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Analysis of Drugs of Abuse using Automated Disposable Pipette Extraction and LC/MS/MS

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Abstract
Solid phase extraction (SPE) is a widely used, proven method for sample preparation and sample clean-up in the field of forensic analysis. A number of SPE products are available that offer various sample preparation functions and cover a wide range of procedures. Most SPE products require relatively large volumes of solvent leading to increased time for sample processing, increased cost per sample and higher limits of detection.

Disposable Pipette Extraction (DPX) was developed as an alternative to traditional SPE, combining efficient and rapid extraction with significantly reduced solvent consumption. DPX is a novel dispersive solid-phase extraction device that uses sorbent loosely contained in a pipette tip to efficiently mix with sample solutions. The main advantages of DPX technology are the extractions are very rapid, recoveries are high, negligible solvent waste is generated, and the extractions can be fully automated and coupled to chromatographic injections.

This study focuses on the automated extraction of small sample volumes coupled to LC/MS/MS in order to provide high throughput analysis of an extended list of benzodiazepines. Using a GERSTEL MPS 2 autosampler, DPX extractions of a variety of biological matrices were performed, using a mixed-mode cation exchange (DPX-CX) sorbent. The resulting eluents from the automated DPX extractions were then introduced into an Agilent 6410 LC/MS/MS instrument.

Coupling DPX to LC/MS/MS provides rapid, just-in-time sample preparation for high throughput analysis. Data show the use of an Agilent 6410 LC/MS/MS instrument to be a highly sensitive procedure for the analysis of benzodiazepines with limits of quantitation of 0.5 ng/mL, and good linearity. The DPX extraction removes potential matrix interferences and ion suppression, and high sensitivity is therefore achieved.

Introduction

To date, the analysis of several benzodiazepines from urine using automated DPX have been reported using GC/MS. Recovery values of 82 % for nordiazepam and 71 % and α-OH-alprazolam have been reported following automated DPX extraction [1]. However, the analysis of benzodiazepines by GC/MS necessitates the use of derivatization, which has been shown to be a limitation for some of the benzodiazepines. The analysis of automated DPX extracted benzodiazepines using LC/MS/MS was chosen in an attempt to eliminate the need for derivatization.

Previous data described in Agilent Application Notes show the use of an Agilent 6410 LC/MS/MS instrument to be a highly sensitive procedure for the analysis of benzodiazepines, with limits of quantitation of 5 ng/mL [2].

This study focuses on the automated extraction of small sample volumes coupled to LC/MS/MS in order to provide high throughput analysis for drugs of abuse such as benzodiazepines. Using a GERSTEL MPS 2 autosampler, the DPX extractions were performed using a mixed-mode cation exchange (DPX-CX) sorbent. The resulting eluents from the automated DPX extractions were then introduced into an Agilent 6410 LC/MS/MS instrument.

Experimental

Materials. Benzodiazepine multi-component mixture-8 (cat.# B-033), containing clonazepam, temazepam, nitrazepam, alprazolam, diazepam, flunitrazepam, lorazepam, and oxazepam at 250 µg/mL each in acetonitrile, nordiazepam (cat.# N-905), clobazam (cat.# C-909), bromazepam (cat.# B-903), estazolam (cat.# E-901), flurazepam (cat.# F-003), midazolam (cat.# M-908), and triazolam (cat.# T-910), at 1.0 mg/mL each in methanol, and α-hydroxyalprazolam (cat.# A-905) at 100 µg/mL in methanol, were purchased from Cerilliant. Intermediate stock solutions of the sixteen benzodiazepines were prepared in water from appropriate dilutions of these stocks.

Deuterated analogues d_5-nordiazepam (cat.# N-903), d_5-α-hydroxyalprazolam (cat.# A-904), d_5-oxazepam (cat.# O-901), d_4-clonazepam (cat.# C-905), and d_5-estazolam (cat.# E-903), at 100 µg/mL each in methanol, were purchased from Cerilliant. These stocks were combined and diluted with water to be used as internal standards during analysis.

ß-Glucuronidase, type-2, from helix pomatia, (cat.# G0876-5 mL) was purchased from Sigma-Aldrich. Fresh urine was obtained from a male volunteer. Hydrolysis of urine consisted of combining 2 mL of urine, 100 µL of ß-glucuronidase, and 500 µL of 0.66 M acetate buffer, pH 4, vortex mixing for 30 seconds, and then incubating at 55°C for 2 hours. All other reagents and solvents used were reagent grade.

