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Automated Hydrolysis, Extraction and Determination of Opioids in Urine using a Novel Robotic Autosampler and LC-MS/MS Platform

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Keywords

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Abstract

The Opioid Epidemic continues to increase throughout the United States. According to the CDC, 66 % of all drug overdose deaths in 2016 involved an opioid [1]. This calculates to roughly 116 deaths every day from opioid related overdoses. After becoming addicted to prescription opioids, users may unfortunately turn to illicit alternatives such as heroin. To compound the issue, heroin has increasingly been found to be mixed with other synthetic opioids such as fentanyl, which is 100 times more potent than morphine. In order to respond effectively to this epidemic, forensic, health care, and law enforcement scientists need access to fast methods for assessing and monitoring which opioids are involved.

Automating the entire hydrolysis, extraction, and subsequent analysis by LC-MS/MS provides the critically needed high throughput analysis for opioids in urine. Using the novel GERSTEL MPS robotic autosampler, syringe transfer of all liquids involved in the enzymatic hydrolysis procedure, controlled incubation of the samples for a defined period of time, as well as extraction of the subsequent hydrolyzed urine samples using dispersive solid phase extraction were performed. The resulting eluents from the automated extractions were then introduced into the new Agilent Ultivo LC-MS/MS instrument.

Introduction

A variety of sample handling steps are required prior to the analysis of urine samples to accurately determine analyte concentrations. These steps typically begin with the enzymatic hydrolysis of analytes from their conjugated forms to the native drug using enzymes such as beta-glucuronidase. The genetically modified, pure beta-glucuronidase *IMCSzyme* can hydrolyze multiple drug classes within 30 minutes with high efficiency [2]. To ensure that the hydrolysis process is complete and reproducible, the pH, temperature and duration must be controlled and optimized for the enzyme used.

To achieve the very low limits of detection necessary for drug compounds and their metabolites, it is often necessary to remove interfering matrix. Interferences can be produced as a result of the hydrolysis procedure or occur naturally from the biological nature of the urine samples themselves. Solid phase extraction (SPE) is a widely used, proven method for sample preparation and sample clean-up of hydrolyzed urine samples in the field of forensic analysis. Disposable Pipette Extraction (DPX) was developed as an alternative to traditional SPE, combining efficient and rapid extraction with significantly reduced solvent consumption. DPX relies

on dispersive solid-phase extraction devices that use sorbent loosely contained in a pipette tip to efficiently mix with the sample solution. The main advantages of DPX technology are: Rapid extraction, high recoveries, negligible solvent waste is generated, and the extractions can be fully automated and coupled to GC/MS or LC/MS injection.

As a result of this study, we were able to show that an automated enzymatic hydrolysis and subsequent solid phase extraction method using the GERSTEL MPS robotic sampler could successfully be used for a variety of opioid compounds in urine. Opioid analytes isolated from hydrolyzed urine samples using the automated cleanup procedure were introduced to the LC-MS/MS system, an Agilent Technologies 1260 HPLC coupled with an Agilent Ultivo triple quadrupole mass spectrometer with Jet Stream electrospray source. The required limits of detection were met.

EXPERIMENTAL

Materials. All 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 solution with methanol, resulting in appropriate concentrations for the method evaluation for the different drug classes.

Deuterated analogues, d_5-fentanyl, d_5-norfentanyl, d_4-meperidine, d_3-morphine, d_9-methadone, d_4-buprenorphine, d_3-norbuprenorphine, and d_3-tramadol, were purchased from Cerilliant. An internal standard stock solution containing the deuterated internal standards was prepared in methanol at a concentration of 10.0 µg/mL. A working internal standard solution was prepared in 10 % methanol in water solution at a concentration of 1541 ng/mL. Table 1 shows which deuterated internal standards were used for the quantitation of the respective analytes.

