AppNote 6/2011

Determination of Pain Management Drugs using Automated Disposable Pipette Extraction and LC-MS/MS

Fred D. Foster, John R. Stuff, Edward A. Pfannkoch
Gerstel, Inc., 701 Digital Dr. Suite J, Linthicum, MD 21090, USA

William E. Brewer
Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter Street, Columbia, SC 29208, USA

Keywords
DPX, LC-MS/MS, Sample Preparation, Lab Automation

Abstract
Solid phase extraction (SPE) is a widely used, proven method for sample preparation and sample clean-up in the field of forensic analysis. Most SPE products, however, are designed in such a way that relatively large volumes of solvent are required for the process. Consequencently, sample processing times, cost per sample, as well as limits of detection are often unnecessarily high, negatively affecting overall method performance and cost.

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 technique that is based on sorbent loosely contained in a pipette tip in which it is efficiently mixed with sample solution. The main advantages of DPX technology are: rapid extractions, high recoveries, negligible solvent waste, and the fact that extractions can be fully automated and combined with direct introduction of the extracts to the chromatography system.

This study focuses on the automated extraction of small sample volumes combined with LC-MS/MS analysis providing high throughput analysis of common pain management drugs. Using a GERSTEL MultiPurpose

Sampler (MPS), DPX extractions of hydrolyzed urine were performed, using a reversed phase sorbent with a proprietary salt additive (DPX-RP-S). The resulting eluents from the automated DPX extractions were introduced into an Agilent 6460 LC-MS/MS instrument.

Coupling DPX to LC-MS/MS provides rapid, just-in-time sample preparation for high throughput analysis. The DPX extraction removes potential matrix interferences, minimizing ion suppression and sample dilution and thereby achieving high overall sensitivity for the target analytes.

Introduction

Several important pain management drugs have been quantified in biological fluids by automated DPX followed by derivatization and GC/MS. However, this approach was limited to compounds amenable to derivatization such as nordiazepam and □-OH-alprazolam. DPX-LC-MS/MS was chosen in order to eliminate the need for derivatization and to address a broader range of pain management drugs.

Data show that using an Agilent 6460 LC-MS/MS instrument results in highly sensitive initial screening of pain management drugs, allowing their respective minimum reporting limits to be met and obtaining good linearity for calibration curves. Combining DPX with LC-MS/MS analysis using the Agilent 6460 enables high throughput while minimizing matrix interference.

Experimental

Materials. All stock solutions for the compounds listed in Table 1 were purchased from Cerilliant. An intermediate analyte stock solution was prepared by diluting the analyte stock solutions with acetonitrile to the required concentrations in order to evaluate the different drug classes.

Deuterated analogues, d3-morphine, d5-fentanyl, d5-nordiazepam, d5-propoxyphene, d7-carisoprodol, d5-amphetamine, d4-ketamine, d4-meperidine, d4-7-aminoclonazepam, and d5-PCP, were purchased from Cerilliant. A working internal standard stock solution containing the deuterated internal standards was prepared at a concentration of 10.989?g/mL and used to represent the drug classes being evaluated. Table 1 shows which deuterated internal standard was used to quantify the respective analytes.

High concentration calibration standard and intermediate QC urine samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solution using analyte free urine to give the concentration listed in Table 1. Calibration standards were then prepared using a dilution ratio strategy from the high concentration sample of 1:5:2:2.5:2:2. The high and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:4:5. Table 1 lists the concentrations for the highest calibration standard, the minimum reportable limit for the analyte, and the limit of quantitation found during analyses.

