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Overcoming Adduct Formation to Develop and Validate an LC-MS/MS Method for the Quantitative Determination of a Human Proislet Peptide (HIP2B) in Human Plasma.

Rhonda L. Johnson, Tara L. Baldwin, Jeffrey L. Jeppson, Ridha Nachi, Chris Kafonek, Rafiqul Islam
Celerion, Lincoln, NE, USA

celerion
Applied Translational Medicine

OBJECTIVE

The identification and mitigation of a concentration dependent interference was necessary to develop and validate an accurate and precise method for the quantitation of HIP2B in human plasma.

INTRODUCTION

Human Proislet Peptide (HIP) is a bioactive peptide that stimulates the differentiation of insulin-producing islets from existing progenitor cells within the pancreas. Islet neogenesis is a novel approach to the treatment of both Type 1 and Type 2 diabetes. Pre-clinical studies demonstrated that treatment with HIP2B (a stabilized form of HIP) increased the formation of new glucose and hormone-responsive islet β-cells, resulting in attenuation of hyperglycemia. A validated bioanalytical method was required to characterize the pharmacokinetics of HIP2B in human studies. The validated method was successfully used to measure concentrations of HIP2B in plasma samples from healthy males participating in a safety and tolerability study.

EXPERIMENTAL

HIP2B and its $^{13}C_{41}{}^{15}N_5$-labeled internal standard (IS; +17 amu) were extracted from 0.0250 mL of human plasma by protein precipitation and dilution. The peptides were chromatographically separated from matrix components on an ACE® C18 (50 x 3.0 mm, 5 µm) column using a gradient composed of aqueous formic acid and acetonitrile. Doubly-charged precursor ions $(M + 2H)^{+2}$ produced by electrospray ionization (ESI) were selected in positive multiple reaction monitoring (MRM) mode.

During early testing, a concentration-dependent interference that significantly affected the linearity of the calibration curve was observed at the retention time and mass-to-charge (*m/z*) transition of the IS.

Table 1 HIP2B and IS Peak Areas for Analytical Run 3

Nominal Concentration	HIP2B Peak Area	IS Peak Area	% Deviation from Nominal Concentration
5.00	1018	135224	-18.8
10.0	1924	131586	13.0
25.0	5769	138091	54.4
100	15397	146159	2.0
200	39731	145365	35.5
500	102755	161209	27.4
2000	277977	194479	-28.5
4000	753338	314834	-40.0
5000	926878	347251	-46.4

Figure 1 Nonlinear Calibration Curve – Analytical Run 3

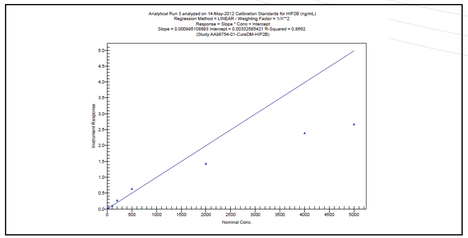
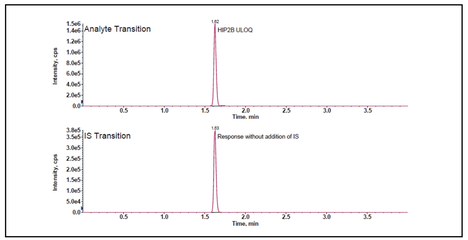
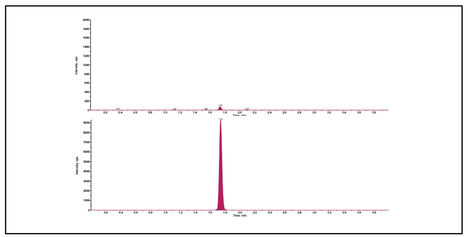


Figure 2 Injection of a HIP2B Solution without IS Confirmed that a Detectable Response was Present at the Retention Time and *m/z* of the IS



The presence of HIP2B with a natural abundance of carbon and nitrogen stable isotopes in the IS reference material prevented the use of a significantly higher concentration of IS in the assay.

Figure 3 Extracted Single Blank (IS only)



Because the monitored *m/z* transitions were doubly charged, the mass difference between the HIP2B and IS precursor ions was approximately 8.5 amu. The interference was hypothesized to be an ammonium adduct of HIP2B as the *m/z* of a doubly charged ammonium adduct would be within the resolution window (± 0.5 amu) of the IS.