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]	MRL [ng/mL]	Limit of Quant. [ng/mL]
Buprenorphine[4]	468.3	396.2	55.1	200	200	39	58	4.08	200	10.0	5.00
Codeine[3]	300.2	165.1	128	158	158	45	60	2.33	1000	50.0	25.0
d_3-Morphine	289.0	165.1	152	153	153	38	66	1.09	-	-	-
d_3-Norbuprenorphine	417.3	152	55.1	190	190	124	76	3.62	-	-	-
d_3-Tramadol	268.2	58.1	-	102	-	14	-	3.16	-	-	-
d_4-Buprenorphine	472.3	400.2	59.1	210	210	40	56	4.06	-	-	-
d_4-Meperidine	252.2	224.2	178.2	140	140	15	15	3.33	-	-	-
d_5-Fentanyl	342.3	188.1	105.1	92	92	20	40	3.67	-	-	-
d_5-Norfentanyl	238.2	84.1	55.1	125	125	20	50	3.06	-	-	-
d_9-Methadone	319.3	268.1	-	118	-	8	-	4.31	-	-	-
Fentanyl[6]	337.2	188.1	105.1	143	143	17	37	3.68	20.0	1.00	0.500
Furanyl Fentanyl[6]	375.2	188.1	105	150	150	15	25	3.67	20.0	n/a	0.500
Hydrocodone[3]	300.2	199	128	159	159	27	63	2.70	1000	50.0	25.0
Hydromorphone[1]	286.2	185	157	159	159	27	43	2.20	1000	50.0	25.0
Meperidine[5]	248.2	220.1	174.1	128	128	17	13	3.33	1000	50.0	25.0
Methadone[8]	310.2	265.1	105	112	112	7	27	4.32	1000	50.0	25.0
Morphine[1]	286.2	165.1	152	158	158	45	64	1.10	1000	50.0	25.0
Norbuprenorphine[2]	414.3	187.1	83.1	205	205	37	53	3.63	200	10.0	5.00
Norfentanyl[7]	233.2	84.1	55.1	112	112	12	36	3.08	20.0	1.00	0.500
Oxycodone[3]	316.2	298.1	241.1	143	143	13	25	2.42	1000	50.0	25.0
Oxymorphone[1]	302.1	227.1	198.1	133	133	24	44	1.29	1000	50.0	25.0
Sufentenil[8]	387.2	238.1	110.9	145	145	10	20	4.07	20.0	n/a	0.500
Tramadol[3]	264.2	58.1	42.1	107	107	14	106	3.16	500	25.0	12.5

1 - Internal Standard d_3-Morphine
2 - Internal Standard d_3-Norbuprenorphine
3 - Internal Standard d_3-Tramadol
4 - Internal Standard d_4-Buprenorphine
5 - Internal Standard d_4-Merperidine
6 - Internal Standard d_5-Fentanyl
7 - Internal Standard d_5-Norfentanyl
8 - Internal Standard d_9-Methadone

A 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 reach the concentrations listed in Table 1. Calibration standards were then prepared using a dilution ratio strategy from the high concentration sample of 1:2:2:2:2.5:2. The high, middle, and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:2:2. Table 1 lists the concentrations for the highest calibration standard and the limit of quantitation found during analyses.

The pure, genetically modified, β-Glucuronidase *IMCSzyme* (cat. #04E1F-010) was purchased from Integrated Micro-Chromatography Systems, LLC, provided with the required rapid hydrolysis buffer (cat. #04-EZ-RHB-020). A master mix solution was prepared by combining 3.70 mL of the *IMCSzyme* β-glucuronidase, 4.44 mL of the rapid hydrolysis buffer, and 1.85 mL of the 1541 ng/mL working internal standard solution. Fresh urine was obtained from a male volunteer. Morphine-6β-D-glucuronide was purchased from Cerilliant. All other reagents and solvents used were reagent grade.

Instrumentation. All automated prep sequences were performed using a MPS robotic^{PRO} sampler with the GERSTEL DPX and mVAP options as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC with an Agilent Poroshell 120 EC-C18 column, (3.0 x 50 mm, 2.7 µm) and an Agilent Ultivo triple quadrupole mass spectrometer with Jet Stream electrospray source. Sample injections were made using the GERSTEL LCMS Tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

Figure 1. MPS robotic^{PRO} Multi-Purpose sampler with the GERSTEL DPX option.

Urine Sample Pretreatment:

1. Pipette 250 µL of urine sample into a clean 2 mL autosampler vial and cap with a magnetically transportable cap.

Automated Prep Sequence:

Hydrolysis

1. The MPS adds 135 µL of the master mix solution to the urine sample.
2. The vial is moved to the mVAP option where the sample is incubated at 55°C for 30 minutes while mixing at 250 rpm.
3. The MPS transfers 250 µL of the hydrolyzed urine sample into a clean, empty shell vial.
4. The MPS adds 100 µL of 2 % formic acid in water to the sample.
5. The MPS adds 125 µL of 100 % acetonitrile to the sample and mixes using the syringe.

