Robust LC-MS/MS Determination of Intact Conjugated Dextrorphan when Hydrolysis is Inefficient

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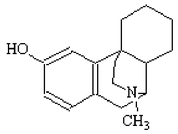
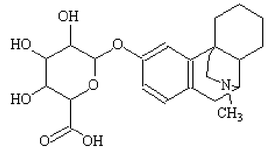
Introduction

Dextromethorphan is a common antitussive drug used in many over-the-counter medicines. During its elimination phase, it is transformed to two major metabolites, dextrorphan and dextrorphan glucuronide. According to the regulatory affairs, analytical methods must have a tracking and an acceptable quantification on every major metabolite. Usually, it is preferred to quantify the glucuronited analyte summed to free form to get a total amount. This is generally achievable using β-glucuronidase or an appropriate hydrolysis. Herein, we describe a case study in which hydrolysis was not efficient using our regular protocol. The root cause was further investigated in terms of enzyme concentration and incubation time.



Dextrorphan
MW: 257.4 g/mole



Dextrorphan Glucuronide
MW: 433.6 g/mole

Method

In order to hydrolyse the glucosidic bond, the β-glucuronidase enzyme from two different sources were used (Helix pomatia and e. coli). In addition, acidic and basic hydrolysis conditions were investigated. Acidic conditionsinvolved 6N HCl heated at 50°C for about 60 minutes. Basic conditions was evaluated using 10% KOH (%w/v) also heated for 60 minutes at approximately 50°C. Dextrorphan glucuronide MSMS parameters were also optimised and monitored during these tests to evaluate the hydrolysis efficiency. The intact metabolite was finally analyzed using an automated protein precipitation, a Betasil-CN column and mass transition of 434→258 by LCMSMS positive TurboIonSpray ionisation.

Extraction Procedure

Matrix	EDTA K_3
Analytical Range	8-8000 ng/mL
Internal Standard	Dextrorphan-d_3
Sample Volume	0.050 mL
Extraction Type	Automated Protein Precipitation
Dilution Factor	60

LC-MS/MS Analysis

	Human Method
Chromatographic Mode	Reverse Phase
Analytical Column	Betasil CN
Elution Mode	Isocratic
Mobile Phase A	Methanol/Water/Ammonium formate/Formic acid 0.1%
Flow Rate	1.00 mL/min
Injection Volume	10 µL
Retention Time	1.16 min for Dextrorphan Glucuronide
	2.16 for Dextrorphan-d_3
Acquisition Time	3.00 min
Detector	API 4000
Source	TurboIonSpray
Ion Monitored	434→258 for Dextrorphan Glucuronide
	261→157 for Dextrorphan-d_3

Results

Using the two different β-glucuronidases sources to cleave the glucuronic acid moiety, recovery was not greater than 10% using the regular procedure when measured against a quantification curve of unconjugated dextrorphan. Dextrorphan glucuronide was mostly intact after the acidic or basic hydrolysis. At this time, it was decided to validate the assay for the determination of intact dextrorphan glucuronide. The method was validated over the dynamic range of 8-8000ng/mL in human plasma. Accuracy and precision was assessed successfully (**Table 1**). Dextrorphan glucuronide was found to be stable in plasma for 22 hours at room temperature and after four freeze/thaw cycles (**Table 2**).

Table 1. Accuracy and Precision of the Validated Method

	LLOQ 8.00 ng/mL		Low QC 24.00 ng/mL		Middle QC 4000.00 ng/mL		High QC 6000.00 ng/mL	
	Measured Conc. (ng/mL)	% Bias	Measured Conc. (ng/mL)	% Bias	Measured Conc. (ng/mL)	% Bias	Measured Conc. (ng/mL)	% Bias
N	24	24	24	24	24	24	24	24
Mean	7.71	-3.61	23.68	-1.32	4045.93	1.15	5969.61	-0.51
SD(±)	0.38		1.13		159.72		213.33	
CV(%)	4.94		4.77		3.95		3.57	

Table 2. Stability Data of Dextrorphan Glucuronide

Parameters	% Bias	
	Low QC 24.00 ng/mL	High QC 6000.00 ng/mL
Freeze/Thaw at -20°C	5.8	3.8
Freeze/Thaw at -80°C	2.9	1.7
Short-Term at RmT (22 hrs)	6.3	6.8
Short-Term at 4°C (22 hrs)	6.9	7.6
Post-Preparative at RmT (95 hrs)	3.2	3.5
Whole Blood at 4°C (230 min)	-2.6	0.2

Further investigation was performed in order to find the root cause of the lack of hydrolysis efficiency with the two different β-glucuronidases. Multiple conditions were tested (**Table 3**).

