Direct Analysis of Urinary Opioids and Metabolites by Mixed-mode µElution SPE Combined with UPLC/MS/MS for Clinical Research

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The analysis of natural and synthetic opioid drugs continues to be an important aspect of clinical research. In the past, analyses were typically conducted by GC/MS after first subjecting the samples to acid or enzymatic hydrolysis to transform the glucuronide metabolites into the parent form [1]. With the advent of modern LC/MS/MS techniques, however, glucuronide metabolites can now be analysed directly [2-5]. Direct analyses of glucuronide metabolites can eliminate the risk of inaccurate quantification due to incomplete hydrolysis, as enzymatic efficiency can vary greatly depending upon the enzyme used and the drug substrate analysed [6].

The sample preparation approach is also an important consideration. Urine samples, unlike some other matrices, can be analysed by 'dilute and shoot' methods in which samples are diluted with an internal standard mix and directly injected onto a LC/MS/MS system [2, 4]. Disadvantages to this type of technique, however, include the fact that urine contains many matrix components that can interfere with MS signals. In addition, this technique does not allow for any sample concentration. This can potentially affect the quantification of some of the glucuronide metabolites that elute in reversed phase conditions under high aqueous conditions, where desolvation efficiency is reduced, as well as many of the opioid drugs, since many of them do not produce intense MS/MS product fragments.

This current work describes a method for the analysis of 26 opioid drugs and metabolites by mixed-mode, strong cation exchange SPE followed by UHPLC/MS/MS. Glucuronide metabolites are directly analysed, eliminating the need for enzymatic or chemical hydrolysis. Direct comparison to 'dilute and shoot' preparation demonstrates that mixed-mode SPE results in improved linearity, greater accuracy and precision, and reduced matrix effects.

Experimental:

Materials:

All compounds and internal standards (IS) were purchased from Cerilliant (Round Rock, TX). Complementary, deuterated internal standards were used for all compounds with the exception of hydromorphone-3-glucuronide, codeine-6-glucuronide, norbuprenorphine-glucuronide, norfentanyl, and buprenorphine-glucuronide. For these compounds, a deuterated IS with the most similar recovery and matrix effect was chosen as a surrogate.

A combined stock solution of all compounds (10 µg/mL; 2.5 µg/mL for fentanyl and norfentanyl) was prepared in methanol. Working solutions were prepared daily by preparing high standards and QCs in matrix (urine) and performing serial dilutions in matrix to achieve the desired concentrations. Calibrator concentrations ranged from 5-500 ng/mL for all analytes with the exception of fentanyl and norfentanyl, which were prepared at 25% of the concentration of the other analytes (1.25-125 ng/mL). A combined internal standard stock solution (5 µg/mL; 1.25 µg/mL for fentanyl) was prepared in methanol. Working IS solutions were prepared daily in MilliQ water at 50 ng/mL.

Separation was performed on a Waters ACQUITY UPLC system using a Waters ACQUITY UPLC BEH C18 column, 1.7µm, 2.1 x 100mm. The column compartment was maintained at 30°C. Mobile phase A (MPA) consisted of 0.1% formic acid in MilliQ water. Mobile phase B (MPB) consisted of 0.1% formic acid in acetonitrile (ACN). The LC gradient program is listed in Table 1.

Table 1: UPLC solvent gradient

Time (min.)	Flow Rate	%A	%B
0	0.4	98.0	2.0
6	0.4	47.2	52.8
6.5	0.4	98.0	2.0
8.0	0.4	98.0	2.0

MS Conditions:

MS System :	Waters XEVO® TQD Mass spectrometer
Ionisation Mode :	ESI Positive
Acquisition Mode :	MRM (See Table 1 for transitions)
Capillary Voltage :	1 kV
Collision Energy (eV) :	Optimised for individual compounds (See Table 1)
Cone Voltage (V):	Optimised for individual compounds (See Table 1)

All data was acquired using Waters MassLynx software v.4.1 and analysed using Waters TargetLynx software.

Table 2: Chemical properties and MS conditions of opiate test compounds [7]