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:

DPX Extraction

1. The MPS conditions a DPX-CX tip using 500 µL of 30 % acetonitrile in water.
2. The MPS aspirates the entire hydrolyzed urine sample and dispersively mixes the sample and sorbent by aspirating air.
3. The sample is equilibrated for 30 seconds before being dispensed back into the shell vial.
4. The extraction of the hydrolyzed urine sample is repeated a second time.
5. The MPS washes the DPX-CX sorbent using 500 µL of 10 % acetonitrile in water.
6. The MPS washes the DPX-CX sorbent using 500 µL of 100 % acetonitrile.
7. The analytes are eluted from the DPX-CX sorbent by dispensing 750 µL of (78:20:2) methylene chloride: isopropyl alcohol: ammonium hydroxide and collecting the eluent in a clean 2 mL autosampler vial.
8. The MPS disposes of the DPX-CX tip at the PipWaste position.
9. The MPS transfers 600 µL of the eluent into a clean, empty, 2 mL autosampler vial fitted with a magnetic cap used to transport the vial.

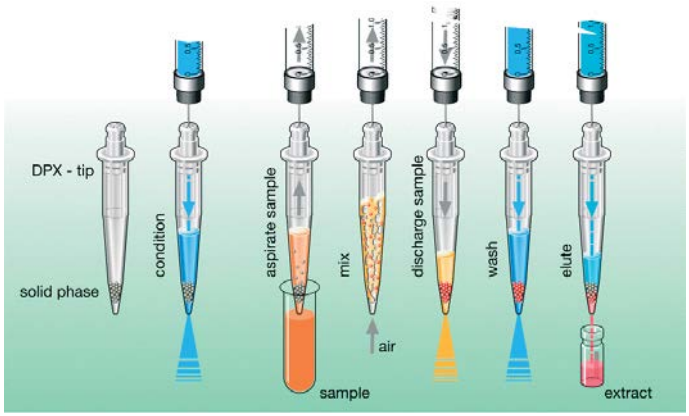


Figure 2. Graphical representation of the DPX extraction process.

Evaporation

1. The MPS transports the vial containing the eluent into the mVAP Option.
2. The sample is evaporated to dryness at 55°C, 250 rpm, and 100 mbar for 10 minutes.
3. The MPS reconstitutes the sample using 250 µL of (90:10) 5 mM ammonium formate with 0.05 % formic acid: 100 % methanol with 0.05 % formic acid and mixes for 18 seconds.
4. The MPS transports the vial back to its original position.

Analysis conditions LC

Pump:	gradient (800 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:	8 minutes	
Injection volume:	2.0 µL (loop over-fill technique)	
Column temperature:	55°C	

Analysis conditions MS

Operation:	ESI positive ion mode
Gas temperature:	350°C
Gas flow (N_2):	5 L/min
Nebulizer pressure:	35 psi
Sheath gas heater:	250°C
Sheath gas flow (N_2):	11 L/min
Capillary voltage:	4000 V
Nozzle voltage:	500 V
delta EMV:	0 V

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

Results and Discussion

To evaluate the automated hydrolysis step of the method, a 150 ng/mL morphine-6β-D-glucuronide sample was prepared in urine. Triplicate 250 µL aliquots of the 150 ng/mL morphine-6β-D-glucuronide urine sample were then hydrolyzed for 0, 15, 30, and 60 minutes, respectively, and extracted and analyzed for morphine using the DPX-LC/MS/MS method. As shown in the graph in Figure 3, morphine reached a maximum response after 15 minutes of incubation, proving that morphine had been deconjugated from the glucuronide. An incubation time of 30 minutes was finally chosen to ensure complete and reproducible hydrolysis of all opioids.