Calibration standards and QC samples were prepared by making appropriate dilutions of the combined benzodiazepine stock solutions using hydrolyzed, analyte free urine to give concentrations equivalent to 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL for the calibration standards and 7.5, 30, and 75 ng/mL for the QC samples.

Unextracted calibration standards in water were prepared to determine extraction recovery at concentrations equivalent to extracted calibration standards.

Instrumentation. All automated DPX PrepSequences were performed using a MultiPurpose Sampler (MPS 2XL), with GERSTEL DPX option as shown in figure 1.

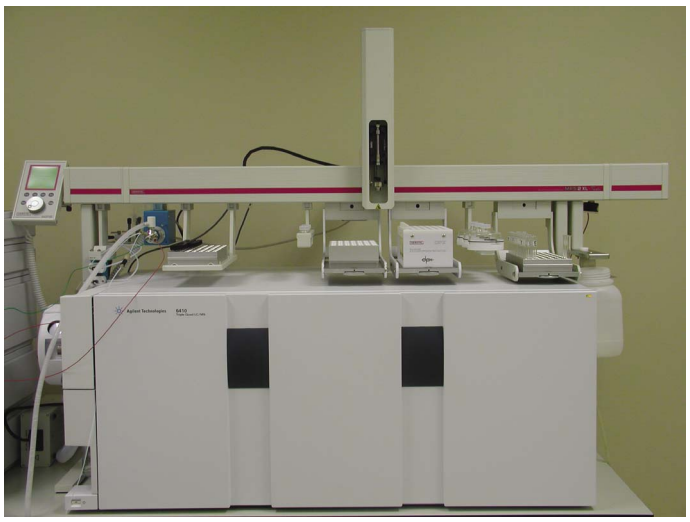

Figure 1. MPS 2XL MultiPurpose Sampler (MPS) with GERSTEL DPX option.

Urine Sample Pretreatment:

- Pipette 260 µL of hydrolyzed urine sample into a clean 12 x 75 mm culture tube.
- Pipette 250 µL of 1.0 M HCl into the tube and vortex mix for a few seconds.
- Pipette 250 µL of acetonitrile into the tube and vortex mix for a few seconds.
- Filter the sample using an Agilent 2 in 1 syringe filter (cat.#5042-1392) and collect the filtrate into a clean 12 x 75 mm culture tube.
- Place the filtered urine sample onto the GERSTEL MultiPurpose Sampler (MPS 2XL) with DPX option.

Whole Blood Sample Pretreatment:

- Pipette 200 µL of whole blood sample into a clean 12 x 75 mm culture tube.
- Pipette 800 µL of acetonitrile into the tube and vortex mix for a few seconds.
- After centrifugation for 10 minutes to pellet the precipitated proteins, transfer the supernatant into a clean 12 x 75 mm culture tube.
- Place the sample onto the GERSTEL MPS 2XL multi-purpose sampler with DPX Option.

Figure 2 shows a graphical representation of the DPX extraction process.

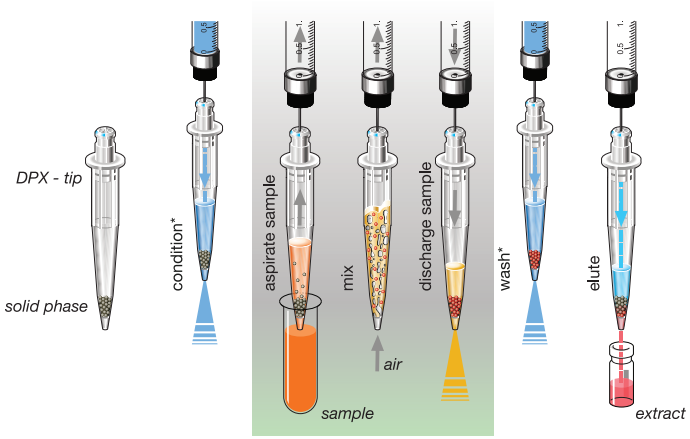

Figure 2. Graphical representation of the DPX extraction process.