	Compound	RT	Formula	Molecular Mass	LogP (predicted)	MRM Transitions	Cone Voltage	Coll. Energy
1	Morphine-3 -D-glucuronide	1.21	$C_{23}H_{27}NO_9$	461.17	-3.48	462.2>286.2 462.2>201.1	58 58	30 52
2	Oxymorphone-3 -D-glucuronide	1.21	$C_{23}H_{27}NO_{10}$	477.16	--	478.1>284.1 478.1>227.1	46 46	28 50
3	Hydromorphone-3 -D-glucuronide	1.34	$C_{23}H_{27}NO_9$	461.17	--	462.2>286.2 462.2>185.1	58 58	28 56
4	Morphine-6 -D-glucuronide	1.47	$C_{23}H_{27}NO_9$	461.17	-2.98	462.2>286.2 462.2>201.1	64 64	38 40
5	Morphine	1.50	$C_{17}H_{19}NO_3$	285.14	0.90	286.2>201.1 286.2>165.1	54 54	28 34
6	Oxymorphone	1.61	$C_{17}H_{19}NO_4$	301.13	0.78	302.1>227.1 302.1>242.1	44 44	28 24
7	Hydromorphone	1.76	$C_{17}H_{19}NO_3$	285.13	1.62	286.2>185.1 286.2>157.1	66 66	32 42
8	Codeine-6 -D-glucuronide	2.00	$C_{24}H_{29}NO_9$	475.18	-2.84	476.2>300.2 476.2>165.2	60 60	36 40
9	Dihydrocodeine	2.07	$C_{18}H_{23}NO_3$	301.17	1.55	302.2>199.1 302.2>128.1	52 52	34 58
10	Codeine	2.14	$C_{18}H_{21}NO_3$	299.15	1.34	300.2>215.2 300.2>165.1	54 54	26 38
11	Oxycodone	2.37	$C_{18}H_{21}NO_4$	315.15	1.03	316.2>256.2 316.2>241.1	44 44	26 26
12	6-Acetylmorphone (6-AM)	2.41	$C_{19}H_{21}NO_4$	327.15	1.31	328.2>165.1 328.2>211.1	60 60	26 36
13	O-desmethyl Tramadol	2.46	$C_{15}H_{23}NO_2$	249.17	1.72	250.2>58.0	26	18
14	Hydrocodone	2.50	$C_{18}H_{21}NO_3$	299.15	1.96	300.2>199.1 300.2>171.0	60 60	30 44
15	Norbuprenorphine-glucuronide	2.83	$C_{31}H_{43}NO_{10}$	589.29	--	590.3>414.3 590.3>101.0	70 70	34 54
16	Norfentanyl	2.93	$C_{14}H_{20}N_2O$	232.16	1.42	233.2>177.2 233.2>150.1	30 30	14 18
17	Tramadol	3.21	$C_{16}H_{25}NO_2$	263.19	2.45	264.2>58.0	24	16
18	Normeperedine	3.58	$C_{14}H_{19}NO_2$	233.1	2.07	234.1>160.1 234.1>188.2	36 36	12 18
19	Meperidine	3.60	$C_{15}H_{21}NO_2$	247.16	2.46	248.2>174.1 248.2>220.2	48 48	22 20
20	Buprenorphine-glucuronide	3.64	$C_{35}H_{49}NO_{10}$	643.34	--	644.3>468.3 644.3>187.1	66 66	42 62
21	Norbuprenorphine	3.77	$C_{25}H_{35}NO_4$	413.26	2.30	414.3>101.0 414.3>187.2	66 66	42 34
22	Fentanyl	4.29	$C_{22}H_{28}N_2O$	336.22	3.82	337.2>188.2 337.2>105.1	48 48	22 38
23	Buprenorphine	4.55	$C_{29}H_{41}NO_4$	467.3	3.55	468.3>101.0 468.3>396.3	72 72	40 48
24	EDDP+	4.79	$C_{20}H_{24}N^+$	278.19	--	278.3>234.2 278.3>249.2	50 50	24 32
25	Propoxyphene	5.18	$C_{22}H_{29}NO_2$	339.3	4.90	340.3>266.2 340.3>143.1	22 22	8 32
26	Methadone	5.25	$C_{21}H_{27}NO$	309.2	5.01	310.2>105.0 310.2>223.1	32 32	22 28

Sample Preparation:

For the dilution method, 100µL of urine was diluted 1:1 with MilliQ water containing internal standards. The samples were vortexed and then loaded into individual wells in the collection plate.

For mixed-mode SPE, urine samples (method blanks, standards, QCs and unknowns) were pretreated by adding equal amounts of 4% H_3PO_4 and a working IS mixture (50 ng/mL) prepared in MilliQ water. Wells in the 96-well Oasis MCX µElution plate were conditioned with 200µL MeOH followed by 200 µL MilliQ water. 300µL of each prepared sample was then added to each well, resulting in a sample load of 100µL

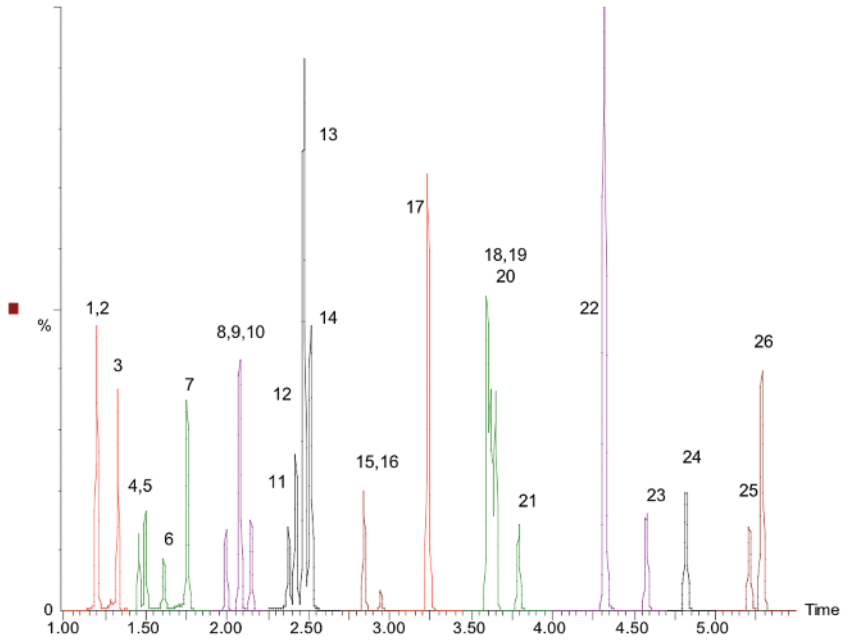

Figure 1: UHPLC separation of opioid and synthetic analgesic compounds. Peak assignments are listed in Table 2.

