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Rapid Cleanup and Comprehensive Screening of Pain Management Drugs in Urine using Automated Disposable Pipette Extraction and LC-MS/MS

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Abstract
This study focuses on the automated extraction of small volumes of urine samples (< 500 µL) using disposable pipette extraction (DPX) for the comprehensive screening for pain management drugs by LC-MS/MS. Using a GERSTEL MPS autosampler, DPX extractions of hydrolyzed urine were performed, using a reversed phase (DPX-RP-S) sorbent. The resulting eluents from the DPX extractions were automatically diluted and injected into an Agilent® Technologies LC-MS/MS system. Sample preparation was performed just-in-time enabling high throughput screenings, averaging a cycle time of 7 min/sample.

Validation results show that the automated DPX-LC-MS/MS screening method provides adequate sensitivity for over 65 analytes and internal standards. Lower limits of quantitation (LLOQ) ranged between 0.5 – 50 ng/mL and % RSDs were below 10% in most cases.

Introduction

Toxicology laboratories are trying to find ways to minimize sample preparation and enhance productivity. The adaptation of LC-MS/MS instrumentation is desired due to the high sensitivity, high selectivity, low detection limits (e.g., 1 ng/mL), smaller sample volumes used, and also due to the fact that LC-MS/MS doesn't require chemical derivatization of analytes. Conventional sample preparation methods involve liquid-liquid or solid-phase extraction (SPE). A different approach is to use SPE to extract the sample matrix. In this case, matrix interferences are bound to the sorbent in order to be removed from the analyte solution. The major advantage of this approach is that no separate wash or elution steps are required, enabling rapid sample preparation while still allowing comprehensive screening of the cleaned sample.

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 uses sorbent loosely contained in a pipette tip enabling highly efficient mixing with the sample solution. The main advantages of DPX technology are: rapid extraction, high recoveries, negligible solvent waste generation, and full automation of the extraction combined with introduction to the chromatographic system.

We have developed a fast automated DPX urine cleanup method using a GERSTEL MultiPurpose Sampler (MPS XL) for comprehensive screening of 49 pain management drugs with LC-MS/MS. The reversed phase sorbent (DPX-RP-S) used in the method allows the removal of salts and proteins present in urine, resulting in reduced matrix effects. The sorbent is chosen to extract the matrix without binding or absorbing the analytes of interest providing high recoveries. The schematic for the DPX "Cleanup" procedure is shown in Figure 2. Since the extraction time (3 min) is less than the analytical LC-MS/MS run time (4 min), the extraction of one sample can be performed during the chromatographic analysis of the previous sample, achieving high throughput while processing each sample "just in time" ensuring that all samples are treated identically.

Experimental

Materials. Stock solutions for the compounds listed in Table 1 were purchased from Cerilliant. An intermediate analyte stock solution was prepared by combining the analyte stock solutions with acetonitrile, at appropriate concentrations, to evaluate the different drug classes.

Deuterated analogues, d_3-morphine, d_4-buprenorphine, d_3-norbuprenorphine, d_9-methadone, d_3-tramadol, d_5-fentanyl, d_5-alpha-hydroxy alprazolam, d_4-clonazepam, d_5-oxazepam, d_5-estazolam, d_3-cocaine, d_5-nordiazepam, d_5-propoxyphene, d_7-carisoprodol, d_5-amphetamine, d_4-ketamine, d_4-7-aminoclonazepam, and d_5-PCP were purchased from Cerilliant. Table 1 shows which deuterated internal standard was used for each respective analyte during quantitation.

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 concentrations listed in Table 1. Calibration standards were then prepared using a dilution ratio strategy from the high concentration sample of 1:2:2:2.5:2. The high, medium and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:1.33:3.33: 8. ß-Glucuronidase, Type-2, from Helix pomatia, (cat.#G0876-5mL) was purchased from Sigma-Aldrich. Fresh urine was obtained from a male volunteer. All other reagents and solvents used were reagent grade.

Instrumentation. All automated DPX PrepSequences were performed using a MultiPurpose Sampler (MPS XL Dual Tower) with GERSTEL DPX Option as shown in Figure 1. All analyses were performed using an Agilent® 1290 Infinity LC with a Zorbax Eclipse Plus C18 column (2.1 x 50 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 Wash Station (AWS). Sample injections were made using a 6 port (0.25mm) Cheminert C2V injection valve fitted with a 2 µL stainless steel sample loop.

