Verification of a LC-MS/MS Research Method for 19 Opioids, Opiates, and their Metabolites in Human Urine without Hydrolysis

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Overview

Purpose: To verify a LC-MS/MS research method for measuring the concentration of opioids, opiates, and their metabolites in urine that does not require hydrolysis prior analysis.

Methods: An LC-MS/MS research method was developed to compare hydrolyzed and nonhydrolzed samples. This research method was used to evaluate whether or not the sum of the glucuronidated metabolites and the parent compounds measured separately before hydrolysis is equal to measuring the concentration of the total parent in the sample after hydrolysis.

Results: The research method was verified for the quantitation of opioids, opiates, and their metabolites without hydrolysis prior to analysis.

Introduction

The use of LC-MS/MS in bioanalysis is often hindered by the need for complex sample preparation and extraction methods which can introduce errors in sample handling. These errors can lead to large sample to sample variability. The development of a rugged system for the sample preparation and chromatographic analysis prior to MS detection that requires minimal sample handling is essential in a clinical research environment. In this work, we present the verification of a panel for 19 opioids, opiates, and their metabolites in urine. This research method alleviates the need for the labor intensive, somewhat variable, and time consuming hydrolysis step that is the typically performed prior to analysis to convert conjugated metabolites back to their parent compounds. The research method quantifies all the glucuronides and their parents separately. The concentrations were summed and compared directly to the concentration obtained from the same samples that were hydrolyzed by conventional method. Individual concentrations of the parent and the metabolites can also be used for pharmacokinetic studies.

Methods

Sample Preparation

The analytes of interest were spiked into human urine at various concentrations to make calibrators and controls. Each sample was divided into two parts; one set was hydrolyzed prior to analysis and the second set was analyzed directly. Hydrolysis was performed by adding 1M ammonium acetate buffer containing β-glucuronidase to samples and incubating overnight. The nonhydrolyzed samples had the same volume of ammonium acetate buffer added but without any b-glucuronidase present and no incubation. Isotopically labeled internal standards were then added to all the samples. The samples were vortex mixed, centrifuged, and the supernatant was removed and transferred into clean vials for LC-MS/MS analysis.

Instrumentation

A Thermo Scientific™ Prelude SPLC™ system was used in LX mode equipped with a 2.1 x 100 mm, 2.6 µm particle size Thermo Scientific™ Accucore™ aQ analytical column. Both a Thermo Scientific™ TSQ Vantage™ triple quadrupole mass spectrometer and a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with HESI-II ionization probes in positive mode were used as detectors.

Method Parameters

The mobile phases consisted of 0.1% formic acid in (A) water and (B) methanol. The LC method is shown in Table 1. The mass spectrometer quantifier and qualifier SRMs are shown in Table 2. The method range for all the analytes is 5-500 ng/mL.

TABLE 1. LC Method Parameters

Step	Start	Sec	Flow	Grad	%A	%B
1	0.00	20	0.40	Step	100.0	-
2	0.33	5	0.40	Step	92.0	8.0
3	0.42	50	0.40	Step	92.0	8.0
4	1.25	5	0.40	Step	75.0	25.0
5	1.33	130	0.40	Ramp	65.0	35.0
6	3.50	45	0.40	Step	-	100.0
7	4.25	100	0.40	Step	100.0	

Results

Four days of accuracy and precision were performed for system verification. Three days on the TSQ Vantage MS for verification and one day cross verification was done on the TSQ Endura MS. A summary of the results is shown in Table 3. The assay precision had RSD values that were less than 20.0% at the LLOQ and low quality control (QC), and 15.0% for all other levels. Additionally, accuracy was ± 20.0% at the LLOQ and low QC, and ±15% for all other levels. The correlation coefficient values for all the compounds ranged from 0.9900 to 0.9991, showing linearity throughout all concentrations and analytes. All the analytes passed carryover, recovery, analytical selectivity, and autosampler stability criterion. Recoveries were all 82% or above for each compound at each level of QC. Example SRM chromatograms at the LOQ are shown in Figure 1. A comparison of hydrolyzed versus nonhydrolyzed metabolite concentrations is shown in Figure 2 along with an illustration of the reaction.