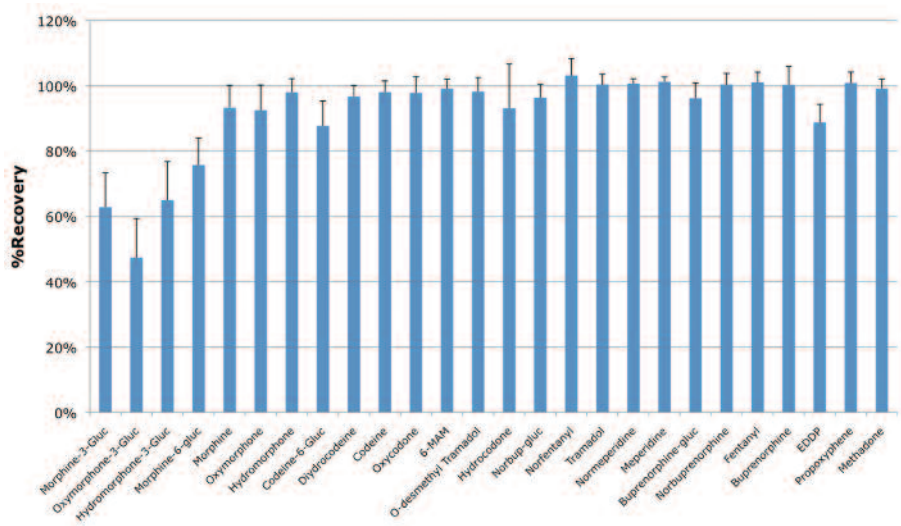

Figure 2: Recovery of opioid compounds from urine using Oasis MCX µElution plates. Bars represent the mean recovery from 6 lots of urine. Error bars indicate standard deviations.

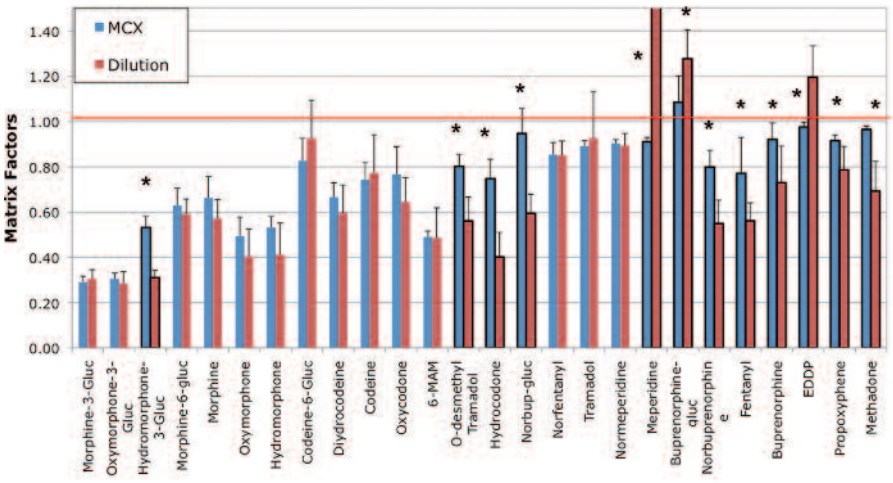

Figure 3. Mean matrix effects of opioid compounds from 6 lots of urine. Blue bars indicate matrix effects measured from Oasis MCX µElution plates. Red bars indicate matrix effects resulting from sample dilution. Error bars indicate standard deviations. Asterisks indicate compounds in which the difference between the two protocols was statistically significant.

urine. After loading, the wells were washed with 200µL water followed by 200µL MeOH. All samples were then eluted with 2 x 50µL of 60:40 MeOH:ACN containing 5% of a concentrated NH_4OH solution (Fisher, 20-22%). After elution, all samples were evaporated under N_2 to dryness (approximately 5 min.) and reconstituted with a solution of 98:2 water:ACN containing 0.1% formic acid and 0.1% human plasma to prevent non-specific binding.

Calibration standards were prepared in urine at concentrations ranging from 5-500 ng/mL (1.25-125 ng/mL for fentanyl and norfentanyl). Quality control samples (N=4) were prepared at 4 concentrations, 7.5, 75, 250, and 400 ng/mL. These samples were then prepared by either sample dilution or mixed-mode SPE.

Results and Discussion:

The 26 compounds and metabolites screened are listed in Table 2 and constitute a comprehensive panel of natural opiate drugs, semi-synthetic opioids, and synthetic narcotic analgesic compounds. Most of the compounds are weak bases, with pKa values of approximately 8-9. They have a wide range of polarities, with LogP values ranging from -3.48 for morphine-3β-d-glucuronide to 5.0 for methadone [7] (see Table 2). MRM transitions used are also listed in Table 2.