Figure 1. MPS 2XL multi-purpose sampler with the GERSTEL DPX option for high throughput pain management drug screening.

Sample pretreatment. Hydrolysis of urine was performed by combining 2 mL of urine, 150 µL of the working internal standard solution, 100 µL of ß-Glucuronidase, and 500 µL of 0.66M acetate buffer, pH 4, vortex mixing for 30 seconds, and then incubating at 55°C for 2 hours. Aliquots of 260 µL of hydrolyzed urine samples were added into clean shell vials for automated cleanup and injection.

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

Automated DPX Prep Sequence - DPX Cleanup procedure

1. Aspirate 750 µL of 100 % acetonitrile from the fast solvent delivery station using the 2.5 mL DPX syringe.
2. Pick up a new DPX tip (DPX-RP-S) located within the tray.
3. Add 500 µL of 100 % acetonitrile through the DPX tip, into the urine sample located on the MPS sample tray.
4. Wait for 6 seconds to allow the acetonitrile to completely wet the DPX sorbent.
5. Aspirate the entire sample followed by 1400 µL of air into the DPX tip.
6. 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.
7. Move the DPX tip to the PipWaste position and dispose of the DPX tip.
8. Transfer 100 µL of the upper liquid layer located within the original shell vial, into a clean, empty, capped autosampler vial with magnetic septum cap located on a VT98 tray.
9. Dilute the extract by adding 900 µL of water into the sample vial.
10. Inject 50 µL of the sample into the HPLC injection valve.

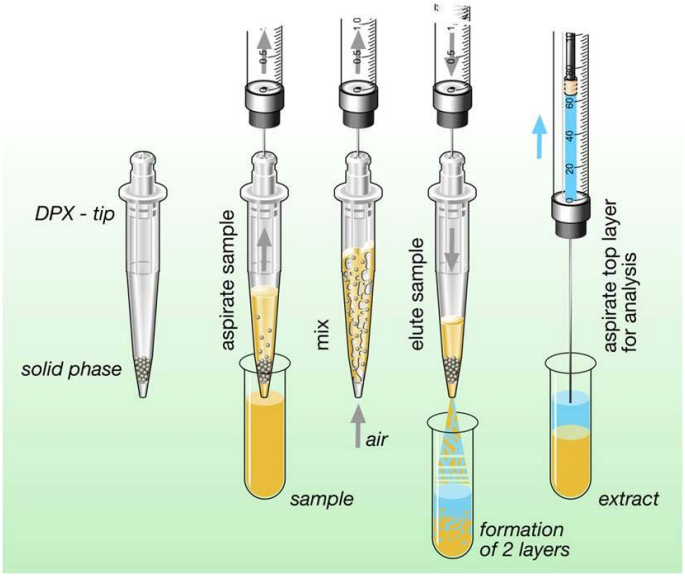

Figure 2. Graphical representation of the automated DPX urine cleanup process.

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
	6.49 min	95 % B
	6.5 min	5 % B
Run time:	6.5 minutes	
Injection volume:	2 µL (loop over-fill technique)	
Column temperature:	55°C	

Analysis condition MS.

Operation:	electrospray positive mode
Gas temperature:	350°C
Gas flow (N_2):	12 L/min
Nebulizer pressure:	35 psi
Capillary voltage:	4400 V

A total of 124 MRM transitions (98 Analyte qualifier/quantifier and 18 internal standard transitions) were monitored in a 4 minute analytical window followed by a column regeneration time of 2.5 minutes. A retention time window value of 30 seconds was used for each positive ion transition being monitored in the dynamic MRM method. Detailed mass spectrometric acquisition parameters are available upon request.

Results and Discussion

Figure 3 shows representative dynamic MRM chromatograms for all 49 pain management drugs and internal standards, from a hydrolyzed urine sample spiked sample at the minimum reporting limit (MRL) concentrations after the automated DPX cleanup procedure.