Table 1. Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]		Fragmentor Voltage [V]		Collision Energy [V]		Ret. Time [min]	High Std Conc. [ng/mL]	Minimum Reporting Limit [ng/mL]	Limit of Quant. [ng/mL]
6-MAM[1]	328.2	165.1	58.1	158	158	41	29	2.106	100	10.0	1.00
7-Aminoclonazepam[2]	286.1	222.1	121.1	138	138	25	33	2.772	500	50.0	5.00
Alprazolam[3]	309	281	205	179	179	25	49	4.115	400	40.0	4.00
Amphetamine[4]	136.1	119.1	91	66	66	5	17	2.223	1000	100	10.0
α-OH-Alprazolam[3]	325	297	216	150	150	28	41	3.991	200	20.0	2.00
Benzoylecgonine[4]	290.1	168.1	105	118	118	17	29	2.624	250	25.0	2.50
Buprenorphine[5]	468.3	414	396.2	220	200	35	41	3.749	100	10.0	1.00
Carisoprodol[6]	261.2	176.1	97.1	80	80	1	10	4.057	500	50.0	5.00
Clonazepam[3]	316	270	214	158	158	25	41	3.856	400	40.0	8.00
Cocaine[4]	304.2	182.1	77	138	138	17	61	2.741	250	25.0	2.50
Codeine[1]	300.2	165.1	128	158	158	45	60	2.082	500	50.0	5.00
d_3-Morphine[1]	289.2	152.1	-	153	-	68	-	1.369	-	-	-
d_4-7-Aminoclonazepam[2]	290.1	121	-	154	-	32	-	2.766	-	-	-
d_4-Ketamine[7]	242.1	129	-	102	-	32	-	2.604	-	-	-
d_4-Meperidine[8]	252.2	105	-	138	-	48	-	2.894	-	-	-
d_5-Amphetamine[4]	141.1	93	-	80	-	15	-	2.208	-	-	-
d_5-Fentanyl[5]	342.3	188.1	-	140	-	20	-	3.265	-	-	-
d_5-Nordiazepam[3]	276	213	-	160	-	30	-	4.323	-	-	-
d_5-PCP[10]	249.3	164.3	-	40	-	15	-	3.172	-	-	-
d_5-Propoxyphene[9]	345.3	271.2	-	120	-	5	-	3.798	-	-	-
d_7-Carisoprodol[6]	268.2	183.1	-	60	-	3	-	4.031	-	-	-
Diazepam[3]	285	257	154	169	169	25	25	4.447	400	40.0	4.00
EDDP[5]	278.2	234.1	219.1	160	160	33	45	3.394	500	50.0	5.00
Fentanyl[5]	337.2	188.1	105.1	143	143	21	41	3.273	10.0	1.00	0.100
Flunitrazepam[3]	314	268	239	153	153	25	37	3.919	400	40.0	4.00
Hydrocodone[1]	300.2	199	128	159	159	29	65	2.078	500	50.0	5.00
Hydromorphone[1]	286.2	185	157	159	159	29	45	1.585	500	50.0	5.00
Ketamine[7]	238.1	220.1	125	105	105	11	11	2.596	1000	100	10.0
Lorazepam[3]	321	275	194	102	102	21	49	4.064	400	40.0	4.00

Table 1. Mass spectrometer acquisition parameters (cont.).