The automated DPX extraction consisted of the following steps:

Automated DPX Prep Sequence:

- Wet the DPX-CX sorbent using 250 µL of 30 % acetonitrile in water.
- Aspirate the entire urine sample and mix the sample using 1.3 mL of air.
- Equilibrate for 20 seconds before dispensing the entire contents to waste.
- Wash the DPX-CX sorbent by dispensing 500 µL of 10 % acetonitrile in water into the top of the tip.
- Wash the DPX-CX sorbent by dispensing 500 µL of 100 % acetonitrile in water into the top of the tip.
- Elute the DPX-CX sorbent by dispensing 700 µL of (78:20:2) methylene chloride: isopropyl alcohol: ammonium hydroxide into the top of the tip and collect the eluent using clean 2 mL autosampler vials.

After evaporating to dryness under a stream of nitrogen at room temperature, all samples were reconstituted using 50 µL of water prior to analysis.

All analyses were performed using an Agilent 1200 HPLC with a Zorbax Eclipse XDB-C18 RRHT column (2.1 x 50 mm, 1.8 µm), an Agilent 6410 Triple Quadrupole Mass Spectrometer with electrospray source and GERSTEL MPS 2XL autosampler configured with an Active Washstation. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 20 µL stainless steel sample loop.

Benzodiazepine LC Method Parameters

Pump:	gradient (600 bar), flowrate = 0.2 mL/min	
Mobile Phase:	A - 20 mM ammonium formate, pH 8.6	
	B – 10 % isopropyl alcohol in methanol	
Gradient:	Initial	45 % B
	2 min	45 % B
	5 min	95 % B
	9 min	90 % B
	9.5 min	45 % B
Run time:	10 minutes	
Injection vol.:	2.5 µL	
Col. temp.:	35°C	

Benzodiazepine Mass Spectrometer Parameters:

Operation:	Electrospray positive mode
Gas Temperature:	350°C
Gas Flow (N_2):	12 L/min
Nebulizer pressure:	35 psi
Capillary voltage:	4500 V

The mass spectrometer acquisition parameters are shown in table 1 with qualifier ions shown in parentheses. A retention time window value of 1 minute was used for each positive ion transition being monitored during the course of the dynamic MRM experiment.

Table 1. Mass spectrometer acquisition parameters.

Analyte	Precursor [m/z]	Product Ion [m/z]	Fragmentor Ions [V]	CE Voltage [V]
Bromazepam	316	182.1 (209)	140 (140)	30 (25)
Nitrazepam	282	236 (180)	160 (160)	25 (35)
d_4-Clonazepam	320	274.1	120	30
Flunitrazepam	314	239 (268)	160 (160)	35 (30)
Clonazepam	316	270 (214)	120 (120)	25 (30)
d_5-α-OH-Alprazolam	330	302	120	30
d_5-Estazolam	300	272	140	25
α-OH-Alprazolam	325	216 (297)	120 (120)	35 (30)
Estazolam	295	205 (267)	160 (160)	40 (25)
Clobazam	301	224 (259)	140 (140)	30 (15)
Triazolam	343	239 (308)	180 (180)	40 (25)
Alprazolam	309	274 (281)	160 (160)	30 (25)
d_5-Oxazepam	292	246	120	20
Oxazepam	287	241 (269)	120 (120)	20 (15)
Lorazepam	321	229 (275)	140 (140)	30 (20)
Temazepam	301	255 (177)	120 (120)	35 (40)
d_5-Nordiazepam	276	213	160	30
Nordiazepam	271	140 (165)	160 (160)	30 (30)
Midazolam	326	249 (291)	180 (180)	40 (25)
Diazepam	285	222 (257)	160 (160)	25 (25)
Flurazepam	388.1	288 (315)	140 (140)	25 (20)

Results and Discussion

Figure 3 shows representative mass chromatograms for all benzodiazepines, along with their respective qualifier ion transitions, from an extracted low QC sample. In light of the world-wide acetonitrile shortage, it was found that the use of 10 % isopropyl alcohol in methanol as the organic modifier of the mobile phase provided an alternative to using acetonitrile. The lower limit of quantitation of this method was 0.5 ng/mL.