Chromatography

A representative chromatogram of all compounds from a 50 ng/mL calibration standard is shown in Figure 1. Peak assignments are listed in Table 2. Using an ACQUITY UPLC BEH C18 column (2.1 x 100mm; 1.7µm) we were able to analyse all analytes in under 5.5 minutes with baseline separation between all critical pairs of isomers, such as between morphine-3-glucuronide, morphine-6-glucuronide and hydromorphone-3-glucuronide (compounds 1, 3, and 4, respectively) and near baseline separation between morphine-6-glucuronide and morphine. Even the most polar analytes were well retained under these conditions, enabling accurate quantification.

Recovery and Matrix Factors

Both mixed-mode SPE and simple dilution were evaluated as possible sample preparation methods. Sample dilution has the advantages of being very simple, inexpensive, and, in the case of urine samples, compatible with reversed-phase chromatographic conditions.

Table 3: Accuracy and coefficients of variation (%CV) from opiate calibration curves prepared using a simple sample dilution protocol. The concentrations of fentanyl and norfentanyl were 25% of the value of other compounds.

		Curve Point (ng/mL)																	
		5		10		20		40		50		100		200		400		500	
	R^2	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV
Morphine-3-β-d-glucuronide	0.986	102.9	9.8%	91.2	14.9%	102.0	1.4%	111.2	0.8%	93.9	3.7%	106.9	5.7%	95.4	4.0%	102.5	8.2%	94.0	9.7%
Oxymorphone-3-b-d-glucuronide	0.985	102.7	7.5%	100.2	3.1%	86.3	2.2%	105.7	11.0%	98.7	8.5%	100.0	6.9%	97.9	6.0%	102.5	7.9%	106.0	20.1%
Hydromorphone-3-b-d-glucuronide	0.987	96.8	8.1%	100.8	4.0%	110.2	4.4%	109.1	8.1%	92.8	5.3%	101.3	6.5%	94.1	4.8%	101.9	12.9%	93.1	9.8%
Morphine-6-gluc	0.979	94.8	18.4%	109.9	3.2%	96.7	10.5%	110.7	16.3%	100.5	3.3%	98.7	6.5%	91.2	4.3%	100.4	2.9%	97.1	16.8%
Morphine	0.954	89.5	29.2%	98.6	18.9%	119.2	28.6%	92.3	15.4%	97.5	29.7%	93.0	10.8%	115.7	20.5%	99.7	16.3%	100.0	27.5%
Oxymorphone	0.989	89.4	2.5%	95.0	8.7%	96.3	8.3%	109.3	3.2%	100.5	11.1%	98.4	2.4%	94.5	9.7%	99.5	12.7%	97.3	17.1%
Hydromorphone	0.996	97.2	1.2%	110.8	8.4%	114.4	14.2%	102.8	3.6%	98.1	9.1%	100.0	1.8%	98.8	6.4%	97.3	1.6%	98.5	4.9%
