Hydrolysis Conditions Investigation for Analysis of Total Dabigatran in Human Plasma

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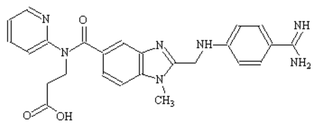
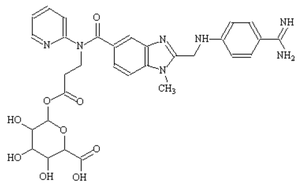
Introduction

Glucuronidation of the carboxylate moiety is the major human metabolic pathway of dabigatran. In order to quantitate precisely and accurately total dabigatran levels in human plasma, a crucial step of deglucuronidation was developed. A careful investigation provided specific and selective conditions for this reaction to be quantitative and its success resided in a specific choice of reagent.



Dabigatran

MW: 471.5 g/mole



Dabigatran Glucuronide

MW: 647.7 g/mole

Method

Dabigatran acylglucuronide, along with an internal standard solution, was hydrolized with a 10% KOH aqueous solution prior extraction onto solid phase cartridges (Oasis HLB 3cc, 60mg). Chromatography was performed on an ACE 3 C18 50x4.6 3µm analytical column using a H_2O/ACN (87/13), ammonium formate 2 mM mobile phase. The triple quadrupole mass spectrometer (AB Sciex), model API 5000, equipped with an electrospray (ESI) ionization source in positive mode, was set up in multiple reaction monitoring mode, using transitions 472.2 → 289.1 and 479.2 → 292.1 for dabigatran and dabigatran-d_7 respectively. The method was validated over the range of 1-800 ng/mL.

Extraction Procedure

Matrix	EDTA K_2
Analytical Range	1-800 ng/mL
Internal Standard	Dabigatran-d_7
Sample Volume	0.050 mL
Extraction Type	Solid-Phase Extraction
Dilution Factor	16

LC-MS/MS Analysis

	Human Method
Chromatographic Mode	Reverse Phase
Analytical Column	ACE 3 C18
Elution Mode	Isocratic
Mobile Phase A	Acetonitrile/Water/Ammonium formate
Flow Rate	1.00 mL/min
Injection Volume	10 µL
Retention Time	1.57 min for Dabigatran 1.52 for Dabigatran-d_7
Acquisition Time	4.00 min
Detector	API 5000
Source	TurboIonSpray
Ion Monitored	472→289 for Dabigatran 479→292 for Dabigatran-d_7

Results

A careful investigation of deglucuronidation conditions was peformed. Complete conversion could not be obtained using β-glucuronidase enzyme solution, the hydrolysis efficiency was of 80% (**Table 1**). Enzyme source (Helix pomatia or Escherichia coli), reaction temperature, reaction pH, enzyme concentration and hydrolysis solution composition were all investigated without success. Dabigatran acylglucuronide MS/MS transition still showed residual peaks after treatment with β-glucuronidase enzyme. Chemical hydrolysis was then experimented using a KOH 10% aqueous solution paired with a heating procedure. This newly developed hydrolysis step provided glucuronide MS/MS transition free of any residual peaks and yielded quantitative analysis of total dabigatran. The hydrolysis step was followed by a SPE or a protein precipitation. The SPE gave the best recovery (**Figures 1 to** 4). The method was validated over the range of 1-800ng/mL using calibration curve prepared with unconjugated dabigatran and quality controls prepared with dabigatran glucuronide. Accuracy and precision were below 6.15% and 6.05%, respectively (**Table 2**). Hydrolysis efficiency was near 100% (**Table 3**).

Table 1. Optimized ConditionsHydrolysis Efficiency (Accuracy) of β-glucuronidase (E-Coli) with Glucuronide Quality Control vs. Calibration Curve in Free Dabigatran

	Low QC 594 ng/mL		Middle QC 99040 ng/mL		High QC 148560 ng/mL	
	Conc. Found (ng/mL)	Accuracy (%)	Conc. Found (ng/mL)	Accuracy (%)	Conc. Found (ng/mL)	Accuracy (%)
	505.40	85.08	84270.29	85.09	120744.33	81.28
	513.33	86.42	85000.30	85.82	130357.11	87.75
	485.61	81.75	80463.42	81.24	124234.12	83.63
	482.73	81.27	79404.41	80.17	132056.37	88.89
	496.97	83.66	77440.03	78.19	132171.44	88.97
	456.60	76.87	84147.98	84.96	116858.48	78.66
Mean	490.11	82.51	81787.74	82.58	126070.31	84.86
SD(±)	20.09	3.38	3110.98	3.14	6450.25	4.34
CV(%)	4.10	4.10	3.80	3.80	5.12	5.12

Table 2. Accuracy and Precision of Dabigatran Glucuronide Quality Controls with KOH Hydrolysis quantified with a Dabigatran Free Calibration Curve