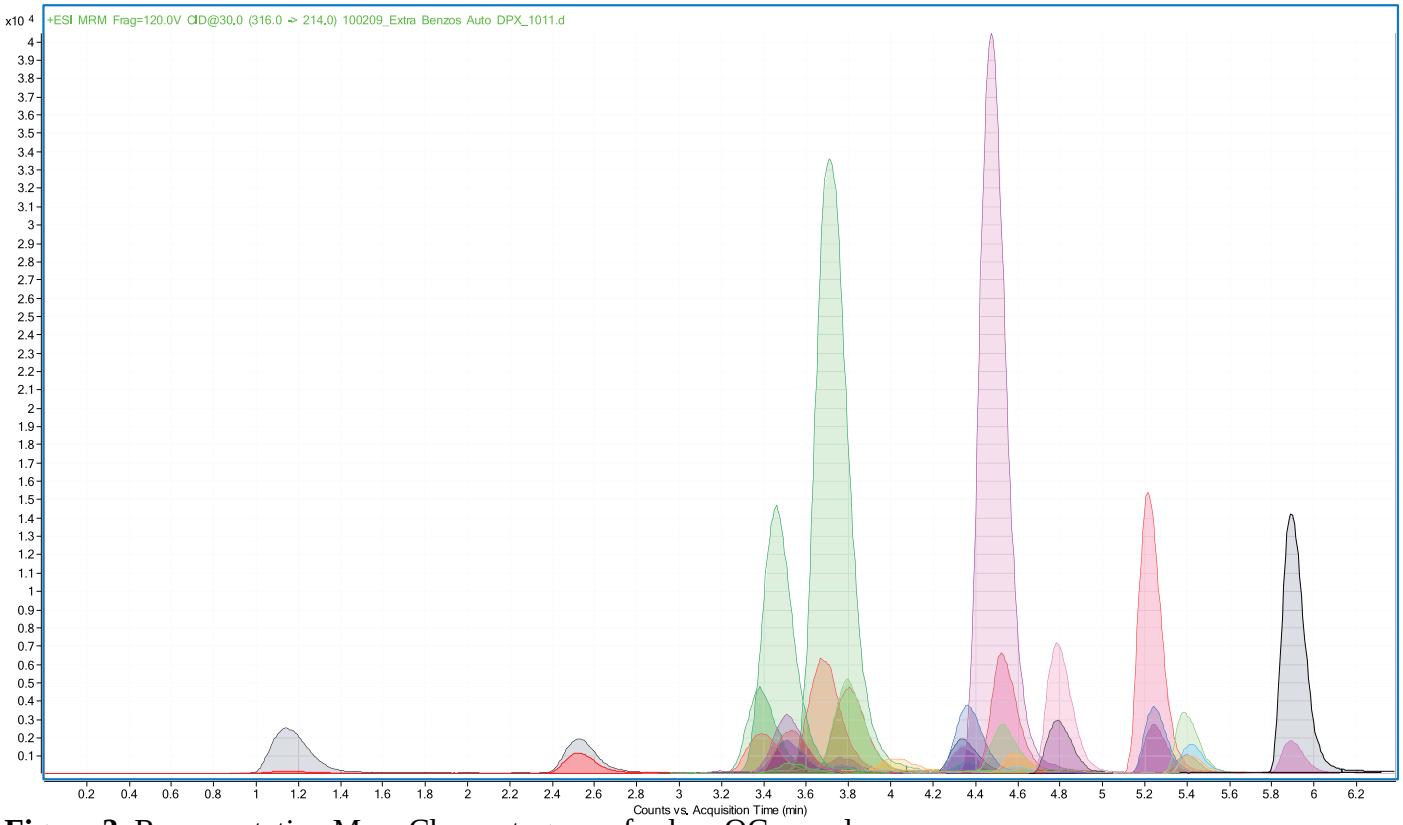

Figure 3. Representative Mass Chromatograms for low QC sample.

Representative calibration curves are shown in figure 4. All calibration curves were found to be linear between 0.5 ng/mL and 100 ng/mL for all benzodiazepines analyzed. Results from all calibration curves can be found in table 2.