Codeine-6-β-d-glucuronide	0.990	94.6	2.3%	107.8	15.2%	106.3	0.9%	104.2	5.8%	96.4	4.5%	98.0	7.5%	95.4	6.0%	98.9	3.2%	98.4	0.3%
Dihydrocodeine	0.997	97.6	1.7%	102.3	6.6%	105.1	6.6%	102.0	2.0%	97.3	2.6%	100.3	4.4%	95.9	4.1%	100.1	5.2%	99.3	5.4%
Codeine	0.990	93.4	11.3%	109.7	2.9%	104.4	8.4%	108.2	10.3%	99.7	5.8%	97.3	5.1%	94.8	6.1%	97.1	3.6%	95.3	2.8%
Oxycodone	0.993	98.6	8.2%	104.1	8.3%	98.0	11.6%	98.3	3.5%	99.4	4.1%	104.6	9.6%	97.0	0.7%	100.7	3.2%	99.3	8.6%
6-Acetylmorphone (6-AM)	0.990	98.4	10.6%	105.1	11.4%	95.8	5.4%	106.9	2.9%	90.6	2.5%	105.2	6.8%	98.1	8.8%	101.9	6.5%	112.6	25.2%
O-desmethyl Tramadol	0.997	96.8	9.0%	104.3	5.0%	102.4	4.1%	104.4	2.1%	100.1	1.0%	101.9	2.1%	94.8	3.8%	99.2	3.5%	96.1	3.0%
Hydrocodone	0.995	95.1	0.4%	113.3	6.0%	103.6	3.7%	105.6	6.0%	100.4	2.1%	99.0	2.1%	96.7	4.8%	97.4	6.8%	95.3	3.7%
Norbuprenorphine-glucuronide	0.992	94.6	13.4%	105.9	5.3%	105.8	5.4%	102.9	1.5%	108.0	6.6%	103.7	1.6%	93.8	5.0%	93.9	2.8%	91.5	1.4%
Norfentanyl	0.995	95.6	4.1%	106.0	4.1%	102.9	6.4%	103.1	1.5%	102.5	3.0%	104.2	2.8%	95.8	4.3%	95.8	5.7%	94.1	3.4%
Tramadol	0.996	95.9	1.6%	104.6	3.0%	103.5	0.8%	107.4	1.4%	101.6	1.1%	101.6	1.4%	95.7	2.1%	96.3	0.4%	93.4	1.8%
Normeperedine	0.996	97.0	3.6%	102.8	3.8%	102.7	3.4%	105.9	2.2%	101.7	1.9%	104.5	1.7%	97.5	3.1%	96.0	1.6%	91.9	5.2%
Meperidine	0.997	96.5	1.5%	105.7	6.0%	100.4	3.1%	104.8	1.6%	100.0	2.0%	100.9	2.9%	96.2	1.8%	98.8	1.8%	96.6	3.9%
Buprenorphine-gluc	0.991	93.3	13.3%	110.0	6.4%	103.4	8.7%	103.9	2.0%	105.8	5.1%	100.0	2.6%	97.4	5.2%	93.8	8.2%	92.4	1.7%
Norbuprenorphine	0.995	95.4	5.5%	104.8	1.4%	105.2	7.5%	105.2	3.9%	103.3	3.6%	102.5	2.7%	94.9	4.5%	94.7	3.8%	94.0	1.6%
Fentanyl	0.997	97.2	0.4%	102.9	3.9%	101.9	4.8%	105.9	0.6%	102.6	1.0%	101.1	3.2%	96.0	3.5%	97.4	5.6%	95.1	1.6%
Buprenorphine	0.994	97.2	8.6%	102.8	9.4%	102.0	8.8%	102.9	0.9%	105.6	4.9%	102.2	2.9%	100.1	5.6%	94.7	7.9%	92.3	1.0%
EDDP+	0.998	97.3	1.2%	103.5	4.3%	101.3	1.2%	104.2	0.8%	101.4	0.9%	100.8	1.7%	97.2	3.2%	98.3	1.1%	95.9	1.7%
Propoxyphene	0.995	95.8	1.0%	105.3	3.0%	101.1	1.1%	105.9	1.7%	105.7	1.0%	102.2	3.1%	99.7	2.7%	94.8	0.8%	89.4	2.4%
Methadone	0.997	98.8	0.9%	101.1	2.1%	98.5	3.4%	105.1	0.5%	103.1	2.5%	102.8	4.0%	101.0	3.0%	98.0	6.4%	91.6	1.2%