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]		Fragmentor Voltage [V]		Collision Energy [V]		Ret. Time [min]	High Std Conc. [ng/mL]	Minimum Reporting Limit [ng/mL]	Limit of Quant. [ng/mL]
MDA[4]	180.1	163	105	61	61	5	21	2.272	1000	100	10.0
MDEA[4]	208	163	135	107	107	9	21	2.455	1000	100	10.0
MDMA[4]	194	163	105	97	97	9	25	2.306	1000	100	10.0
Meperidine[8]	248.2	220.1	174.1	128	128	21	17	2.935	500	50.0	5.00
Meprobamate[6]	219.1	158	97	60	65	0	7	3.289	500	50.0	5.00
Methadone[5]	310.2	265.1	105	112	112	9	29	3.877	500	50.0	5.00
Methamphetamine[4]	150.2	119	91	92	92	5	17	2.313	1000	100	10.0
Methylphenidate[4]	234.1	84.1	56.1	112	112	21	53	2.786	500	50.0	5.00
Morphine[1]	286.2	165.1	152.1	158	158	41	60	1.374	500	50.0	5.00
Nitrazepam[3]	282	236	180	148	148	25	41	3.908	400	40.0	4.00
Norbuprenorphine[5]	414.3	187.1	83.1	205	205	41	57	3.263	100	10.0	1.00
Nordiazepam[3]	271	165	140	138	138	25	29	4.378	400	40.0	4.00
Norfentanyl[5]	233.1	150.1	84.1	100	112	20	16	2.688	10.0	1.00	0.100
Norketamine[7]	224	207	125	92	92	8	24	2.690	1000	100	10.0
Normeperidine[8]	234.2	160.1	56.1	138	138	12	20	2.942	500	50.0	5.00
Norpropoxyphene[9]	326.2	252.1	44.1	90	66	5	13	3.830	1000	100	10.0
o-Desmethyltramadol[9]	250.2	58.1	42.1	97	97	16	104	2.313	250	25.0	2.50
Oxazepam[3]	287	269	241	133	133	20	21	4.060	400	40.0	8.00
Oxycodone[1]	316.2	298.1	241.1	143	143	17	29	2.080	500	50.0	5.00
Oxymorphone[1]	302.2	227.1	198.1	133	133	28	48	1.464	500	50.0	5.00
PCP[10]	244.2	91	86.1	86	86	41	9	3.249	50.0	5.00	0.500
Propoxyphene[9]	340.2	266.2	58.1	92	92	5	25	3.800	1000	100	10.0
Temazepam[3]	301	255	177	117	117	29	45	4.264	400	40.0	4.00
Tramadol[9]	264.2	58.1	42.1	107	107	16	108	2.724	250	25.0	2.50

1 - Internal Standard d_3-Morphine
2 - Internal Standard d_4-7-Aminoclonazepam
3 - Internal Standard d_5-Nordiazepam
4 - Internal Standard d_5-Amphetamine
5 - Internal Standard d_5-Fentanyl
6 - Internal Standard d_7-Carisoprodol
7 - Internal Standard d_4-Ketamine
8 - Internal Standard d_4-Meperidine
9 - Internal Standard d_5-Propoxyphene
10 - Internal Standard d_5-PCP

ß-Glucuronidase, Type-2, from Helix pomatia, (cat.#G0876-5mL) was purchased from Sigma-Aldrich. Fresh urine was obtained from a male volunteer. Hydrolysis of urine consisted of combining 2 mL of urine, 28.4 µL of the working internal standard solution, 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.

Instrumentation. All automated DPX PrepSequences were performed using a dual-head GERSTEL MPS XL with DPX Option as shown in Figure 1. All analyses were performed using an Agilent 1290 HPLC with a Zorbax Eclipse Plus C18 column (2.1 x 100 mm, 1.8 µm, 600 bar), an Agilent 6460 Triple Quadrupole Mass Spectrometer with Jet stream electrospray source and GERSTEL MPS XL autosampler configured with an Active Washstation. Sample injections were performed using a 6 port (0.25 mm) Cheminert C2V injection valve fitted with a 2 µL stainless steel sample loop.

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

Urine Sample Pretreatment.

- Pipette 260 µL of hydrolyzed urine sample into a clean shell vial.

Figure 2 shows a graphical representation of the general DPX extraction process. The automated DPX extraction used for this method consisted of the following steps:

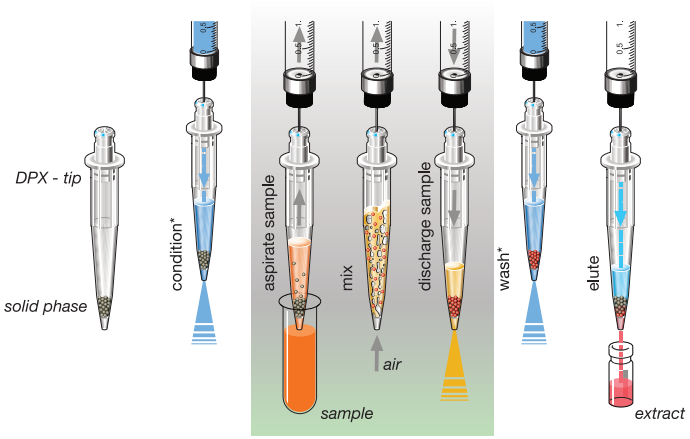

Figure 2. Graphical representation of the DPX extraction process.