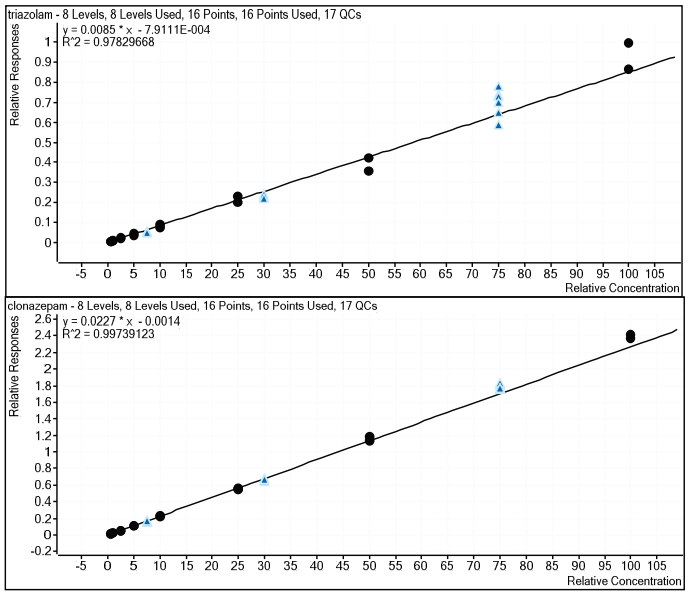

Figure 4. Representative calibration curves: triazolam and clonazepam.

Table 2. Calibration curve results.

Compound	R^2	LOQ [ng/mL]	CV* [%]	Accuracy* [%]
Temazepam[C]	0.9917	0.5	2.29	101
Alprazolam[C]	0.9901	0.5	4.25	97.9
Oxazepam[C]	0.9950	0.5	1.68	101
Lorazepam[C]	0.9908	0.5	8.48	98.3
Estazolam[D]	0.9955	0.5	1.01	102
α-OH-Alprazolam[A]	0.9958	0.5	1.40	99.3
Flurazepam[B]	0.9937	0.5	10.3	92.5
Midazolam[B]	0.9919	0.5	9.37	92.4
Flunitrazepam[E]	0.9959	0.5	2.85	99.6
Diazepam[B]	0.9969	0.5	3.95	100
Clonazepam[E]	0.9974	0.5	2.05	97.5
Clobazam[C]	0.9947	0.5	5.67	105
Bromazepam[E]	0.9973	0.5	15.5	103
Triazolam[C]	0.9783	0.5	3.69	89.9
Nordiazepam[B]	0.9941	0.5	2.10	97.8
Nitrazepam[E]	0.9910	0.5	4.59	98.4

* of 30 ng/mL QC sample
A - Calculated using d_5-α-Hydroxyalprazolam
B - Calculated using d_5-Nordiazepam
C - Calculated using d_5-Oxazepam
D - Calculated using d_5-Estazolam
E - Calculated using d_4-Clonazepam

The accuracy and precision of the method was determined for all benzodiazepines using QC samples at concentrations of 7.5, 30, and 75 ng/mL. Table 3 shows the resulting accuracy and precision data for all benzodiazepines. Accuracy data averaged 102 % (range: 84.9 % - 136 %) and precision data averaged 5.48 %RSD (range: 1.01 % -26.4 %) for all benzodiazepines analyzed.

Table 3. QC samples accuracy and precision table.

Compound	QC 1 7.5 ng/mL n=6	QC 2 30 ng/mL n=5	QC 3 75 ng/mL n=6
Alprazolam			
mean	6.65	29.4	86.8
SD	0.335	1.25	2.82
% CV	5.03	4.25	3.24
% Accuracy	93.7	97.9	116
α-OH-Alprazolam			
mean	7.50	29.8	78.7
SD	0.322	0.417	1.81
% CV	4.30	1.40	2.31
% Accuracy	101	99.3	105
Bromazepam			
mean	8.20	31.0	102.0
SD	1.21	4.81	27.0
% CV	14.8	15.5	26.4
% Accuracy	104	103	136
Clobazam			
mean	7.54	31.6	66.4
SD	1.03	1.79	10.2
% CV	13.7	5.67	15.3
% Accuracy	101	105	88.5
Clonazepam			
mean	7.30	29.3	79.0
SD	0.139	0.600	1.38
% CV	1.91	2.05	1.75
% Accuracy	97.7	97.5	105
Diazepam			
mean	6.96	30.1	83.7
SD	0.255	1.19	1.83
% CV	3.67	3.95	2.19
% Accuracy	97.7	100	112
Estazolam			
mean	7.31	30.5	80.6
SD	0.111	0.308	1.25
% CV	1.52	1.01	1.55
% Accuracy	99.3	102	108
Flunitrazepam			
mean	7.33	29.9	80.4
SD	0.123	0.851	1.09
% CV	1.67	2.85	1.36
% Accuracy	98.5	100	107

Table 3. QC samples accuracy and precision table (cont.).