TABLE 2. SRM Transitions

Analyte	Precursor Ion (Q1)	Product Ions (Q3)	Collision Energy	S-lens
normorphine	272.001	165	59	95
		209	40	95
morphine 3b glucuronide	462.1	286.1	52	148
		185.2	58	139
oxymorphone 3b glucuronide	478.1	284.1	47	147
		302.1	42	147
hydromorphone 3b glucuronide	462.1	185.2	58	139
		286.1	52	148
morphine 6b glucuronide	462.1	286.1	52	148
		185.2	58	139
codeine 6b glucuronide	476.2	300.2	31	114
		215.2	39	114
6acetylmorphine	328.1	165	58	112
		211	39	112
6acetylcodeine	342.1	225.1	27	109
		165.1	47	109
dihydromorphine	288.132	185.05	48	95
		165	59	95
morphine	286.102	165.1	64	90
		185	44	119
oxymorphone	302.004	227	40	116
		199.1	55	116
hydromorphone	286.105	185	44	119
		165.1	64	90
codeine	300.001	171	40	119
		199.07	43	119
dihydrocodeine	302.003	201.08	42	93
		199	52	93
norcodeine	286.102	165.1	64	90
		181.6	49	90
oxycodone	316	241.1	41	119
		256	40	119
noroxycodone	302.1	227	41	116
		187	40	116
Norhydrocodone	286.107	199	39	119
		241.09	35	119
hydrocodone	300.001	171	40	119
		181	51	94
noroxycodone-d3	305.1	190.1	25	116
norhydrocodone-d3	289.1	152.1	62	116
6acetylmorphine-d6	334.1	165.1	38	116
morphine 6b glucuronide-d3	465.1	289.1	32	140
morphine-d3	289.1	152.1	61	116
dihydrocodeine-d6	308.1	202.1	34	116
codeine-d6	306.1	165.1	43	116
hydromorphone-d6	292.1	185.1	32	116
morphine-3b-glucuronide-d3	465.1	289.1	31	140
oxycodone-d6	322.1	218.1	43	116

FIGURE 1. Representative Chromatograms at the LOQ

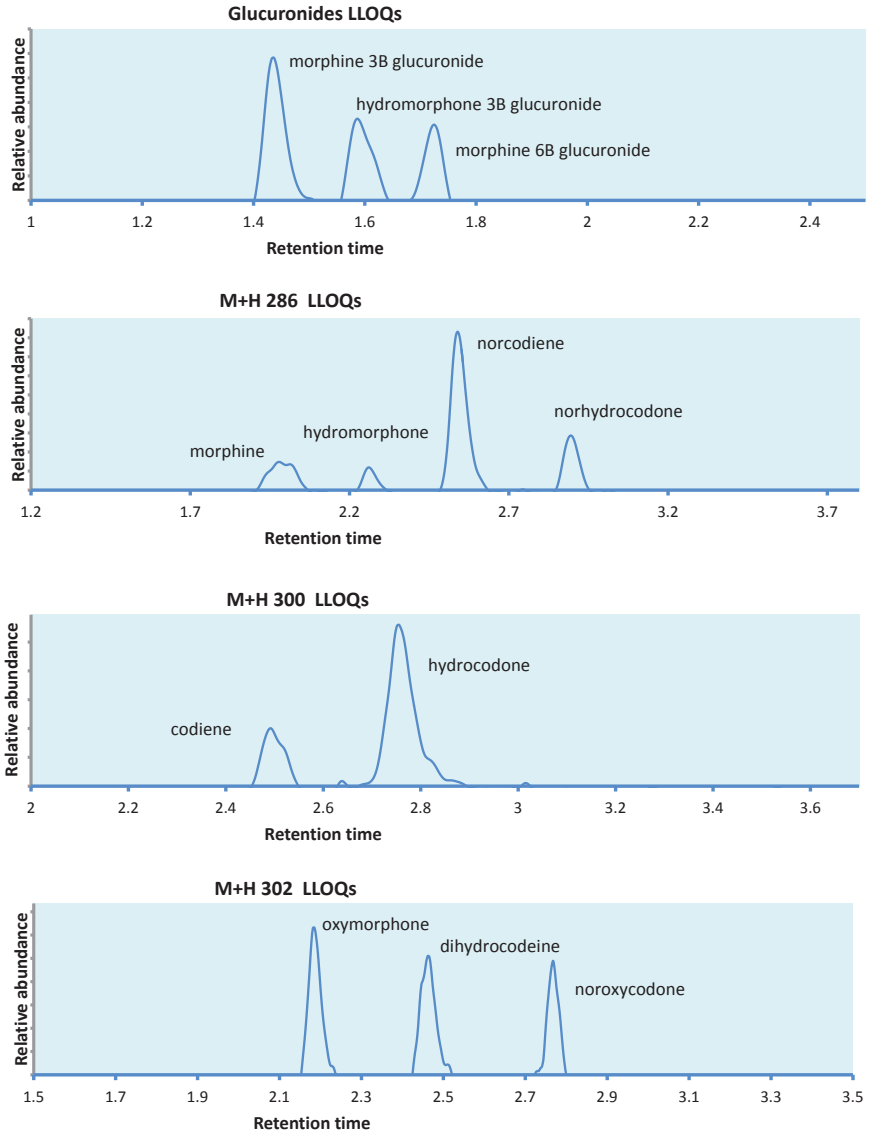
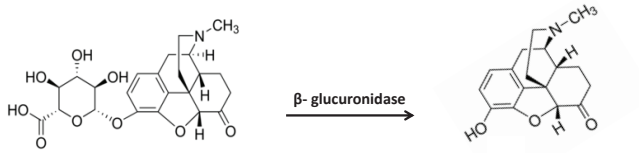