Automated DPX Prep Sequence.
DPX Extraction.

- The MPS aspirates 750 µL of 100 % acetonitrile into the 2.5 mL DPX syringe.
- Pick up a new DPX tip (DPX-RP-S) located on the MPS tray.
- The MPS adds 500 µL of 100 % acetonitrile through the DPX tip, into the urine sample found on the MPS sample tray.
- Wait 6 seconds to allow acetonitrile to completely wet the DPX sorbent.
- Aspirate the entire sample and then air into DPX tip.
- After equilibrating for 5 seconds, dispense the contents of the DPX tip, as well as the remaining acetonitrile found within the DPX syringe, back into the original shell vial in the tray.
- Move the DPX tip to the PipWaste position and dispose of the DPX tip.

Evaporation.

- Transfer 450 µL of the upper liquid layer located within the original shell vial, into a clean, empty, magnetically capped autosampler vial with septum located on a VT98 tray.
- Transport the vial to the SPESampl position of the Evaporation Station Option.
- Transport the Evaporation Station Tool to the SPEVial position.
- Evaporate the sample to dryness under a stream of nitrogen for 4 minutes at 70°C
- Transport the Evaporation Station Tool to the SPEWaste position.
- Transfer 250 µL of 10 % methanol in water into the vial and mix for 10 seconds.
- Transport the vial back to the original position on the VT98 tray.

Analysis conditions LC.

Pump:	gradient (600 bar), flowrate = 0.5 mL/min	
Mobile Phase:	A - 5 mM ammonium formate with 0.05 % formic acid	
	B - 0.05 % formic acid in methanol	
Gradient:	Initial	5 % B
	0.5 min	5 % B
	1.5 min	30 % B
	3.5 min	70 % B
	4.5 min	95 % B
	6.5 min	95 % B
	7.5 min	5 % B
Run time:	12 minutes	
Inj. volume:	2.0 µL (loop over-fill technique)	
Column temp.:	55°C	

Analysis conditions MS.

Operation:	electrospray positive mode
Gas temperature:	350°C
Gas flow (N_2):	12 L/min
Nebulizer pressure:	35 psi
Capillary voltage:	4000 V
delta EMV:	+ 400 V
delta RT (min):	0.5 min

The mass spectrometer acquisition parameters are shown in Table 1 including qualifier ions. A retention time window value of 0.5 minute was used for each positive ion transition being monitored during the course of the dynamic MRM experiment.

Results and Discussion

Figure 3 shows representative mass chromatograms for all pain management drugs, along with their respective qualifier ion transitions, from an extracted low QC sample.