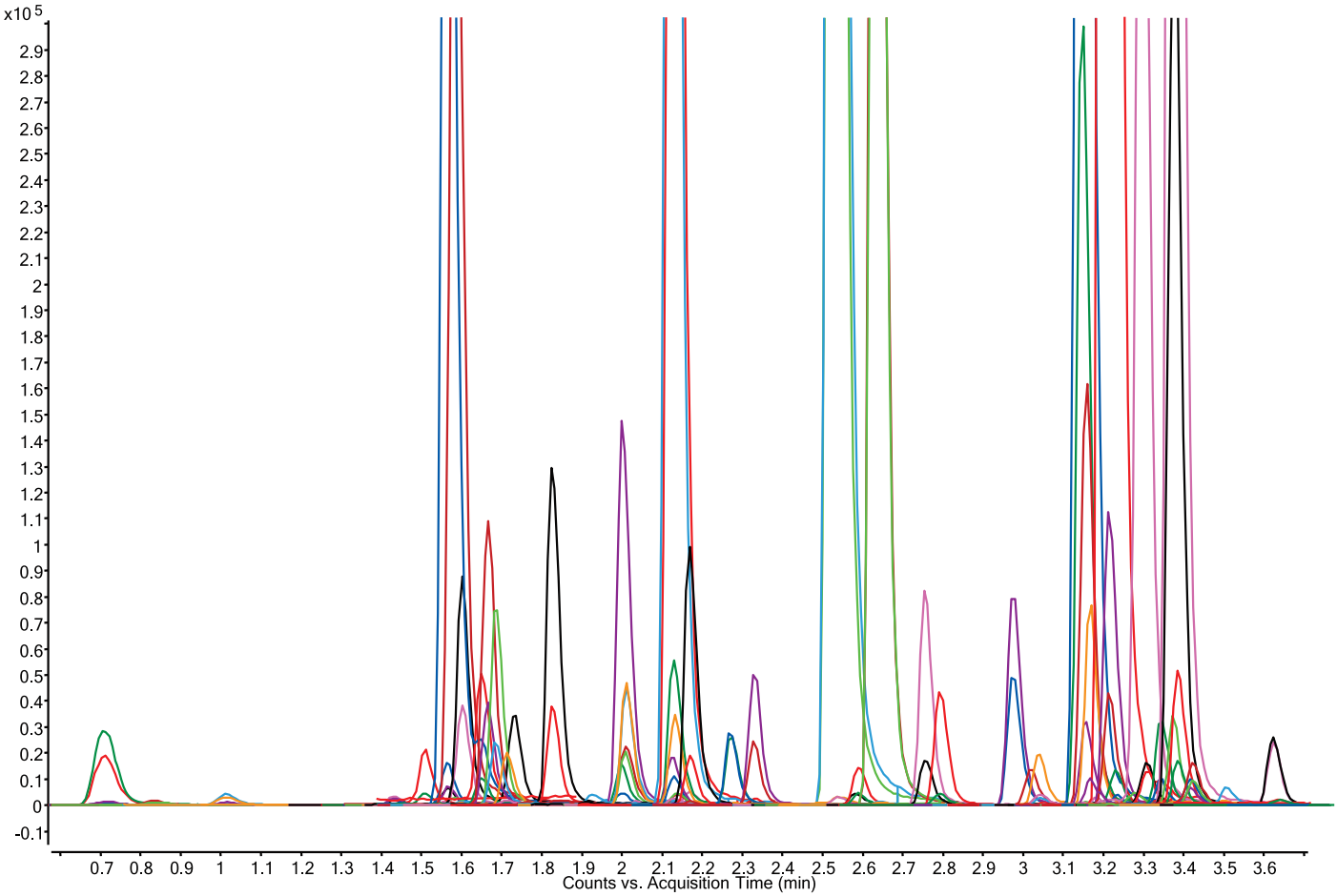

Figure 3. Overlaid chromatograms for all 124 dynamic MRM transitions from an extracted urine sample at the MRL.

Table 1 lists the column retention times, concentrations for the highest calibration standard, MRLs and LLOQs for the 49 analytes in this screening assay. LLOQ concentrations are higher (5 fold factor increase) in comparison to those listed in our previous work performed with an automated concentration step using a solvent evaporation station [1]. However, the LLOQs of this modified cleanup method are still below the original MRLs. Representative calibration curves are shown in Figure 4. Regression analysis for all pain management drugs analyzed within this method resulted in R^2 values of 0.99 or greater.

Table 1. Retention times, high calibration standard concentrations, MRLs and LOQs for all pain management drugs analyzed.

