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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*

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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. Most SPE products, however, are designed in such a way that relatively large volumes of solvent are required for the process. Consequently, 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 α -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 μ 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	158	41	2.106	100	10.0	1.00
7-Aminoclonazepam ²	286.1	222.1	138	25	2.772	500	50.0	5.00
Alprazolam ³	309	281	179	25	4.115	400	40.0	4.00
Amphetamine ⁴	136.1	119.1	66	5	2.223	1000	100	10.0
α -OH-Alprazolam ³	325	297	150	28	3.991	200	20.0	2.00
Benzoyllecgonine ⁴	290.1	168.1	118	17	2.624	250	25.0	2.50
Buprenorphine ⁵	468.3	414	220	35	3.749	100	10.0	1.00
Carisoprodol ⁶	261.2	176.1	80	1	4.057	500	50.0	5.00
Clonazepam ³	316	270	158	25	3.856	400	40.0	8.00
Cocaine ⁴	304.2	182.1	138	17	2.741	250	25.0	2.50
Codeine ¹	300.2	165.1	158	45	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	169	25	4.447	400	40.0	4.00
EDDP ⁵	278.2	234.1	160	33	3.394	500	50.0	5.00
Fentanyl ⁵	337.2	188.1	143	21	3.273	10.0	1.00	0.100
Flunitrazepam ³	314	268	153	25	3.919	400	40.0	4.00
Hydrocodone ¹	300.2	199	159	29	2.078	500	50.0	5.00
Hydromorphone ¹	286.2	185	159	29	1.585	500	50.0	5.00
Ketamine ⁷	238.1	220.1	105	11	2.596	1000	100	10.0
Lorazepam ³	321	275	102	21	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	61	5	2.272	1000	100	10.0
MDEA ⁴	208	163	107	9	2.455	1000	100	10.0
MDMA ⁴	194	163	97	9	2.306	1000	100	10.0
Meperidine ⁸	248.2	220.1	128	21	2.935	500	50.0	5.00
Meprobamate ⁶	219.1	158	60	0	3.289	500	50.0	5.00
Methadone ⁵	310.2	265.1	112	9	3.877	500	50.0	5.00
Methamphetamine ⁴	150.2	119	92	5	2.313	1000	100	10.0
Methylphenidate ⁴	234.1	84.1	112	21	2.786	500	50.0	5.00
Morphine ¹	286.2	165.1	158	41	1.374	500	50.0	5.00
Nitrazepam ³	282	236	148	25	3.908	400	40.0	4.00
Norbuprenorphine ⁵	414.3	187.1	205	41	3.263	100	10.0	1.00
Nordiazepam ³	271	165	138	25	4.378	400	40.0	4.00
Norfentanyl ⁵	233.1	150.1	100	20	2.688	10.0	1.00	0.100
Norketamine ⁷	224	207	92	8	2.690	1000	100	10.0
Normeperidine ⁸	234.2	160.1	138	12	2.942	500	50.0	5.00
Norpropoxyphene ⁹	326.2	252.1	90	5	3.830	1000	100	10.0
o-Desmethyiltramadol ⁹	250.2	58.1	97	16	2.313	250	25.0	2.50
Oxazepam ³	287	269	133	20	4.060	400	40.0	8.00
Oxycodone ¹	316.2	298.1	143	17	2.080	500	50.0	5.00
Oxymorphone ¹	302.2	227.1	133	28	1.464	500	50.0	5.00
PCP ¹⁰	244.2	91	86	41	3.249	50.0	5.00	0.500
Propoxyphene ⁹	340.2	266.2	92	5	3.800	1000	100	10.0
Temazepam ³	301	255	117	29	4.264	400	40.0	4.00
Tramadol ⁹	264.2	58.1	107	16	2.724	250	25.0	2.50

- 1 - Internal Standard d₃-Morphine
2 - Internal Standard d₄-7-Aminoclonazepam
3 - Internal Standard d₅-Nordiazepam
4 - Internal Standard d₅-Amphetamine
5 - Internal Standard d₅-Fentanyl
6 - Internal Standard d₇-Carisoprodol
7 - Internal Standard d₄-Ketamine
8 - Internal Standard d₄-Meperidine
9 - Internal Standard d₅-Propoxyphene
10 - Internal Standard d₅-PCP

β -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 μ L of the working internal standard solution, 100 μ L of β -Glucuronidase, and 500 μ L 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 μ m, 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 μ L stainless steel sample loop.

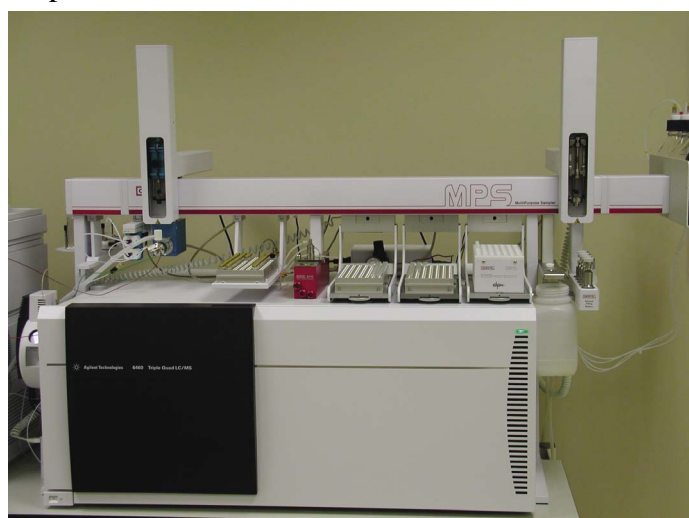


Figure 1. MPS 2XL MultiPurpose Sampler (MPS) with GERSTEL DPX option.

Urine Sample Pretreatment.

- Pipette 260 μ L 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:

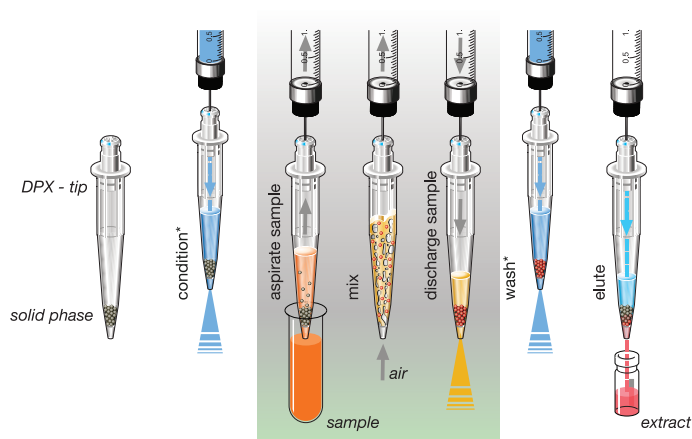


Figure 2. Graphical representation of the DPX extraction process.

Automated DPX Prep Sequence.

DPX Extraction.

- The MPS aspirates 750 μ L 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 μ L 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 μ L 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 μ L 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 (N₂): 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.