TABLE 3. Inter and Intra Day Accuracy and Precision Summary

Analyte	Level	Expected conc	Day1		Day 2		Day 3		Day 4		Average	Stdev	%RSD
			Avg	%RSD	Avg	%RSD	Avg	%RSD	Avg	%RSD			
normorphine	Low	12	13.2	8.3	11.7	14.3	12.5	8.1	11.7	9.4	12.3	0.7	5.7
	Mid	225	229	3.4	225	5.2	232	5.5	220	1.5	227	5.2	2.3
	High	400	383	3.9	402	4.5	407	3.0	404	3.3	399	10.9	2.7
dihydromorphine	Low	12	12.1	9.5	11.7	14.1	11.2	8.3	12.3	9.5	11.8	0.5	4.2
	Mid	225	225	6.3	215	4.7	195	3.6	237	3.9	218	17.8	8.2
	High	400	384	5.1	374	3.9	362	4.1	409	10.2	382	20	5.2
morphine	Low	12	10.5	5.2	11.4	17.5	10.4	8.8	11.3	4.0	10.9	0.5	4.6
	Mid	225	226	5.2	228	4.1	205	3.4	222	5.6	220	10.5	4.8
	High	400	402	5.9	387	4.9	386	3.0	402	6.3	394	9	2.3
oxymorphone	Low	12	11.8	10.3	11.4	2.6	10.8	9.0	11.4	7.3	11.4	0.4	3.5
	Mid	225	220	5.5	214	7.3	217	1.6	218	4.5	217	2.5	1.2
	High	400	391	3.7	375	2.5	370	2.4	390	6.3	382	10.6	2.8
hydromorphone	Low	12	11.8	6.1	11.7	14.1	11.2	8.3	11.6	5.9	11.6	0.3	2.6
	Mid	225	217	2.9	215	4.7	195	3.6	224	4.3	213	12.4	5.8
	High	400	389	4.2	374	3.9	362	4.1	401	2.8	382	17.1	4.5
norcodeine	Low	12	12.7	7.3	12.2	4.4	11.6	9.6	12.5	4.7	12.3	0.5	4.1
	Mid	225	232	4.2	238	7.1	232	4.2	218	8.6	230	8.5	3.7
	High	400	391	6.1	400	3.8	401	3.8	423	5.2	404	13.6	3.4
dihydrocodeine	Low	12	12.4	11.1	11.9	7.4	11.9	3.4	12.1	8.9	12.1	0.2	1.7
	Mid	225	233	3.6	225	2.1	205	3.3	223	4.5	222	11.8	5.3
	High	400	398	1.8	383	2.2	380	1.6	403	5.3	391	11.2	2.9
codeine	Low	12	11.6	3.3	11.2	5.6	11.4	7.2	12	13.6	11.6	0.3	2.6
	Mid	225	215	4.2	228	1.6	207	1.7	221	3.4	218	8.9	4.1
	High	400	427	2.9	4.06	4.4	376	4.6	386	2.2	298	197.4	66.2
norhydrocodone	Low	12	12	5	11.5	2.8	11	2.5	13.5	3	12	1.1	9.2
	Mid	225	224	5.9	220	4	222	1.9	227	1.5	223	3	1.3
	High	400	382	1.8	398	4.1	373	1.3	400	4.9	388	13	3.3
oxycodone	Low	12	12.1	9.5	11.7	14.1	12.1	9.1	12.1	8.8	12	0.2	1.7
	Mid	225	225	6.3	215	4.7	205	2.5	221	5.1	217	8.7	4
	High	400	384	5.1	374	3.9	366	5.1	417	2.7	385	22.4	5.8
noroxycodone	Low	12	10.6	6.3	10.7	11.6	11.4	7.9	13.1	7.3	11.5	1.2	10.4
	Mid	225	215	4	217	2.9	202	4.7	226	1.9	215	9.9	4.6
	High	400	404	9.1	392	5.5	392	6.8	407	1.9	399	7.9	2
hydrocodone	Low	12	12.5	7.4	12.3	4.2	11.2	4.7	12	4.5	12	0.6	5
	Mid	225	222	6.6	228	2.9	222	5.7	233	6.3	226	5.3	2.3
	High	400	374	4.1	416	3.6	416	1.3	409	4.8	404	20.1	5
6acetylmorphine	Low	12	11.8	8.1	11.5	2.4	12.3	2.4	11.5	9.7	11.8	0.4	3.4
	Mid	225	221	2.1	221	3.2	231	2	242	3.5	229	10	4.4
	High	400	399	2.3	398	2.4	388	3.5	398	4.5	396	5.2	1.3
codeine 6β gluc	Low	12	11.5	8.5	12	4.8	12.7	5.7	12.6	4.3	12.2	0.6	4.9
	Mid	225	2.8	4.2	220	2.3	215	3.2	229	3.1	167	109.4	65.6
	High	400	417	2.2	404	3.1	388	4	394	3.9	401	12.7	3.2
oxymorphone 3β gluc	Low	12	11.3	13.5	12	14.4	11.5	12.1	10.9	10.4	11.4	0.5	4.4
	Mid	225	222	6	233	4.9	222	3.2	213	2.4	223	8.2	3.7
	High	400	399	2.1	415	5.3	418	1.5	393	2.7	406	12.1	3
hydromorphone 3β gluc	Low	12	13.2	4.9	11.8	7.9	11.7	5.6	12.3	4.5	12.3	0.7	5.7
	Mid	225	231	4.9	214	4.8	222	4.5	211	3.6	220	9	4.1
	High	400	412	4.5	374	3.8	397	3.3	390	2.8	393	15.8	4
morphine 3β gluc	Low	12	11.7	14.9	12.1	12.4	12.8	10.5	12	10.7	12.2	0.5	4.1
	Mid	225	229	4.8	238	2	228	1.9	227	2.3	231	5.1	2.2
	High	400	402	7.1	422	4	416	4.3	394	3.8	409	12.8	3.1
morphine 6β gluc	Low	12	11.5	1	11.5	10.3	11.6	5.3	12	10.8	11.7	0.2	1.7
	Mid	225	220	8.5	216	3.8	233	6	224	1.7	223	7.3	3.3
	High	400	395	4.8	388	7.9	421	4.8	394	3.8	400	14.7	3.7
6acetylcodeine	Low	12	12.2	4.7	11.8	3.8	11.9	6.1	10.5	5.8	11.6	0.8	6.9
	Mid	225	213	5.2	233	2.1	223	3.2	206	4.3	219	11.8	5.4
	High	400	395	4.1	397	3.4	393	4.7	397	3.2	396	1.9	0.5