	LLOQ 1.09 ng/mL		Low QC 3.27 ng/mL		Middle QC 436.00 ng/mL		High QC 654.00 ng/mL	
	Measured Conc. (ng/mL)	% Bias	Measured Conc. (ng/mL)	% Bias	Measured Conc. (ng/mL)	% Bias	Measured Conc. (ng/mL)	% Bias
N	30	30	30	30	30	30	30	30
Mean	1.12	3.09	3.31	1.19	462.83	6.15	682.54	4.36
SD(±)	0.07		0.16		21.16		29.95	
CV(%)	6.05		4.83		4.57		4.39	

Table 3. Efficiency of the Hydrolysis with KOH 10% followed by SPE

	LLOQ-QC 1.09 ng/mL		Low QC 3.27 ng/mL		Middle QC 436 ng/mL		High QC 654 ng/mL		ULOQ-QC 872 ng/mL	
	Measured Conc. (ng/mL)	Hydrolysis Efficiency (%)	Measured Conc. (ng/mL)	Hydrolysis Efficiency (%)	Measured Conc. (ng/mL)	Hydrolysis Efficiency (%)	Measured Conc. (ng/mL)	Hydrolysis Efficiency (%)	Measured Conc. (ng/mL)	Hydrolysis Efficiency (%)
	1.15	105.50	3.30	100.92	467.08	107.13	623.37	95.32	838.57	96.17
	1.11	101.83	3.49	106.73	481.42	110.42	653.85	99.98	945.15	108.39
	1.07	98.17	2.83	86.54	457.08	104.83	658.09	100.63	906.33	103.94
	1.14	104.59	3.24	99.08	467.92	107.32	665.86	101.81	931.93	106.87
	1.03	94.50	3.15	96.33	462.23	106.02	670.01	102.45	870.75	99.86
	1.25	114.68	3.19	97.55	458.54	105.17	649.34	99.29	969.89	111.23
N	6	6	6	6	6	6	6	6	6	6
Mean	1.125	103.21	3.200	97.86	465.712	106.81	653.420	99.91	910.437	104.41
SD(±)	0.0758		0.2169		8.8494		16.5628		48.9048	
CV(%)	6.74		6.78		1.90		2.53		5.37	

Chromatography

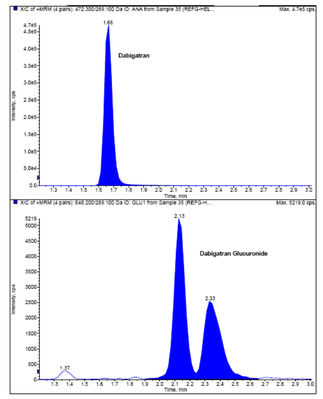


Figure 1. Chromatogram of Dabigatran Glucuronide QC with Helix Pomatia Hydrolysis followed by Protein Precipitation

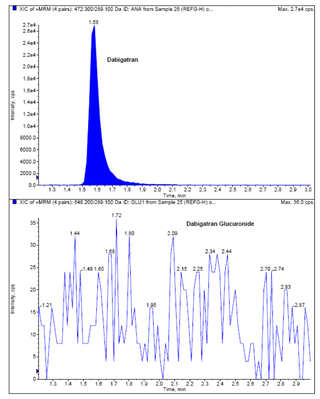


Figure 2. Chromatogram of Dabigatran Glucuronide QC with KOH 10% Hydrolysis followed by Protein Precipitation

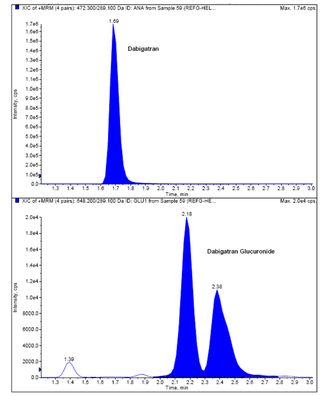


Figure 3. Chromatogram of Dabigatran Glucuronide QC with Helix Pomatia Hydrolysis followed by SPE

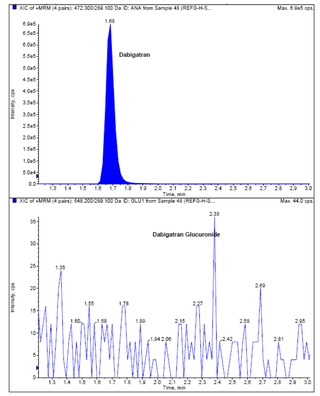


Figure 4. Chromatogram of Dabigatran Glucuronide QC with KOH 10% Hydrolysis followed by SPE

Conclusion

Overall, quantitative conversion of dabigatran acylglucuronide to its free form could not be obtained using β-glucuronidase solution. A careful investigation of deglucuronidation reaction conditions was successful in obtaining precise and accurate analysis of total dabigatran in human plasma.

TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY