The Separation and Analysis of Opiates and their Glucuronide Metabolites in Urine Matrix Negating the Need for Hydrolysis During Sample Preparation Using Tandem LCMS/MS.

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Introduction

Opiates and their metabolites can be challenging to analyze using LC/MS due to a relatively large number of their analytes having the identical empirical mass and subsequent similar fragmentation patterns in MS/MS. The need for good chromatographic separation is therefore paramount as is the need to reduce complicated sample preparation techniques.

Opiate glucuronides pose the greater separation challenge in reverse phase chromatography due to their highly polar nature and are, therefore, routinely hydrolyzed during sample preparation, a process that removes any sugar group returning the metabolite to its original parent form.

Acid and enzyme hydrolysis stages of sample preparation can be relatively lengthy processes so this research project set out to investigate the scientific feasibility of separating and measuring common opiates, metabolites and glucuronide metabolites directly and in one quick analytical method.

A five minute LC/MS MRM method was developed which chromatographically separated each of the isobaric analytes outlined in table 1. Results were obtained for five batches of spiked urine samples over the concentration range of 0.5-1000ng/ml. Precision data obtained and calibration accuracies will be reported for each analyte

Table 1 – Isobaric analytes used for research method development.

Name	$[M+H]^+$ m/z
Hydromorphone	286.2
Morphine	286.2
norhydrocodone	286.2
Codeine	300.2
Hydrocodone	300.2
Noroxycodone	302.1
Oxymorphone	302.1
Oxycodone	316.2
hydromorphone 3beta-D-glucuronide	462.2
morphine 3beta-D-glucuronide	462.2
morphine 6beta-D-glucuronide	462.2
codeine 6beta-D-glucuronide	476.2
oxymorphone 3beta-D-glucuronide	478.2

Sample Preparation

Spiked Urine Sample Preparation (Dilute & Shoot):

1. Internal Standard Diluent Preparation:
   a) Take 36ml of deionized water;
   b) Add 10uL of each internal standard (100ug/ml) or 1uL (1mg/ml) to the water;
   c) Vortex the ~ 25ng/ml ISTD solution.
2. Negative Urine Pre-treatment:
   a) Split 5ml of neat negative urine between 10x nanosep 3K centrifuge filters;
   b) Centrifuge @ 12000 for 20 min;
   c) Pool filtered urine into single tube or vessel;
   d) Draw 4ml of the filtered urine and add to the 36ml ISTD solution (1);
   e) This solution was used as calibrator diluent containing 25ng/ml of each ISTD.

Table 2 - Internal Standards Utilized.

Codeine-D3	
Hydrocodone-D6	Norhydrocodone-D3
Hydromorphone-D3	Noroxycodone-D3
morphine 3beta-D-glucuronide-D3	Oxycodone-D3
morphine 6beta-D-glucuronide-D3	oxymorphone 3beta-D-glucuronide-D3
Morphine-D3	Oxymorphone-D3

Experimental & Instrument Parameters

HPLC Method Conditions (Agilent 1290):

Column:	Aquity HSS T3 C18, 2.1 x 100mm (1.7µm)
Column temperature:	55°C
Injection volume:	1 uL
Autosampler temp:	4°C
Needle wash:	flushport (100% acetonitrile), 5 sec
Mobile phase:	A = 5mM NH4formate/0.1% formic acid in water
	B = 0.1% formic acid in acetonitrile
Gradient flow rate:	0.6 mL/min

Table 3 - HPLC Gradient Profile.

Time (min)	% A	%B
0.00	98	2
0.90	98	2
3.00	85	15
3.50	85	15
3.51	5	95
4.00	5	95
4.01	98	2
5.00	98	2

Mass Spectrometer Conditions & Configuration:

Configuration:
Agilent 6460 QqQ Mass Spectrometer.