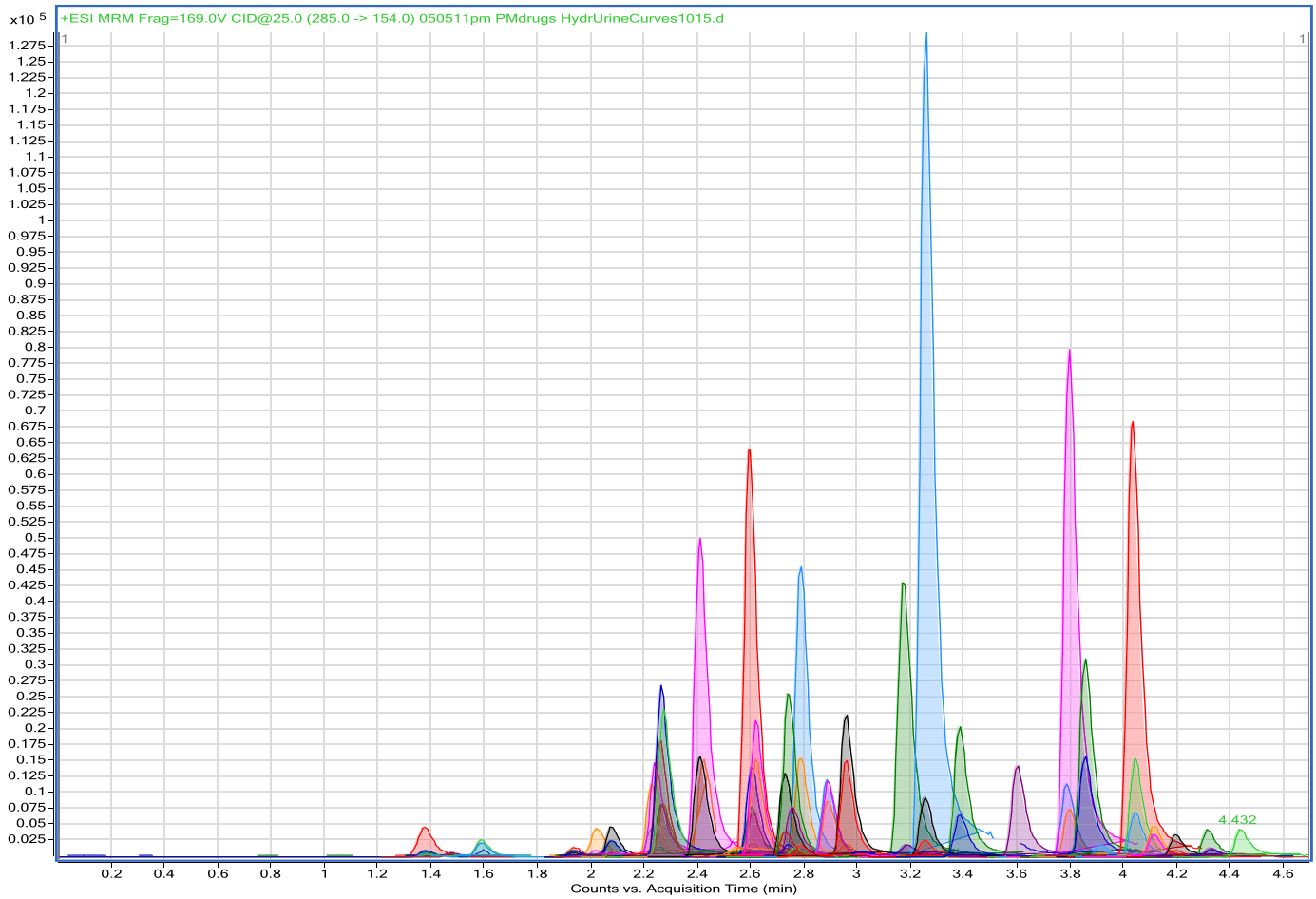

Figure 3. Representative Mass Chromatograms for low QC sample.

The lower limits of quantitation of this method are shown in Table 1. Representative calibration curves are shown in Figure 4. Regression analysis for all pain management drugs analyzed using this method resulted in R^2 values of 0.99 or greater.