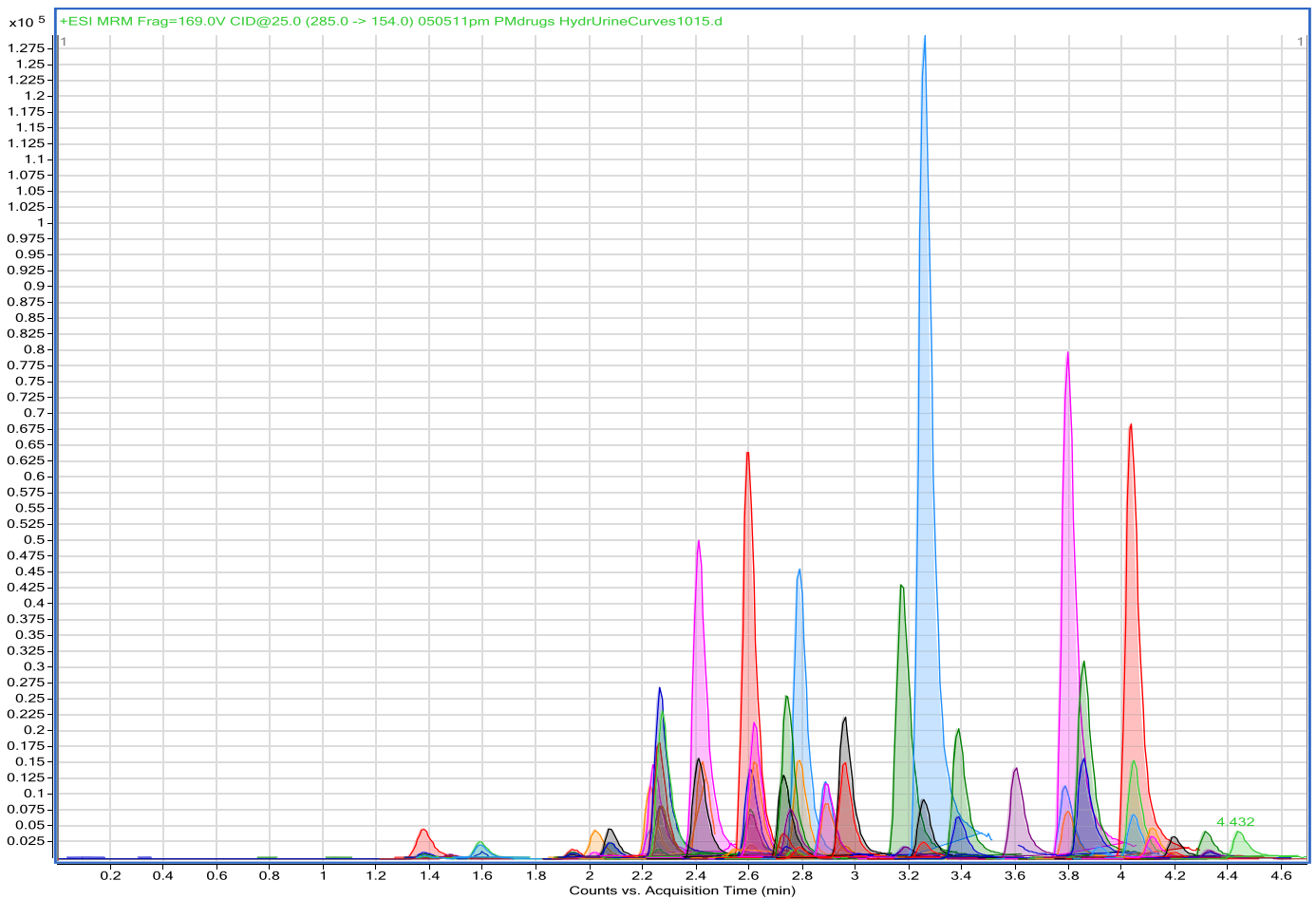


Figure 3. Representative Mass Chromatograms for low QC sample.

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 R² values of 0.99 or greater.