Ion Source Conditions:

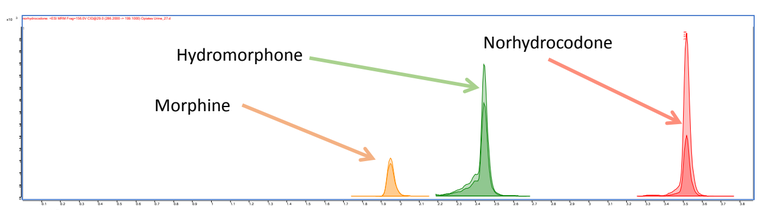
Ion Mode:	ESI/ Positive.
Capillary Voltage:	3750 V
Nozzle Voltage:	0 V
Drying gas (nitrogen):	9 L/min
Drying gas temperature:	325 ºC
Nebulizer gas (nitrogen):	27 psi
Sheath Gas temperature:	380 ºC
Sheath Gas flow:	11 L/min

Dynamic MRM acquisition (QqQ):

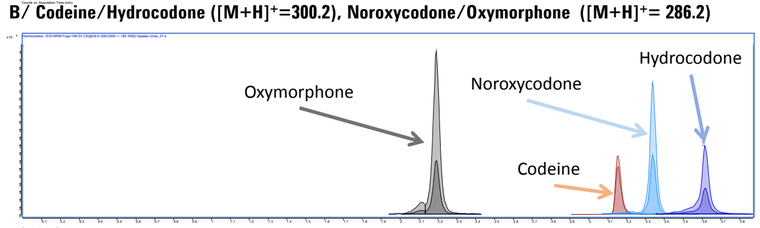
Cycle Time:	330 ms
Total dynamic MRMs:	48
Retention Time Window:	30 sec
Q1 and Q2 Resolution:	0.7 amu [autotune]

Results 1 – Isobar Chromatographic Separation

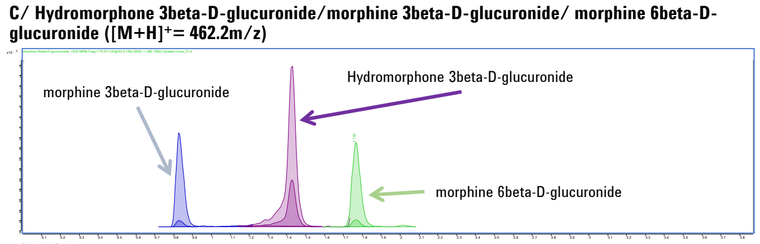
A/ Morphine, Hydromorphone & Norhydrocodone (Nominal Mass $[M+H]^+$= 286.2m/z)



B/ Codeine/Hydrocodone ($[M+H]^+$=300.2), Noroxycodone/Oxymorphone ($[M+H]^+$= 286.2)



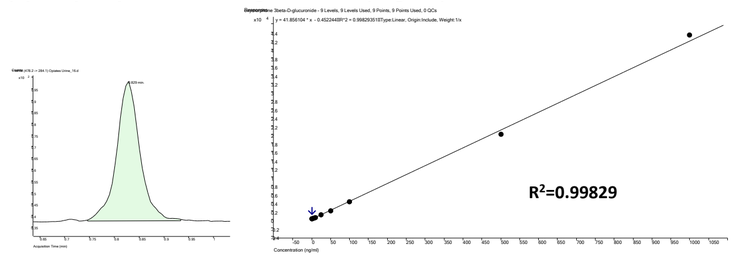
C/ Hydromorphone 3beta-D-glucuronide/morphine 3beta-D-glucuronide/ morphine 6beta-D-glucuronide ($[M+H]^+$= 462.2m/z)