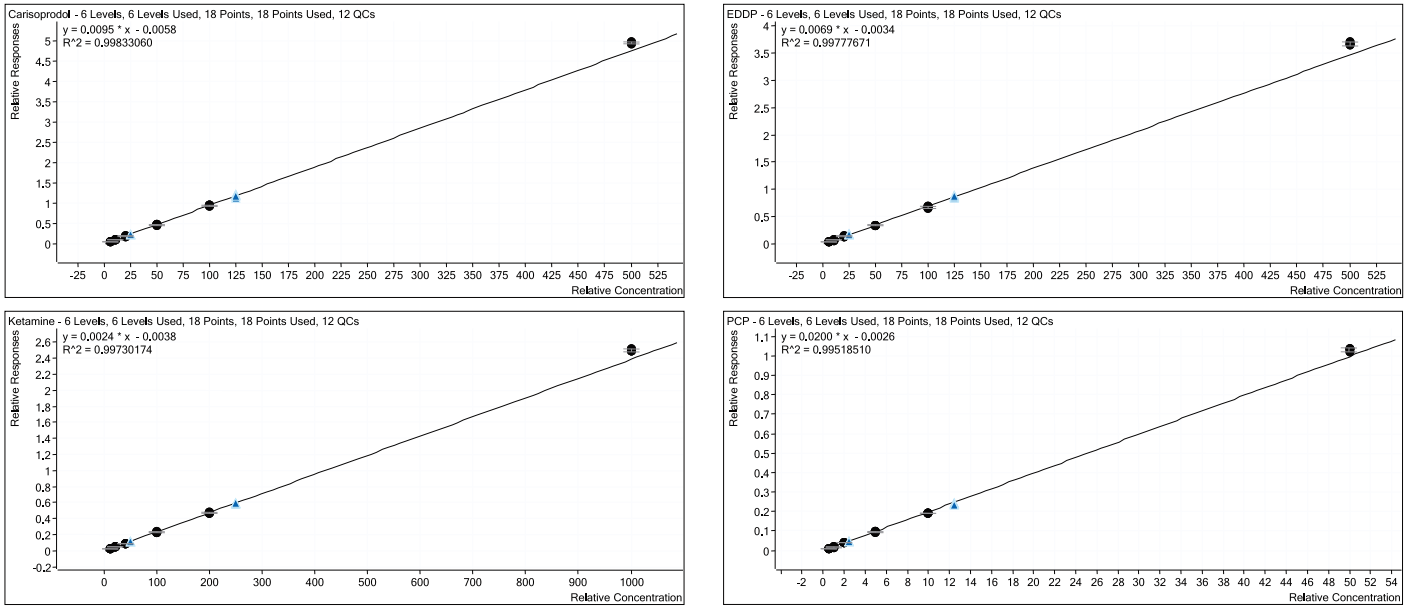

Figure 4. Representative calibration curves: Carisoprodol, EDDP, Ketamine, and PCP.

The accuracy and precision of the method was determined for all pain management drugs analyzed using QC samples at high and low concentrations. Table 2 shows the resulting accuracy and precision data for all pain management drugs. Accuracy data averaged 98.9 % (range: 91.2 % - 108 %) and precision data averaged 5.48 % CV (range: 1.06 % -17.0 %) for all pain management drugs analyzed.

Table 2. QC samples accuracy and precision table.

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Precision [%]	Ave. Accuracy [%]
6-MAM	QCL	5.00	4.80	8.60	96.1
	QCH	25.00	24.88	2.24	99.5
7-Aminoclonazepam	QCL	25.00	23.77	6.97	95.1
	QCH	125.00	121.38	3.62	97.1
Alprazolam	QCL	20.00	18.42	4.80	92.1
	QCH	100.00	92.30	2.73	92.3
Amphetamine	QCL	50.00	50.31	9.74	101
	QCH	250.00	260.62	2.96	104
α-OH-Alprazolam	QCL	10.00	9.59	8.36	95.9
	QCH	50.00	48.99	11.0	98.0
Benzoylecgonine	QCL	12.50	11.91	6.47	95.2
	QCH	62.50	67.46	5.36	108
Buprenorphine	QCL	5.00	4.73	10.8	94.7
	QCH	25.00	26.05	7.64	104
Carisoprodol	QCL	25.00	24.40	1.06	97.6
	QCH	125.00	125.45	2.57	100
Clonazepam	QCL	20.00	19.18	12.8	95.9
	QCH	100.00	103.75	8.90	104
Cocaine	QCL	12.50	12.33	7.71	98.7
	QCH	62.50	65.83	5.88	105

Table 2. QC samples accuracy and precision table (cont.).

