Title: Pharmacokinetics of glepaglutide, a long-acting glucagon-like peptide-2 analogue: a study in healthy subjects

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Quantitative method for parent, M1 and M2:

Parent drug and metabolites (M1 and M2) were quantified at the Charles River Laboratories, Edinburgh Ltd (UK), using immunoaffinity extraction followed by liquid chromatography–tandem mass spectroscopy (LC MS/MS).

Calibration samples, linearity, accuracy, and precisions

Calibration standards, ranging from 25.0 to 2500 pmol/L for the parent and M1 and 50 to 5000 pmol/L for M2, were prepared by spiking blank human plasma with appropriate amounts of stock solutions followed by serial dilution. Similarly, quality control (QC) samples at 75.0, 300 and 2000 pmol/L for parent and M1, and at 150, 600 and 4000 pmol/L for M2, were prepared by spiking blank human plasma with stock solutions followed by serial dilution. The quantification of test samples and QC samples were performed using linear regression with a $1/x^2$ weighting factor, and the correlation coefficient was in general greater than 0.99. The overall bias and precision for 58 analytical batches were below ± 1.8% and 8.8% for all analytes, respectively. For a run to be considered acceptable, the measured concentrations of at least 66% of QC samples were to be within ±15% of their actual concentrations with a minimum of 50% of QC samples accepted at each concentration.

Sample preparation

For the immunoaffinity extraction, a mix of two antibodies (1:1) directed against the N-terminal- and mid-region of parent were immobilized (26.6 mg/g) to magnetic Dynabeads™ (M270 Epoxy, ThermoFisher Scientific). The antibody-coated beads were added to 400 µL K_2EDTA plasma (calibration, QC or test samples) spiked with 50 µL (2-propanol:water, 50:50 v/v) stable isotope labeled internal standards of parent, M1 and M2 (all containing four hexa^{13}C-^{15}N-Leucine residues in amino acid position 10, 14, 17 and 26) and 600 µL coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH7.2) and mixed (approx. 750 rpm) for 2 hours at room temperature. Following incubation, the beads were washed 3 times for 30 seconds using 500 µL phosphate buffer (50 mM, pH 7.0) and the analytes extracted by addition of 100 µL Acetonitrile:Water:Trifluoroacetic acid (40:60:0.25, v/v/v) for 30 seconds. The supernatant was transferred and centrifuged (3500 rpm for 30 minutes at ca. 4°C) prior to injection to the LC MS/MS system using the settings presented in Table 1. The recoveries of the

analytes ranged from approximately 40 to 50% and were similar for the corresponding internal standards.

Table 1 LC MS/MS instrumentation and settings

Liquid chromatography system	Acquity UPLC System, I-class (Waters)		
Mass spectrometer	API5000 (Sciex)		
Analytical column	POROS R2 10 µm, 30x 2.1 mm (Applied biosystems)		
Column temperature	60°C		
Autosampler temperature	4°C		
Injection volume	30 µL		
Flow rate	300 µL/min		
Mobile Phase A	Acetonitrile/deionized water/formic acid (90/10/0.2, v/v/v)		
Mobile Phase B	Deionized water/formic acid (100/0.2, v/v)		
Run time (10 min)	Time (min)	A (%)	B (%)
	0	0	100
	6	55	45
	7	100	0
	8	0	100
	10	0	100
Retention time	Parent + IS	Ca. 4.3 min	
	M1 + IS	Ca. 4.8 min	
	M2 + IS	Ca. 5.0 min	
MS/MS detection	Ionisation mode	Turbo ion spray positive	
	Parent	720.6 → 801.3 + 819.1	
	Parent IS	724.9 → 806.3	
	M1	762.0 → 920.7 + 897.1	
	M1 IS	767.6 → 928.1	
	M2	736.4 → 878.1 + 854.6	
	M2 IS	742.0 → 884.7	