Compound	Ret. Time [min]	High Cal Std. [ng/mL]	MRL [ng/mL]	LOQ [ng/mL]
6-MAM[1]	1.60	100	10	5
Codeine[1]	1.43	500	50	25
Hydrocodone[1]	1.56	500	50	25
Hydromorphone[1]	1.01	500	50	25
Oxycodone[1]	1.51	500	50	25
Morphine[1]	0.71	500	50	25
Oxymorphone[1]	0.83	500	50	25
Meperidine[1]	2.27	500	50	25
Normeperidine[1]	2.33	500	50	25
Buprenorphine[2]	3.02	100	10	5
Norbuprenorphine[3]	2.60	100	10	5
EDDP[4]	2.76	500	50	25
Methadone[4]	3.22	500	50	25
Norpropoxyphene[5]	2.98	1000	100	50
Propoxyphene[5]	3.16	1000	100	50
o-Desmethyl-cis-Tramadol[6]	1.71	250	25	12.5
cis-Tramadol[6]	2.13	250	25	12.5
Fentanyl[7]	2.64	10	1	0.5
Norfentanyl[7]	2.04	10	1	0.5
Meprobamate[8]	2.58	500	50	25
Carisoprodol[8]	3.38	500	50	25
7-Aminoclonazepam[9]	2.12	400	40	20
Clonazepam[10]	3.17	400	40	20
Oxazepam[11]	3.38	400	40	20
Estazolam[12]	3.30	400	40	20
Alprazolam[13]	3.42	400	40	20
Diazepam[13]	3.75	400	40	20
Flunitrazepam[13]	3.23	400	40	20
Lorazepam[12]	3.39	400	40	20
Nitrazepam[13]	3.15	400	40	20
Temazepam[13]	3.50	400	40	20
α-OH-Alprazolam[14]	3.29	400	40	20
Nordiazepam[13]	3.63	400	40	20
Bromazepam[12]	3.05	400	40	20
Clobazam[13]	3.34	400	40	20
Midazolam[13]	3.08	400	40	20
Triazolam[13]	3.41	400	40	20
Flurazepam[13]	2.79	400	40	20
Ketamine[15]	2.01	1000	100	50
Norketamine[15]	2.01	1000	100	50
Amphetamine[16]	1.60	1000	100	50
MDA[16]	1.64	1000	100	50
MDEA[16]	1.82	1000	100	50
MDMA[16]	1.69	1000	100	50
Methamphetamine[16]	1.66	1000	100	50
Methylphenidate[16]	2.16	1000	100	50
PCP[17]	2.54	50	5	2.5
Benzoylecgonine[18]	1.99	250	25	12.5
Cocaine[18]	2.13	250	25	12.5

Internal Standards			
1) d3-morphine	*6) d3-cistramadol*	*11) d5-oxazepam*	*16) d5-amphetamine*
2) d4-buprenorphine	*7) d5-Fentanyl*	*12) d5-estazolam*	*17) d5-PCP*
3) d3-norbuprenorphine	*8) d7-Carisoprodol*	*13) d5-nordiazepam*	*18) d3-cocaine*
4) d9-methadone	*9) d4-7aminoclonazepam*	*14) d5- -OH-alprazolam*	
5) d5-propoxyphene	*10) d4-Clonazepam*	*15) d4-ketamine*	