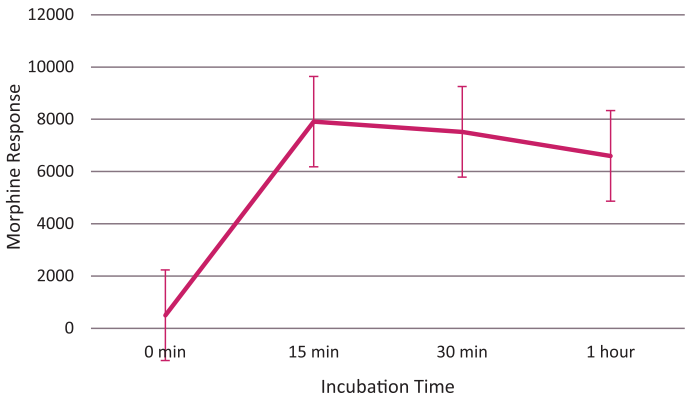


Figure 3. Results from evaluation of hydrolysis time for morphine.

Figure 4 shows representative mass chromatograms for all drugs of abuse, along with their respective qualifier ion transitions, from an extracted low QC sample.

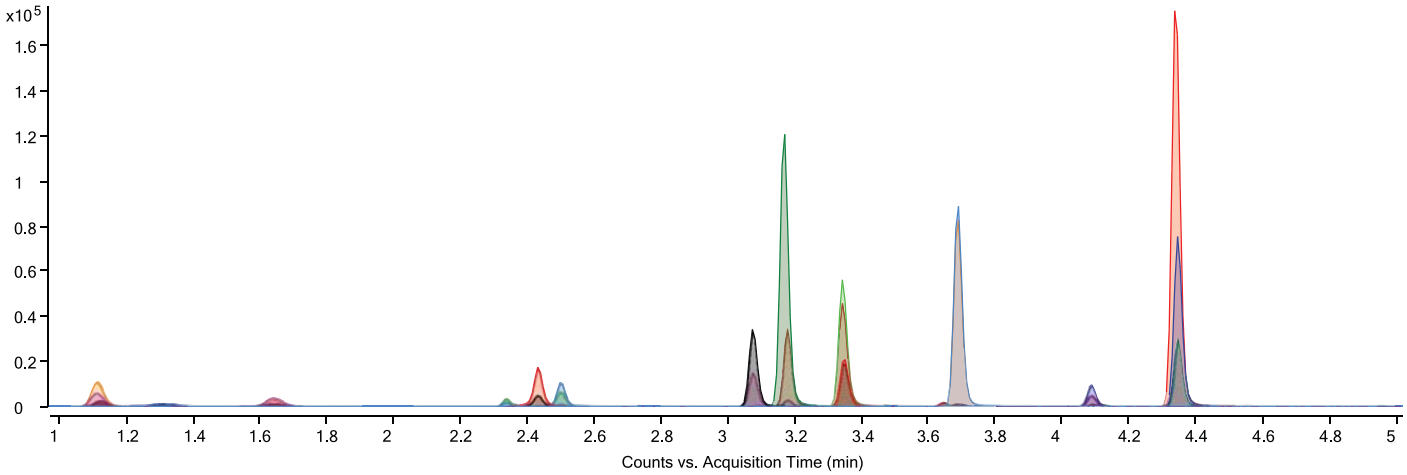
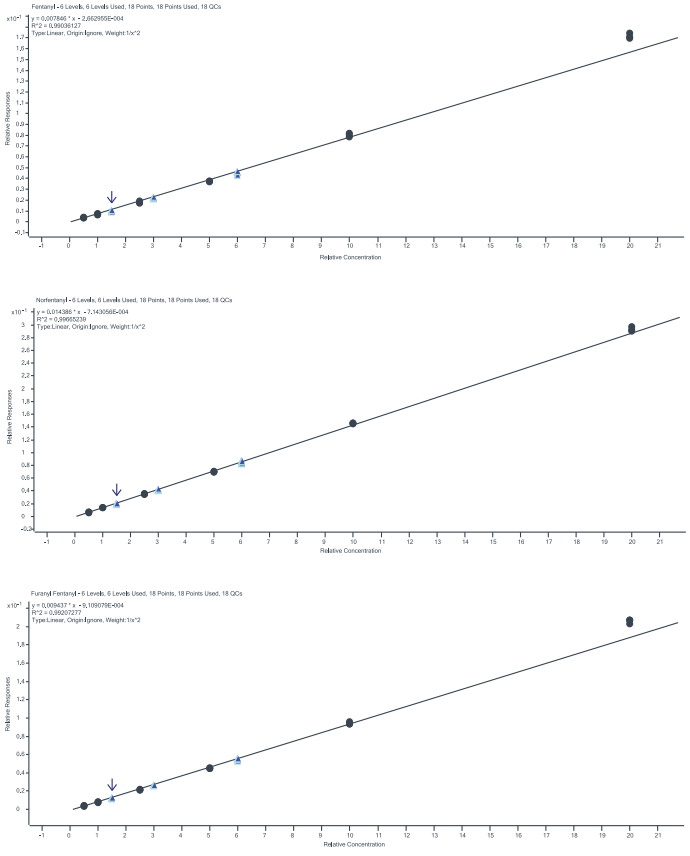


Figure 4. Mass chromatogram overlay for hydrolyzed, extracted low QC sample.