Figure 2. Comparison of Hydromorphone (H) and Hydromorphone Glucuronide (H3G) Concentration from Hydrolyzed and Nonhydrolyzed Samples



Hydromorphone 3βD glucuronide (mw 461) — Hydromorphone (mw 285)

	H3G	H3G	H	H
Prepared concentration (ng/mL)	20	100	20	100
Measured concentration prior to hydrolysis (ng/mL)	18.6	112.4	0	0
Measured concentration after hydrolysis (ng/mL)	0	0	12.4	64.2
Expected % converted to parent based on molecular weight (285/461=62)	62		62	
Actual % measured based on results	66		57	
% difference	6.3		8.4	

Conclusion

A LC-MC/MS research method for quantification of opioids, opiates, and their metabolites without requiring hydrolysis of the glucuronides prior to analytes has been verified. The research method takes less time and is less costly than those that require hydrolysis because it eliminates the long sample preparation steps.

- Quantitation of opiods,opiates, and their metabolites without hydrolysis prior to analysis
- Verification of a research method for opioids, opiates, and their metabolites on a Prelude SPLC system
- The research method is more accurate, easier to perform, takes less time, and is less costly then those that require hydrolysis
- The research method was cross verified on both an TSQ Endura and a TSQ Vantage mass spectrometer without changes.

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