Highlighted values indicate %CV ≥10% or deviate from expected values by >10%

Disadvantages include reduced analytical sensitivity resulting from sample dilution and potential interference from matrix components remaining in the sample. SPE, on the other hand, can reduce potential matrix effects because of its selective nature. In addition, the ability of SPE to concentrate the sample can help improve analytical sensitivity of the assay. For this application, evaporation of the organic eluate and reconstitution in a high aqueous solution (2% ACN) was necessary to prevent solvent effects that otherwise interfered with the chromatography of many of the glucuronide metabolites. Figure 2 shows the average recovery of all compounds from 6

Table 4: Accuracy and coefficients of variation (%CV) from opiate calibration curves extracted using Oasis MCX µElution plates. The concentrations of fentanyl and norfentanyl were 25% of the value of other compounds.

		Curve Point (ng/mL)																	
		5		10		20		40		50		100		200		400		500	
	R^2	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV
Morphine-3-β-d-glucuronide	0.996	98.8	8.9%	99.0	7.9%	103.7	5.0%	103.2	4.7%	104.7	5.6%	99.5	1.1%	100.7	4.9%	95.9	3.4%	96.2	2.7%
Oxymorphone-3-b-d-glucuronide	0.997	101.7	0.1%	97.3	4.9%	97.6	3.6%	101.5	1.0%	103.3	7.6%	103.4	3.6%	101.2	2.3%	98.5	5.6%	95.7	7.1%
Hydromorphone-3-b-d-glucuronide	0.998	98.5	1.1%	100.7	2.9%	103.4	4.4%	102.3	1.0%	98.5	7.1%	102.9	3.0%	100.9	3.1%	98.7	4.7%	95.6	3.0%
Morphine-6-gluc	0.994	97.3	11.6%	104.0	7.3%	95.9	6.3%	107.5	2.2%	104.5	2.8%	104.4	2.2%	101.6	6.5%	94.1	5.8%	92.3	2.9%
Morphine	0.992	102.0	5.1%	93.9	11.3%	102.2	8.3%	107.0	9.9%	99.6	2.6%	99.0	4.9%	92.5	4.4%	104.8	9.7%	100.8	12.3%
Oxymorphone	0.998	99.7	0.7%	98.9	2.3%	103.1	2.9%	100.5	2.8%	101.1	3.2%	102.0	7.7%	102.0	1.3%	97.9	3.0%	95.9	3.3%
Hydromorphone	0.998	98.9	7.7%	101.3	2.9%	97.2	6.2%	106.2	0.9%	100.5	0.8%	101.3	2.5%	99.3	0.6%	98.7	3.1%	97.4	2.0%
Codeine-6-β-d-glucuronide	0.998	100.5	0.5%	100.9	4.7%	96.8	2.0%	102.1	0.4%	96.5	2.8%	99.1	6.6%	100.9	3.1%	100.9	2.3%	102.4	1.3%
Dihydrocodeine	0.997	96.7	6.4%	102.0	1.0%	101.5	0.2%	107.0	0.0%	103.5	0.7%	102.0	0.7%	100.6	1.8%	95.3	1.0%	93.1	1.5%
Codeine	0.995	95.6	4.1%	102.2	3.5%	105.8	0.9%	108.0	2.1%	101.4	1.5%	104.8	0.9%	100.3	2.2%	93.6	0.5%	91.4	2.3%
Oxycodone	0.996	96.9	4.4%	101.6	3.0%	101.7	5.0%	105.7	0.1%	104.8	1.0%	102.9	1.4%	100.1	1.2%	96.0	3.6%	91.8	4.2%
6-Acetylmorphone (6-AM)	0.997	95.5	4.9%	105.7	1.3%	99.5	3.1%	103.6	4.0%	100.1	2.4%	98.8	2.9%	101.6	0.9%	100.1	0.9%	94.7	4.5%
O-desmethyl Tramadol	0.999	99.2	3.3%	100.2	0.2%	99.1	0.2%	105.0	1.3%	101.0	1.6%	102.0	0.4%	100.4	0.5%	97.6	1.0%	96.6	0.5%
Hydrocodone	0.999	99.4	0.6%	101.5	2.5%	96.7	1.1%	103.5	1.4%	98.8	0.6%	101.7	1.5%	101.2	0.5%	98.0	1.5%	99.1	1.2%
Norbuprenorphine-glucuronide	0.998	99.6	7.7%	100.6	2.1%	98.6	2.5%	103.1	1.9%	100.7	3.4%	96.8	3.6%	101.0	5.8%	101.0	1.1%	99.0	1.3%
Norfentanyl	0.998	97.9	5.6%	102.4	6.3%	99.9	3.5%	100.9	3.9%	101.8	0.5%	100.2	1.2%	101.7	1.3%	98.0	2.1%	96.8	1.1%
Tramadol	0.995	95.0	0.1%	103.0	0.4%	104.0	1.8%	109.7	0.1%	104.4	0.9%	103.3	1.0%	99.0	0.5%	92.6	1.0%	92.1	0.7%
Normeperedine	0.997	97.0	1.9%	101.2	1.7%	102.1	3.4%	107.4	0.7%	104.3	1.1%	102.8	0.5%	99.7	2.3%	94.2	0.9%	93.5	1.2%
Meperidine	1.000	98.7	0.1%	100.8	1.3%	101.3	0.6%	103.2	1.1%	99.9	0.6%	100.9	1.1%	100.1	1.4%	97.6	1.0%	98.5	1.2%
Buprenorphine-gluc	0.997	104.1	2.2%	97.9	5.0%	94.5	0.8%	95.4	2.4%	94.8	2.4%	100.2	2.3%	101.5	2.3%	105.2	2.4%	104.5	1.0%
Norbuprenorphine	0.997	96.5	0.7%	102.0	1.8%	102.7	1.7%	109.3	1.6%	102.7	2.1%	99.6	2.4%	101.2	3.1%	95.8	0.6%	93.1	3.0%
Fentanyl	0.998	98.1	1.8%	100.9	1.6%	100.7	1.0%	105.8	1.1%	102.0	0.9%	102.7	0.4%	101.1	0.4%	95.9	1.8%	94.4	0.5%
Buprenorphine	0.998	100.9	0.5%	98.1	2.6%	98.3	1.5%	105.1	1.1%	101.4	0.8%	103.7	0.9%	101.8	1.3%	97.4	0.5%	94.9	1.0%
EDDP+	0.999	99.5	0.2%	100.6	0.9%	98.2	0.8%	104.2	0.8%	99.6	1.1%	101.3	0.3%	101.8	0.9%	97.9	0.3%	97.6	0.4%
Propoxyphene	0.996	96.9	2.6%	101.0	0.8%	102.2	0.0%	108.7	0.3%	105.2	0.4%	103.0	0.9%	100.2	1.6%	94.6	1.1%	90.8	1.2%
Methadone	0.998	99.3	1.8%	99.3	1.5%	100.0	0.2%	106.5	2.0%	102.8	0.9%	102.4	1.8%	100.4	1.6%	97.4	0.4%	93.9	1.0%

Highlighted values indicate %CV ≥10%

Table 5: Quality control statistics for opioid compounds prepared using a simple sample dilution protocol. For each concentration, mean, %CV and % bias are listed (N=4)