The lower limits of quantitation for this method were chosen to be two times lower than industry minimum reporting limits and are shown in Table 1. Representative calibration curves are shown in Figures 5 A-C. Regression analysis for all drugs of abuse analyzed within this method resulted in R^2 values of 0.99 or greater.



Figures 5a-c. Representative calibration curves: fentanyl, norfentanyl, and furanyl fentanyl.

The accuracy and precision of the method were determined for all opioids analyzed using QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for all drug compounds. Accuracy data averaged 95.2 % (range: 78.3 % - 102 %) and precision data averaged 3.91 %RSD (range: 0.871 % -12.5 %) for all drugs of abuse analyzed.

Table 2. QC sample accuracy and precision table.

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Prec. [%]	Ave. Acc. [%]
Buprenorphine	low	15.0	11.7	78.3	4.03
	middle	30.0	29.9	99.8	3.05
	high	60.0	59.2	98.7	2.01
Codeine	low	75.0	74.5	99.3	3.84
	middle	150	151	100	12.2
	high	300	282	94.1	12.1
Fentanyl	low	1.50	1.33	88.7	4.94
	middle	3.00	2.84	94.8	2.36
	high	6.00	5.84	97.3	2.88
Furanyl Fentanyl	low	1.50	1.39	92.5	2.23
	middle	3.00	2.87	95.7	1.21
	high	6.00	5.88	98.0	2.03
Hydrocodone	low	75.0	70.3	93.7	4.49
	middle	150	142	94.6	4.91
	high	300	281	93.6	12.5
Hydromorphone	low	75.0	67.3	89.8	10.4
	middle	150	148	98.6	3.33
	high	300	292	97.4	4.25
Meperidine	low	75.0	70.1	93.5	1.64
	middle	150	145	96.4	2.11
	high	300	293	97.7	1.40

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

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Prec. [%]	Ave. Acc. [%]
Methadone	low	75.0	67.8	90.4	1.66
	middle	150	141	94.2	0.998
	high	300	293	97.7	1.45
Morphine	low	75.0	68.5	91.4	6.13
	middle	150	146	97.5	1.89
	high	300	292	97.2	1.78
Norbuprenorphine	low	15.0	14.5	96.8	5.72
	middle	30.0	27.8	92.7	6.11
	high	60.0	57.6	96.0	3.77
Norfentanyl	low	1.50	1.43	95.4	4.21
	middle	3.00	2.95	98.5	1.94
	high	6.00	5.94	98.9	2.28
Oxycodone	low	75.0	70.0	93.3	2.47
	middle	150	144	96.2	5.79
	high	300	273	90.9	2.01
Oxymorphone	low	75.0	69.7	92.9	6.76
	middle	150	153	102	5.10
	high	300	300	99.9	4.23
Sufentenil	low	1.50	1.32	88.2	6.07
	middle	3.00	2.88	96.0	2.77
	high	6.00	5.90	98.3	1.35
Tramadol	low	37.5	35.4	94.4	1.08
	middle	75	72.5	96.7	1.38
	high	150	145	96.8	0.871

Conclusions

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

- Over 20 opioids and internal standards in urine samples can be successfully hydrolyzed, extracted using an automated SPE procedure, and determined using the Agilent Ultivo triple quadrupole mass spectrometer.
- This method was readily automated using the GERSTEL MPS robotic^{PRO} sampler.
- Evaluation of the hydrolysis method showed morphine to be deconjugated within the 30-minute incubation period.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved for the opioids analyzed.
- The hydrolysis-SPE-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 95.2 % (range: 78.3 % - 102 %) and precision data averaged 3.91 %RSD (range: 0.871 % -12.5 %) for all opioids analyzed.

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