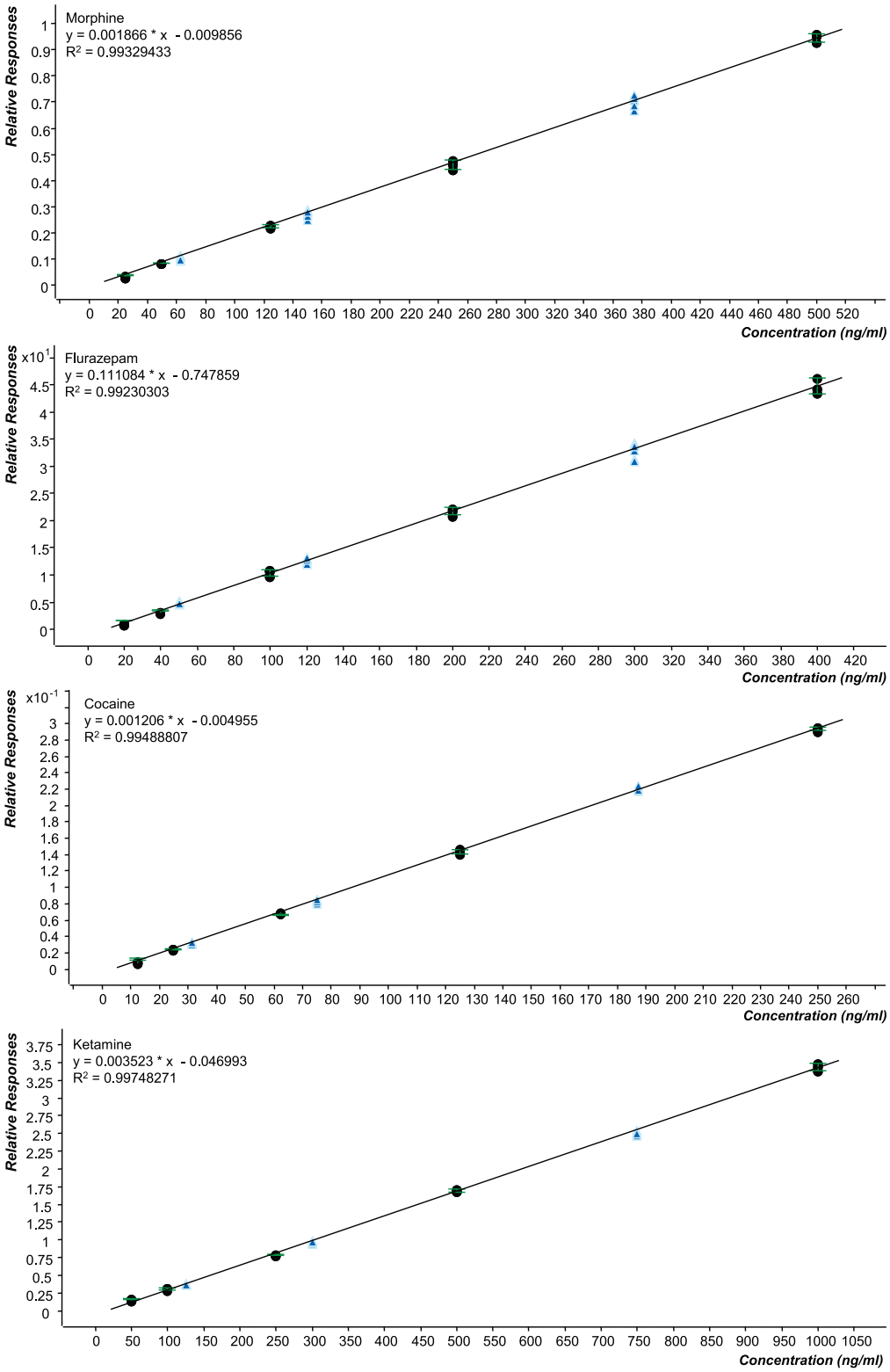

Figure 4. Representative calibration curves: morphine, flurazepam, cocaine and ketamine.

The DPX automated sample cleanup time was reduced from 7 to 3 min/sample; the total cycle time per sample for the extraction process and injection was reduced from 13 to 7 min/sample, fitting with the “just in time” sample preparation strategy available using the Maestro software and increasing throughput. Using this automated procedure for extraction and analysis over 200 samples can be processed per day.

The accuracy and precision of the method was measured for all pain management drugs analyzed extracting replicate (n=4) QC samples at high and low concentrations. Table 2 shows the resulting accuracy and precision data for all pain management drugs. Accuracy data averaged 98.0 % (range: 77 % - 108 %) and precision data (% RSD) averaged 4.2 % (range: 1.0 % -12.8 %) for all pain management drugs analyzed.

Table 2. Extracted QC sample % accuracies and % RSDs.

