lorazepam	22.2	312.3	0.998	1.0	3.2

Accuracy and precision, expressed in terms of recovery from blank urine samples, were studied by analyzing spiked samples at five different concentration levels (10, 20, 50, 100, and 200 ng/mL). The recovery values ranged from 89 to 121 %, with a relative standard deviation not greater than 6.0 % for triplicate analyses. Table 3 summarizes these results.

Conclusion

This study shows that CE-MS/MS is suited for the analysis of benzodiazepines in urine. The proposed method presented a linear response to benzodiazepines in the concentration range from 5 to 500 μg/mL with excellent precision for replicate injections. The use of a PVA-coated silica capillary provided EOF suppression and increased separation efficiency with no peak tailing effects. The proposed method is fast, simple, uses a small amount of sample with low reagent consumption, and has good sensitivity and precision. The modified QuEChERS extraction at high pH was simple and efficient, with recoveries from 89 to 121 % in urine matrix. Moreover, the combination of the efficient cleanup step and a selective and sensitive CE-MS/MS makes the method promising for other complex matrixes with small modifications in forensic laboratories.

References

1. Szatkowska, P.; *et al.* Analytical methods for determination of benzodiazepines. A short review. *Central European Journal of Chemistry* **2014**, *12(10)*, 994-1007.

2. Tomita, M.; Okuyama, T. Application of capillary electrophoresis to the simultaneous screening and quantitation of benzodiazepines. *Journal of Chromatography B* **1996**, *678*, 331-337.

3. Scientific working group for the analysis of seized drugs (SWGDRUG) recommendations. *United States Department of Justice – Drug Enforcement Administration, Third Edition* **2007**, 14-16.

4. Hudson, J.; *et al.* Benzodiazepine and Z-drug Quantitation using an Agilent 6430 LC/MS/MS, *Agilent Technologies Application Note*, publication number 5991-2291EN, **2013**.

Table 3. Concentration (ng/mL) of benzodiazepines spiked into urine samples, RSD (%) and recovery tests carried out in these samples (n = 3).

Benzodiazepine	Concentration added	Concentration found	RSD(%)	Recovery (%)
Diazepam	10	8.9	2.0	89
	20	19.4	6.0	97
	50	48.7	1.2	97
	100	99.9	1.6	100
	200	200.7	1.5	100
Nitrazepam	10	10.1	4.5	101
	20	21.0	1.7	105
	50	50.0	0.2	100
	100	100.5	1.1	101
	200	198.0	0.3	99
Oxazepam	10	9.6	2.6	96
	20	23.2	1.2	116
	50	51.5	0.7	103
	100	103.6	3.3	104
	200	199.0	2.8	100
Clonazepam	10	11.2	5.2	112
	20	18.7	2.6	93
	50	51.8	4.0	104
	100	100.6	3.9	101
	200	201.1	0.8	101
Temazepam	10	12.1	3.7	121
	20	22.8	3.0	114
	50	51.7	3.1	103
	100	102.7	2.2	103
	200	208.2	0.3	104
Lorazepam	10	11.3	5.1	113
	20	23.1	0.7	115
	50	54.0	0.2	108
	100	100.3	1.6	100
	200	201.0	0.2	101

www.agilent.com/chem

Agilent Technologies