	QC Concentration (ng/mL)											
	7.5			75			250			400		
	Mean	%CV	Bias	Mean	%CV	Bias	Mean	%CV	Bias	Mean	%CV	Bias
Morphine-3-β-d-glucuronide	7.08	10.3%	-5.7%	73.3	6.1%	-2.3%	239.4	2.3%	-4.2%	380.0	6.2%	-5.0%
Oxymorphone-3-β-d-glucuronide	6.85	18.1%	-8.7%	72.9	6.8%	-2.8%	229.7	4.0%	-8.1%	365.9	7.0%	-8.5%
Hydromorphone-3-β-d-gluc	7.75	14.5%	3.3%	78.1	4.5%	4.1%	236.5	6.9%	-5.4%	362.7	5.8%	-9.3%
Morphine-6-gluc	7.85	23.1%	4.7%	74.0	17.5%	-1.4%	249.1	9.3%	-0.4%	358.6	3.5%	-10.4%
Morphine	5.28	26.9%	-29.7%	76.0	7.9%	1.3%	267.4	9.4%	7.0%	410.7	16.6%	2.7%
Oxymorphone	8.98	23.3%	19.7%	82.4	9.7%	9.9%	251.9	5.8%	0.7%	360.1	5.4%	-10.0%
Hydromorphone	8.13	14.1%	8.3%	79.3	5.0%	5.7%	251.8	5.1%	0.7%	381.1	3.4%	-4.7%
Codeine-6-β-d-glucuronide	6.45	11.5%	-14.0%	71.6	7.0%	-4.5%	226.7	8.0%	-9.3%	358.6	4.4%	-10.4%
Dihydrocodeine	8.25	9.4%	10.0%	86.1	8.0%	14.8%	244.8	5.6%	-2.1%	387.3	5.3%	-3.2%
Codeine	7.90	10.5%	5.3%	76.5	4.7%	2.0%	236.2	8.0%	-5.5%	366.0	3.9%	-8.5%
Oxycodone	7.53	20.4%	0.3%	79.2	6.8%	5.6%	243.0	3.4%	-2.8%	380.3	3.4%	-4.9%
6-Acetylmorphone (6-AM)	6.50	7.7%	-13.3%	68.3	9.5%	-8.9%	215.6	2.8%	-13.8%	371.6	5.2%	-7.1%
O-desmethyl Tramadol	7.45	3.6%	-0.7%	79.5	4.9%	5.9%	240.2	3.3%	-3.9%	369.0	2.5%	-7.8%
Hydrocodone	6.75	8.2%	-10.0%	71.9	3.6%	-4.2%	227.2	6.4%	-9.1%	341.2	5.8%	-14.7%
Norbuprenorphine-glucuronide	7.25	5.3%	-3.3%	77.1	2.7%	2.8%	234.5	5.0%	-6.2%	350.2	3.0%	-12.4%
Norfentanyl	1.53	11.2%	-18.7%	20.1	3.7%	6.9%	60.3	3.7%	-3.6%	92.1	0.6%	-7.9%
Tramadol	6.53	1.5%	-13.0%	69.8	3.6%	-6.9%	218.1	1.3%	-12.8%	335.5	0.8%	-16.1%
Normeperedine	7.45	4.6%	-0.7%	79.3	5.1%	5.7%	234.6	3.1%	-6.2%	356.8	0.7%	-10.8%
Meperidine	7.33	1.7%	-2.3%	77.4	7.0%	3.2%	236.3	2.1%	-5.5%	367.0	2.7%	-8.2%
Buprenorphine-gluc	4.80	4.5%	-36.0%	65.8	3.6%	-12.3%	211.1	4.9%	-15.6%	327.1	2.1%	-18.2%
Norbuprenorphine	7.15	9.2%	-4.7%	79.6	2.8%	6.2%	242.6	5.6%	-3.0%	364.2	1.7%	-9.0%
Fentanyl	1.75	3.3%	-6.7%	19.5	2.9%	3.9%	60.0	3.9%	-4.1%	91.9	1.4%	-8.2%
Buprenorphine	6.80	6.4%	-9.3%	75.5	3.8%	0.6%	231.1	3.7%	-7.6%	356.6	2.3%	-10.9%
EDDP+	7.45	1.7%	-0.7%	78.3	3.3%	4.4%	239.2	1.0%	-4.3%	365.2	2.1%	-8.7%
Propoxyphene	7.00	8.2%	-6.7%	75.9	2.2%	1.2%	229.7	2.8%	-8.1%	349.9	4.5%	-12.5%
Methadone	6.98	6.0%	-7.0%	75.6	2.5%	0.7%	232.8	3.4%	-6.9%	349.5	4.4%	-12.6%

Bias or %CV > 10%

different lots of urine using the Oasis MCX µElution protocol detailed above. With the exception of the 4 earliest eluting glucuronide metabolites, all compounds demonstrated recoveries of 89% or greater. In addition, when peak areas from extracted 50 ng/mL samples were compared, the areas resulting from the mixed-mode SPE protocol ranged from 2.1 to more than 6 times greater than the dilution protocol. Thus, the ability to concentrate the samples more than made up for the limited recovery seen for a few analytes.

In addition to recovery, matrix factors were evaluated for both protocols. Matrix factors were calculated according to the following equation:

Matrix Factor = (See Table 6). With very few

Table 6: Quality control statistics for opioid compounds extracted using Oasis MCX µElution plates. For each concentration, mean, %CV and % bias are listed (N=4).