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Precision [%]	Ave. Accuracy [%]
Codeine	QCL	25.00	23.57	6.72	94.3
	QCH	125.00	125.35	4.02	100
Diazepam	QCL	20.00	19.20	4.20	96.0
	QCH	100.00	98.43	2.91	98.4
EDDP	QCL	25.00	24.23	5.79	96.9
	QCH	125.00	124.84	2.18	99.9
Fentanyl	QCL	0.50	0.46	9.09	91.7
	QCH	2.50	2.54	4.00	101
Flunitrazepam	QCL	20.00	18.24	1.13	91.2
	QCH	100.00	100.09	5.71	100
Hydrocodone	QCL	25.00	23.57	6.72	94.3
	QCH	125.00	125.35	4.02	100
Hydromorphone	QCL	25.00	23.54	5.84	94.2
	QCH	125.00	128.61	2.62	103
Ketamine	QCL	50.00	47.44	2.62	94.9
	QCH	250.00	247.41	1.11	99.0
Lorazepam	QCL	20.00	19.66	9.49	98.3
	QCH	100.00	103.91	5.90	104
MDA	QCL	50.00	48.68	10.0	97.4
	QCH	250.00	248.23	2.19	99.3
MDEA	QCL	50.00	49.63	8.48	99.3
	QCH	250.00	254.70	3.50	102
MDMA	QCL	50.00	48.80	10.8	97.6
	QCH	250.00	255.70	3.15	102
Meperidine	QCL	25.00	23.54	2.88	94.2
	QCH	125.00	126.71	1.63	101
Meprobamate	QCL	25.00	24.27	2.76	97.1
	QCH	125.00	127.82	3.74	102
Methadone	QCL	25.00	23.97	7.03	95.9
	QCH	125.00	123.46	3.76	98.8
Methamphetamine	QCL	50.00	48.95	10.2	97.9
	QCH	250.00	255.22	4.90	102
Methylphenidate	QCL	25.00	24.51	7.06	98.1
	QCH	125.00	129.98	6.09	104
Morphine	QCL	25.00	23.70	3.07	94.8
	QCH	125.00	126.66	2.00	101
Nitrazepam	QCL	20.00	19.04	6.27	95.2
	QCH	100.00	107.18	7.28	107
Norbuprenorphine	QCL	5.00	5.32	9.42	106
	QCH	25.00	24.57	5.93	98.3
Nordiazepam	QCL	20.00	19.22	3.46	96.1
	QCH	100.00	101	2.73	101
Norfentanyl	QCL	0.50	0.51	5.52	101
	QCH	2.50	2.54	4.32	102

Table 2. QC samples accuracy and precision table (cont.).

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Precision [%]	Ave. Accuracy [%]
Norketamine	QCL	50.00	49.63	1.97	99.3
	QCH	250.00	249.28	2.03	99.7
Normeperidine	QCL	25.00	24.18	3.97	96.7
	QCH	125.00	123.89	2.04	99.1
Norpropoxyphene	QCL	50.00	48.57	3.37	97.1
	QCH	250.00	251.74	2.98	101
o-Desmethyltramadol	QCL	12.50	12.75	5.55	102
	QCH	62.50	65.20	9.63	104
Oxazepam	QCL	20.00	19.72	17.0	98.6
	QCH	100.00	106.30	9.60	106
Oxycodone	QCL	25.00	23.20	7.50	92.8
	QCH	125.00	129.15	5.22	103
Oxymorphone	QCL	25.00	23.61	6.02	94.4
	QCH	125.00	126.16	5.40	101
PCP	QCL	2.50	2.35	2.89	94.0
	QCH	12.50	12.13	1.65	97.0
Propoxyphene	QCL	50.00	47.14	4.83	94.3
	QCH	250.00	249.99	3.88	100
Temazepam	QCL	20.00	19.56	5.81	97.8
	QCH	100.00	101.90	8.87	102
Tramadol	QCL	12.50	12.13	2.97	97.0
	QCH	62.50	64.03	5.47	102

Robustness of the method was evaluated by extracting and analyzing multiple hydrolyzed urine samples at the minimum reportable limits of the pain management drugs over three separate days. Table 3 shows the resulting precision data for the responses of representative pain management drugs. Precision data averaged 6.04 % CV (range: 0.788 % -14.2 %) for all pain management drugs analyzed over the three day period.