Compound	QCL [ng/mL]	Avg. Accuracy [%] [n = 4]	% RSD	QCH [ng/mL]	Avg. Accuracy [%] [n = 4]	% RSD
6-MAM	12.5	100.95	12.69	75.0	107.08	4.92
Codeine	62.5	98.24	3.01	375.0	105.02	2.31
Hydrocodone	62.5	96.07	3.78	375.0	103.65	2.74
Hydromorphone	62.5	98.57	2.26	375.0	101.83	1.62
Oxycodone	62.5	98.37	2.90	375.0	103.07	2.84
Morphine	62.5	96.84	6.46	375.0	102.07	1.49
Oxymorphone	62.5	100.46	2.57	375.0	100.52	3.04
Meperidine	62.5	97.32	3.15	375.0	104.16	4.48
Normeperidine	62.5	98.07	2.59	375.0	102.64	3.95
Buprenorphine	12.5	103.48	10.78	75.0	106.05	4.17
Norbuprenorphine	12.5	90.59	9.28	75.0	97.44	7.72
EDDP	62.5	106.42	1.47	375.0	106.77	1.72
Methadone	62.5	106.76	2.99	375.0	108.11	2.21
Norpropoxyphene	125.0	96.58	1.85	750.0	96.86	1.51
Propoxyphene	125.0	96.30	2.18	750.0	96.57	0.95
o-Desmethyl-cis-Tramadol	31.3	94.28	2.06	187.5	93.31	1.26
Tramadol	31.3	94.13	2.55	187.5	94.07	0.89
Fentanyl	1.3	108.68	6.97	7.5	101.72	3.97
Norfentanyl	1.3	98.98	12.11	7.5	98.20	2.84
Meprobamate	62.5	87.40	3.29	375.0	87.78	1.12
Carisoprodol	62.5	89.53	2.84	375.0	84.49	1.08
7-Aminoclonazepam	50.0	89.73	10.12	300.0	91.96	6.18
Clonazepam	50.0	88.94	9.13	300.0	101.38	2.64
Oxazepam	50.0	83.90	12.85	300.0	97.32	5.89
Estazolam	50.0	86.68	3.65	300.0	89.56	3.18
Alprazolam	50.0	100.33	3.28	300.0	103.97	5.54
Diazepam	50.0	97.87	3.81	300.0	98.22	4.48
Flunitrazepam	50.0	108.99	6.88	300.0	94.41	4.31
Lorazepam	50.0	91.83	6.68	300.0	97.05	8.71
Nitrazepam	50.0	97.22	12.63	300.0	96.97	4.97
Temazepam	50.0	94.79	7.76	300.0	98.30	2.22
α-OH-Alprazolam	50.0	76.57	4.13	300.0	79.37	6.21
Nordiazepam	50.0	109.88	10.59	300.0	95.81	5.82
Bromazepam	50.0	103.72	11.95	300.0	89.70	2.32
Clobazam	50.0	101.55	5.26	300.0	97.33	4.35
Midazolam	50.0	101.55	5.26	300.0	97.33	4.35
Triazolam	50.0	99.65	4.17	300.0	104.24	6.29
Flurazepam	50.0	100.66	3.15	300.0	97.94	4.20
Ketamine	125.0	88.19	1.43	750.0	88.72	1.04
Norketamine	125.0	90.52	2.05	750.0	87.67	1.43
Amphetamine	125.0	100.11	2.37	750.0	102.14	1.47
MDA	125.0	99.34	2.48	750.0	103.44	1.37
MDEA	125.0	101.75	2.49	750.0	102.59	1.67
MDMA	125.0	100.78	2.39	750.0	101.49	1.39
Methamphetamine	125.0	101.31	2.59	750.0	102.65	1.43
Methylphenidate	125.0	99.32	2.33	750.0	103.49	1.95
PCP	6.3	106.94	7.32	37.5	105.36	2.99
Benzoylecgonine	31.3	96.94	3.38	187.5	97.79	2.51
Cocaine	31.25	98.42	3.34	187.5	99.87	1.19

Conclusions

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

- The automated DPX cleanup method using the GERSTEL MPS XL Dual Tower robotic sampler for pain management drug screenings in urine was modified to provide cycle times of approximately 7 min/sample allowing throughput of over 200 samples per day
- 49 pain management drugs can be rapidly and reproducibly isolated from hydrolyzed urine samples using an automated DPX cleanup procedure coupled to LC/MS/MS analysis using the Agilent 6460 Triple Quadrapole Mass Spectrometer.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved with LOQs lower than the minimum reportable limits for the majority of pain management drugs analyzed.
- The DPX-LC/MS/MS method provided good accuracy and precision averaging 98.0 % (range: 77 % - 110 %) accuracy with 4.2 % RSD (range: 0.89 % -12.8 %) for all pain management drugs analyzed.

References

[1] Determination of Pain Management Drugs using Automated Disposable Pipette Extraction and LC-MS/MS, Gerstel AppNote AN-2011-06

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