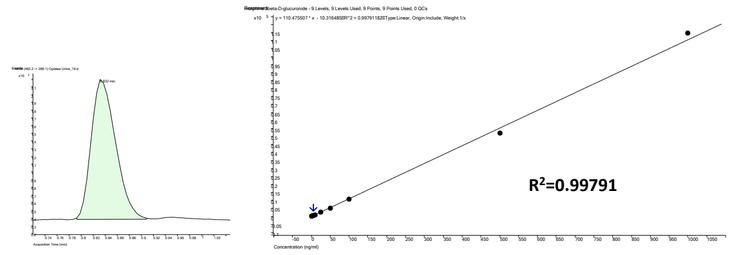
Results 2 & Discussion

Quantitative Linearity of Opiate Glucuronides (500pg/ml→1000ng/ml)

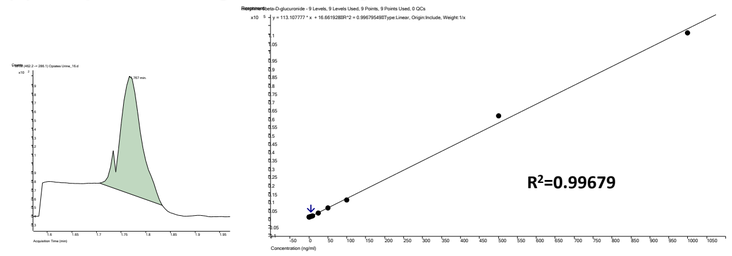
(a) Oxymorphone 3beta-D-glucuronide (1ng/ml)



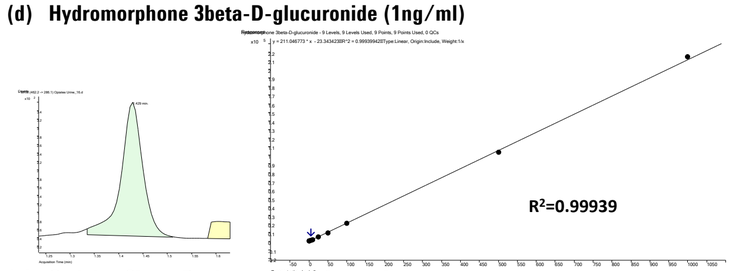
(b) Morphine 3beta-D-glucuronide (1ng/ml)



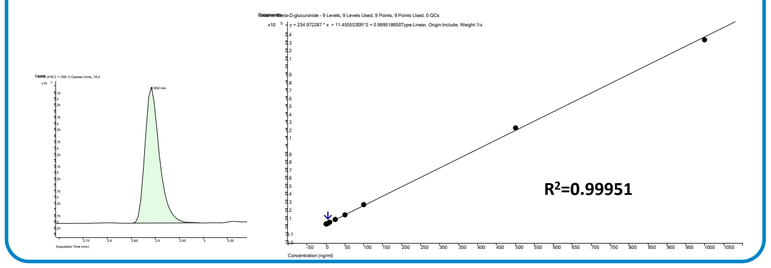
(c) Morphine 6beta-D-glucuronide (5ng/ml)



(d) Hydromorphone 3beta-D-glucuronide (1ng/ml)



(e) Codeine 6beta-D-glucuronide (1ng/ml)



Analytical Method Summary of Opiate Glucuronide Results:

i. LOQ values for all opiate glucuronides in this study were typically <1ng/ml (except Morphine 6beta-D-glucuronide which was 5ng/ml.) These equate to a Urine equivalent of 10ng/ml since there was a 1/10 Urine dilution.
ii. All other opiate analytes in this study showed similar and better LOQ values of less than 1ng/ml (<10ng/ml Urine equivalents.)
iii. Over five separately spiked batches of samples, %RSD values were <10% across the Linear range.

Conclusions

The analytical method developed herein has demonstrated the feasibility of:
• Analyzing opiate/opioid glucuronide metabolites directly and individually without the necessity of hydrolysis sample preparation;
• Effectively demonstrated the ability to baseline-separate multi-isobaric groups of analyte in one rapid chromatographic method;
• Illustrated that low limits of quantitation can be achieved for opiates and their glucuronide metabolites with minimal sample preparation;
•Wide linear dynamic ranges of > 4 orders can be achieved for all analytes in this study.

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