Table 3. Method robustness data.

Compound	Response [% CV]
6-MAM	12.9
Alprazolam	6.40
Amphetamine	13.4
α-OH-Alprazolam	1.56
Benzoylecgonine	2.64
Buprenorphine	3.99
Carisoprodol	3.04
Clonazepam	4.73
Cocaine	7.77
Codeine	1.06
Diazepam	10.3
EDDP	7.03
Fentanyl	2.13

Compound	Response [% CV]
Flunitrazepam	14.2
Hydrocodone	2.96
Hydromorphone	3.83
Lorazepam	9.38
MDA	6.91
MDEA	4.18
MDMA	5.42
Meprobamate	2.32
Methadone	7.68
Methamphetamine	5.06
Methylphenidate	7.58
Morphine	2.80
Nitrazepam	4.19

Compound	Response [% CV]
Norbuprenorphine	6.99
Nordiazepam	8.20
Norfentanyl	2.49
Norpropoxyphene	8.02
o-Desmethyltramadol	3.30
Oxazepam	8.62
Oxycodone	0.788
Oxymorphone	5.99
PCP	8.59
Propoxyphene	6.79
Temazepam	11.1
Tramadol	5.21

The total cycle time per sample for the DPX extraction, sample concentration, reconstitution and injection was 13 minutes, enabling "just in time" sample preparation using the MAESTRO software PrepAhead function. Using this automated procedure for extraction and analysis over 100 samples can be processed per day.

Conclusions

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

- Over 40 pain management drugs can be successfully extracted from hydrolyzed urine samples using an automated DPX procedure followed by LC-MS/MS analysis using the Agilent 6460 Triple Quadrapole Mass Spectrometer.
- This DPX method proved to be rapid and readily automated using the dual head GERSTEL MPS XL robotic sampler.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved with limits of quantitation ten (10) times lower than the minimum reportable limits for the majority of pain management drugs analyzed.
- The DPX-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 98.9 % (range: 91.2 % - 108 %) and precision data averaged 5.48 % CV (range: 1.06 % - 17.0 %) for all pain management drugs analyzed.
- Method robustness data averaged 6.04 % CV (range: 0.788 % -14.2 %) for all pain management drugs analyzed over a three day period.

GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
45473 Mülheim an der Ruhr
Germany
+49 (0) 208 - 7 65 03-0
+49 (0) 208 - 7 65 03 33
gerstel@gerstel.com
www.gerstel.com

GERSTEL Worldwide

GERSTEL, Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
USA
+1 (410) 247 5885
+1 (410) 247 5887
sales@gerstelus.com
www.gerstelus.com

GERSTEL AG
Wassergrabe 27
CH-6210 Sursee
Switzerland
+41 (41) 9 21 97 23
+41 (41) 9 21 97 25
swiss@ch.gerstel.com
www.gerstel.ch

GERSTEL K.K.
1-3-1 Nakane, Meguro-ku
Tokyo 152-0031
SMBC Toritsudai Ekimae Bldg 4F
Japan
+81 3 5731 5321
+81 3 5731 5322
info@gerstel.co.jp
www.gerstel.co.jp

GERSTEL LLP
10 Science Park Road
#02-18 The Alpha
Singapore 117684
+65 6779 0933
+65 6779 0938
SEA@gerstel.com
www.gerstel.com

GERSTEL (Shanghai) Co. Ltd
Room 206, 2F, Bldg.56
No.1000, Jinhai Road,
Pudong District
Shanghai 201206
+86 21 50 93 30 57
china@gerstel.com
www.gerstel.cn

GERSTEL Brasil
Av. Pascoal da Rocha Falcão, 367
04785-000 São Paulo - SP Brasil
+55 (11)5665-8931
+55 (11)5666-9084
gerstel-brasil@gerstel.com
www.gerstel.com.br

Awarded for the active pursuit of environmental sustainability