Compound	QC 1 7.5 ng/mL n=6	QC 2 30 ng/mL n=5	QC 3 75 ng/mL n=6
Flurazepam			
mean	6.15	27.8	89.0
SD	0.613	2.87	4.67
% CV	9.96	10.3	5.25
% Accuracy	86.6	92.5	119
Lorazepam			
mean	6.04	29.5	91.4
SD	0.542	2.50	10.5
% CV	8.97	8.48	11.5
% Accuracy	89.3	98.3	122
Midazolam			
mean	6.02	27.7	87.9
SD	0.549	2.60	6.51
% CV	9.12	9.37	7.40
% Accuracy	84.9	92.4	117
Nitrazepam			
mean	7.15	29.5	80.2
SD	0.155	1.35	1.15
% CV	2.17	4.59	1.44
% Accuracy	97.6	98.4	107
Nordiazepam			
mean	7.38	29.3	77.8
SD	0.194	0.615	1.37
% CV	2.63	2.10	1.77
% Accuracy	99.5	97.8	104
Oxazepam			
mean	7.21	30.3	79.6
SD	0.125	0.510	0.968
% CV	1.74	1.68	1.22
% Accuracy	98.6	101	106
Temazepam			
mean	6.97	30.3	78.5
SD	0.136	0.692	1.35
% CV	1.96	2.29	1.72
% Accuracy	97.2	101	105
Triazolam			
mean	6.13	27.0	81.7
SD	0.396	0.996	8.00
% CV	6.46	3.69	9.79
% Accuracy	86.7	89.9	109

The extraction efficiency was evaluated by comparing the resulting peak area ratios from extracted calibration standards to the resulting peak area ratios from unextracted calibrations standards prepared at equivalent concentrations. Table 4 shows the % recovery data.

Table 4. Calculated % recovery for 16 benzodiazepines using DPX-LC/MS/MS.

Compound	Recovery [%]
Alprazolam	65
α-OH-Alprazolam	100
Clonazepam	91
Diazepam	80
Flunitrazepam	86
Lorazepam	49
Nitrazepam	93
Nordiazepam	91
Oxazepam	91
Temazepam	84
Estazolam	90
Clobazam	6
Triazolam	54
Flurazepam	82
Midazolam	57
Bromazepam	43

Interestingly, the lowest extraction efficiency was obtained for Clobazam, which unlike the other benzodiazepines does not contain a tertiary amine in its structure. These results support the specificity of the DPX-CX sorbent for tertiary amines during the extraction of benzodiazepines using cation exchange mechanisms.

The effects of matrix on the ionization of compounds during analysis were evaluated for benzodiazepines extracted from either hydrolyzed urine or from whole blood extracts by comparing the resulting peak area ratios from a neat standard containing the benzodiazepines and deuterated internal standards in water, to extracted matrix blanks that were reconstituted using this same neat standard. Figure 5 shows a graphical representation of the effects of matrix on the ionization of compounds, as a percentage of the neat standard for each benzodiazepine monitored.