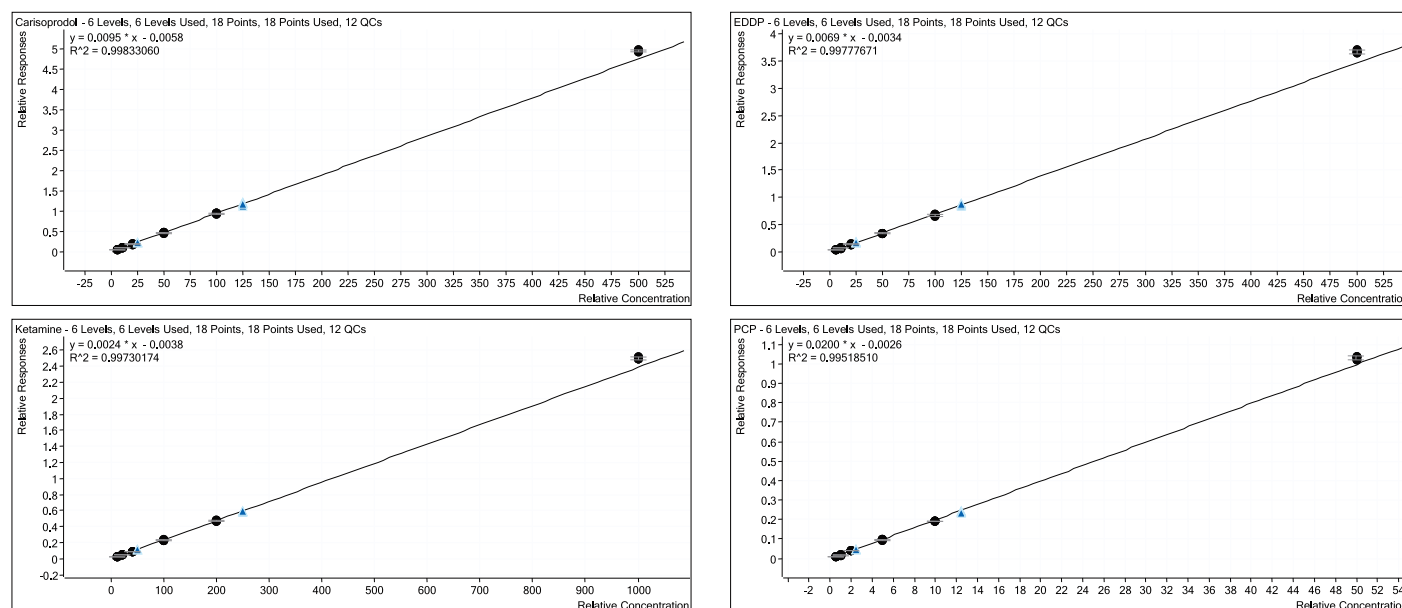


Figure 4. Representative calibration curves: Carisoprodol, EDDP, Ketamine, and PCP.

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
Benzoylcegonine	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]	Compound	Response [% CV]	Compound	Response [% CV]
6-MAM	12.9	Flunitrazepam	14.2	Norbuprenorphine	6.99
Alprazolam	6.40	Hydrocodone	2.96	Nordiazepam	8.20
Amphetamine	13.4	Hydromorphone	3.83	Norfentanyl	2.49
α -OH-Alprazolam	1.56	Lorazepam	9.38	Norpropoxyphene	8.02
Benzoyllecgonine	2.64	MDA	6.91	o-Desmethyltramadol	3.30
Buprenorphine	3.99	MDEA	4.18	Oxazepam	8.62
Carisoprodol	3.04	MDMA	5.42	Oxycodone	0.788
Clonazepam	4.73	Meprobamate	2.32	Oxymorphone	5.99
Cocaine	7.77	Methadone	7.68	PCP	8.59
Codeine	1.06	Methamphetamine	5.06	Propoxyphene	6.79
Diazepam	10.3	Methylphenidate	7.58	Temazepam	11.1
EDDP	7.03	Morphine	2.80	Tramadol	5.21
Fentanyl	2.13	Nitrazepam	4.19		

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 Quadrupole 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 R² 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.

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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 Toritsuudai Ekimae Bldg 4F
Japan

☎ +81 3 5731 5321
☎ +81 3 5731 5322
@ info@gerstel.co.jp
🌐 www.gerstel.co.jp

GERSTEL LLP

Level 25, North Tower
One Raffles Quay
Singapore 048583

☎ +65 6622 5486
☎ +65 6622 5999
@ SEA@gerstel.com
🌐 www.gerstel.com

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

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