Table 3. Hydrolysis Efficiency with different Conditions

Hydrolysis Efficiency (%)				
Hydrolysis Conditions	Incubation Time			
	1h	2h	3h	20h
NaOH 10%	0.4	0.9	2.7	1.4
HCL 6N	0.8	2.6	3.3	21.8
HCL 6N in Methanol	0.8	1.9	2.4	21.8
Helix Prometia pH 5 1427U/mL	67.7	72.6	79.5	84.7
Beta Glucuronidase pH 7, 483U/mL	72.9	63.4	81.1	85.9
Beta Glucuronidase pH 7, 943U/mL	87.7	85.6	85.9	87.2
Beta Glucuronidase pH 7, 1800U/mL	88.9	88.6	87.3	90.0
Boron Trifluoride (BF_3)	0.0	0.1	0.1	0.5
Hydrazine Buffer pH 7	0.3	0.5	0.7	1.1
Hydrazine Buffer 50mM in AcOH	0.2	0.6	0.5	1.2

The rate of conversion using different concentrations of β-glucuronidase E. Coli was studied. Concentration from 27 U/mL to 2583 U/mL was assessed. Incubation time was set at 1 hour at 50°C. The hydrolysis efficiency was directly related to the enzyme concentration (**Figure 1**).

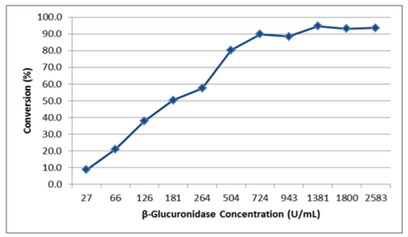


Figure 1. Hydrolysis Efficiency of β-glucuronidase E. Coli in Function of the Enzyme Concentration over 1 hour Incubation at 50°C

Chromatography

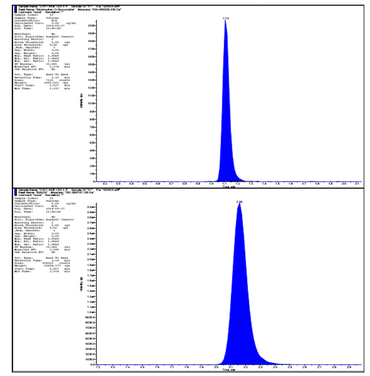


Figure 2. Chromatogram of Dextrorphan Glucuronide LLOQ (8 ng/mL)

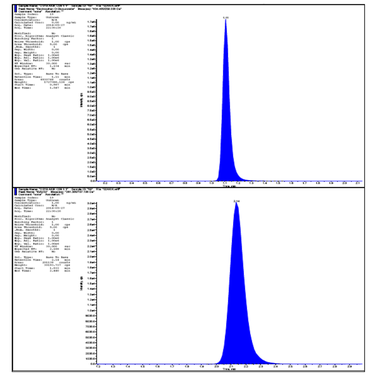


Figure 3. Chromatogram of Dextrorphan Glucuronide ULOQ (8000 ng/mL)

Conclusion

This work describes the method validation of dextrorphan-O-glucuronide in human plasma. During original method development, hydrolysis efficiency was not very high. It was decided to measure dextrorphan-O-glucuronide in its intact form. Dextrorphan-O-glucuronide was found stable in matrix and all over the extraction process. This method was used for study sample analysis and was found to be accurate, precise and reproducible with ISR results near 100%. However, it was observed after further investigation that the concentration of the β-glucuronidase impacted directly the rate of hydrolysis. Therefore, the hydrolysis step must be carefully studied during method development and it is strongly suggested to evaluate the hydrolysis efficiency thoroughly.

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