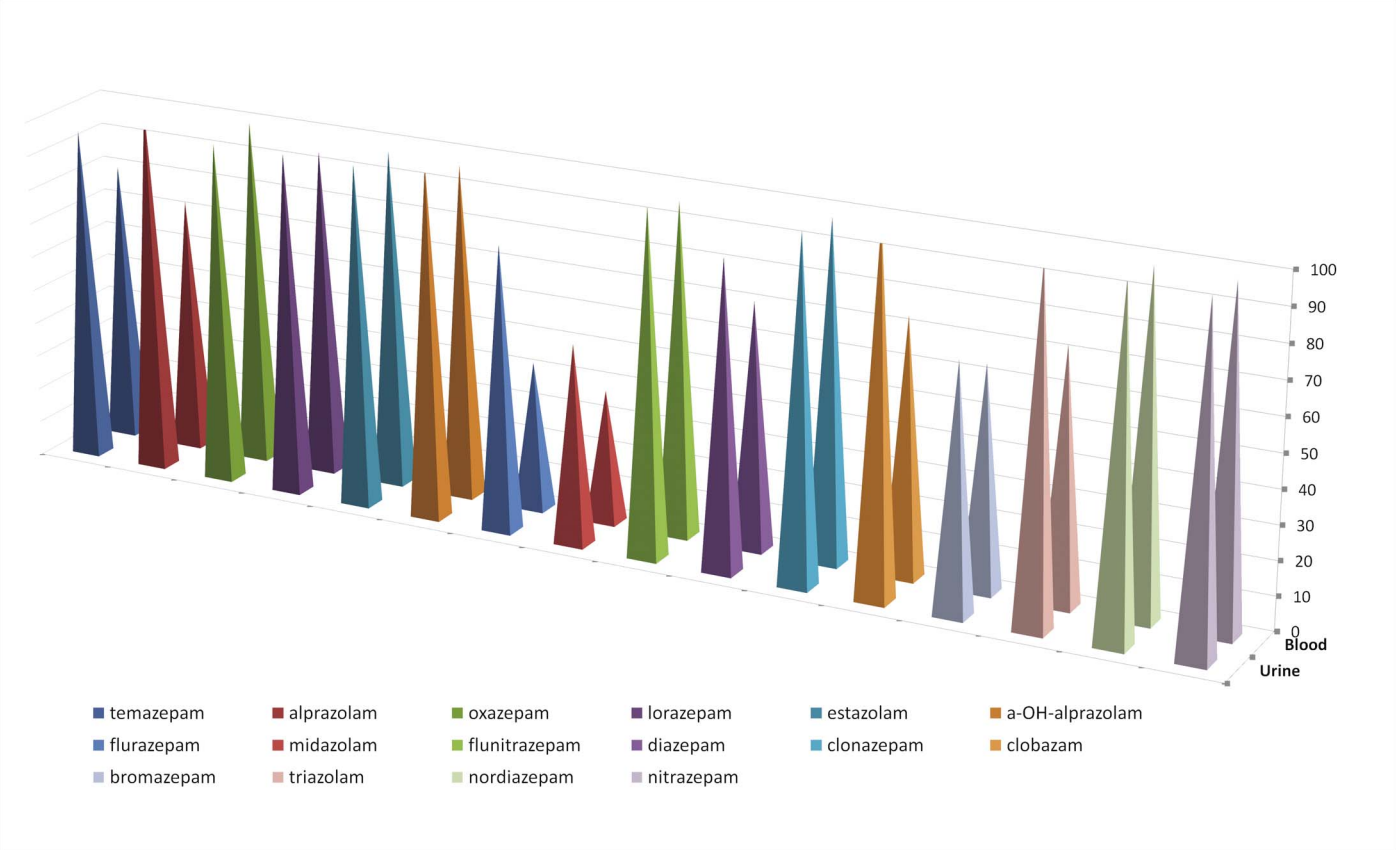

Figure 5. Effects of matrix on the ionization of compounds.

The total DPX extraction time per sample was 6.5 minutes which means that "just in time" parallel sample preparation can be performed automatically without reducing LC/MS throughput. Samples are prepared in parallel to the LC/MS run of the preceding sample using MAESTRO software and PrepAhead. Whenever the LC/MS becomes ready after a run the next sample is prepared and ready for introduction. Future studies will include this approach.

Conclusions

As a result of this study, we were able to show:

- 16 benzodiazepines can be successfully extracted from hydrolyzed urine samples using an automated DPX procedure coupled to LC/MS/MS analysis using the Agilent 6410 Triple Quadrapole Mass Spectrometer.
- This DPX method proved to be rapid and readily automated using the GERSTEL MPS 2XL robotic sampler.
- High recovery of all benzodiazepines was achieved using this automated DPX procedure.
- Linear calibration curves resulting in R^2 values 0.98 or greater were achieved with limits of quantitation of 0.5 ng/mL for all benzodiazepines.
- The DPX-LC/MS/MS method proved to be accurate and precise. Accuracy data averaged 102 % (range: 84.9 % - 136 %) and precision data averaged 5.48 % RSD (range: 1.01 % - 26.4 %) for all benzodiazepines analyzed.

References

[1] Analysis of Drugs and Metabolites in Blood and Urine using Automated Disposable Pipette Extraction, Sparkle Ellison, Stephen Morgan, William Brewer, University of South Carolina, Columbia, SC, Fred Foster, Edward Pfannkoch, John Stuff, and Jacqueline Whitecavage, GERSTEL, Inc., Linthicum, MD, EAS 2008 Poster Presentation

[2] Agilent Technologies, Application Note – Determination of Benzodiazepines in Urine and Blood Using Rapid Resolution Liquid Chromatography/Triple Quadrupole Mass Spectrometry, Christine Moore, Cynthia Coulter, and Katherine Crompton, Immunalysis Corporation, Pomona, CA, Michael Zumwalt, Agilent Technologies Inc., Englewood, CO, www.agilent.com/chem

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