	QC Concentration (ng/mL)											
	7.5			75			250			400		
	Mean	%CV	Bias	Mean	%CV	Bias	Mean	%CV	Bias	Mean	%CV	Bias
Morphine-3-β-d-glucuronide	7.10	8.3%	-5.3%	74.5	5.2%	-0.7%	250.0	2.2%	0.0%	386.3	3.6%	-3.4%
Oxymorphone-3-β-d-glucuronide	7.43	9.7%	-1.0%	76.9	3.0%	2.5%	239.9	4.9%	-4.0%	372.1	3.7%	-7.0%
Hydromorphone-3-β-d-gluc	7.98	7.8%	6.3%	76.4	5.8%	1.9%	252.4	2.9%	0.9%	398.1	3.7%	-0.5%
Morphine-6-gluc	8.30	8.7%	10.7%	74.9	6.7%	-0.1%	240.9	5.1%	-3.7%	376.8	4.0%	-5.8%
Morphine	8.15	10.1%	8.7%	75.6	7.7%	0.8%	217.1	5.1%	-13.2%	391.2	4.3%	-2.2%
Oxymorphone	7.85	5.1%	4.7%	73.3	4.2%	-2.3%	243.6	4.7%	-2.6%	385.5	4.5%	-3.6%
Hydromorphone	7.93	1.6%	5.7%	75.7	3.0%	0.9%	247.8	3.7%	-0.9%	388.9	1.2%	-2.8%
Codeine-6-β-d-glucuronide	7.78	4.0%	3.7%	73.6	3.8%	-1.9%	257.3	5.0%	2.9%	421.7	2.6%	5.4%
Dihydrocodeine	7.65	0.8%	2.0%	75.8	1.1%	1.1%	243.8	0.6%	-2.5%	377.9	2.8%	-5.5%
Codeine	7.68	4.7%	2.3%	75.8	0.6%	1.1%	245.2	1.9%	-1.9%	385.4	0.9%	-3.7%
Oxycodone	7.58	5.2%	1.0%	75.5	2.3%	0.7%	244.5	3.4%	-2.2%	378.0	2.8%	-5.5%
6-Acetylmorphone (6-AM)	7.70	5.3%	2.7%	76.2	4.3%	1.6%	245.9	2.3%	-1.7%	391.5	0.7%	-2.1%
O-desmethyl Tramadol	7.83	1.9%	4.3%	75.0	1.3%	0.0%	247.1	0.7%	-1.2%	384.6	0.7%	-3.8%
Hydrocodone	7.60	1.9%	1.3%	74.5	1.3%	-0.7%	244.2	1.6%	-2.3%	381.3	0.9%	-4.7%
Norbuprenorphine-glucuronide	7.80	3.6%	4.0%	76.4	3.1%	1.8%	255.0	3.9%	2.0%	401.9	1.3%	0.5%
Norfentanyl	1.90	0.0%	1.3%	19.4	2.3%	3.3%	62.7	1.2%	0.4%	101.7	2.2%	1.7%
Tramadol	7.60	0.0%	1.3%	76.8	0.3%	2.4%	240.5	0.8%	-3.8%	369.2	0.5%	-7.7%
Normeperedine	7.48	2.0%	-0.3%	75.3	1.6%	0.4%	238.7	1.2%	-4.5%	371.4	1.4%	-7.2%
Meperidine	7.43	0.7%	-1.0%	73.2	0.5%	-2.5%	242.4	2.4%	-3.1%	388.1	1.7%	-3.0%
Buprenorphine-gluc	8.08	2.7%	7.7%	77.8	1.8%	3.7%	267.0	1.6%	6.8%	441.1	1.3%	10.3%
Norbuprenorphine	7.73	1.2%	3.0%	77.7	3.8%	3.6%	246.1	1.5%	-1.6%	377.2	1.0%	-5.7%
Fentanyl	1.90	0.0%	1.3%	19.2	1.1%	2.4%	60.8	1.0%	-2.7%	96.8	1.0%	-3.2%
Buprenorphine	7.55	2.3%	0.7%	77.2	1.9%	2.9%	247.2	1.9%	-1.1%	397.1	1.3%	-0.7%
EDDP+	7.65	1.3%	2.0%	75.0	1.1%	0.0%	243.2	0.9%	-2.7%	387.7	1.1%	-3.1%
Propoxyphene	7.55	0.8%	0.7%	78.4	0.5%	4.5%	243.4	0.9%	-2.6%	378.9	1.9%	-5.3%
Methadone	7.58	0.7%	1.0%	78.2	1.5%	4.3%	246.4	1.0%	-1.4%	386.4	1.2%	-3.4%

Bias or %CV > 10%

exceptions, nearly all accuracy and precision values are less than 10%. In addition, only 3 QC points show a deviation from expected values of more than 10% and all are within 15%.

Conclusions:

The method presented here demonstrates the advantages of mixed-mode SPE for the analysis of 26 opioid compounds and metabolites of interest in urine. All compounds are analysed in under 5.5 minutes with complete resolution of all isobaric compound pairs, and even the most polar glucuronide metabolites were well retained. The use of mixed-mode SPE resulted in improved linearity and significantly reduced matrix effects compared to a simple dilution method. Accuracy and precision for quality control samples and calibration standards were also improved using mixed-mode SPE. While 13 QC points exceeded the recommended %CVs when prepared by sample dilution, only a single point out of 104 in the SPE prepared samples (morphine at 7.5 ng/mL; %CV=10.1%) exceeded the suggested %CV of 10%. This dramatically improved accuracy and precision, the ability to achieve LOQs of 5 ng/mL for nearly all analytes, and the ability to measure glucuronide metabolites directly without hydrolysis make this method well suited for the analysis of these compounds.

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