Modification of the ionization parameters, specifically increasing the declustering potential (DP) from 61 to 110 V, sufficiently mitigated formation of the adduct.

Figure 4 Injection of a HIP2B Solution without IS after Increasing the Declustering Potential

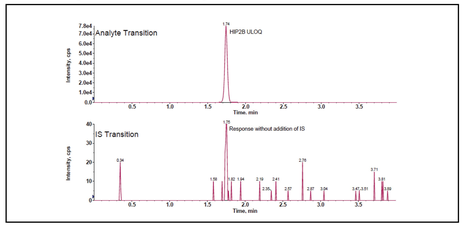
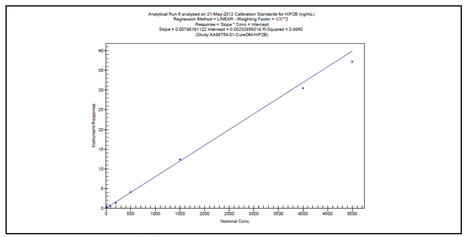


Table 2 HIP2B and IS Peak Areas for Analytical Run 6

Nominal Concentration	HIP2B Peak Area	IS Peak Area	% Deviation from Nominal Concentration
5.00	2090	15649	0.2
10.0	2950	13959	-4.5
25.0	8721	14002	8.4
100	20771	13098	7.9
200	57728	14020	-9.0
500	144448	14032	3.6
2000	444819	14411	4.0
4000	1423823	15299	-4.3
5000	1441435	14090	-6.6

Figure 5 Linear Calibration Curve – Analytical Run 6



RESULTS

The validated concentration range was 10.0 to 1000 ng/mL. Inter-batch accuracy (%Bias) and precision (%CV) for quality control samples ranged from -0.9 to 3.0 and 6.1 to 9.5, respectively. The mean (n=4) correlation coefficient was 0.9967 ± 0.0025.

Table 3 Inter-batch Precision and Accuracy

Batch	LLOQ QC 10.0 ng/mL	QC A 30.0 ng/mL	QC B 100 ng/mL	QC C 750 ng/mL
Inter-Batch Mean	10.3	30.1	102	743
Inter-Batch % CV	9.5	8.7	6.1	6.3
Inter-Batch % Bias	3.0	0.3	2.0	-0.9
n	30	30	30	30

Table 4 Calibration Curve Parameters

Batch	Slope	Intercept	r^2
20	0.00951	0.00537	0.9959
21	0.00892	-0.00017	0.9984
22	0.00986	0.00300	0.9935
24	0.00972	0.00779	0.9990

Figure 6 HIP2B LLOQ (10.0 ng/mL)

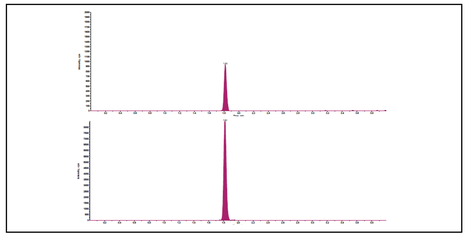
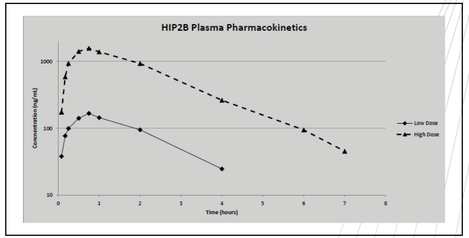


Table 5 Summary of Stability and Selectivity Data

Sample Collection and Handling	120 minutes in human whole blood (EDTA) polypropylene / ambient / white light
Short-term Stability (1 thaw cycle)	24 hours - polypropylene / ice bath / UV-shielded light
Short-term Stability (6 thaw cycles)	75 hours - polypropylene / ice bath / UV-shielded light
Freeze-Thaw Stability	6 freeze (-80°C)-thaw (ice water bath) cycles under UV-shielded light
Long-Term Storage Stability	87 days - polypropylene / -20°C and -80°C
Processed Stability	221 hours in polypropylene at 5°C (fresh calibrators) 160 hours in polypropylene at 5°C (re-injection)
Dilution Integrity	Up to 37,500 ng/mL, diluted 50-fold
Selectivity	No significant interference at the retention time and mass transition of HIP2B or the IS was observed in 10 lots
Matrix Factor	Mean (IS normalized) 1.0; CV 2.5 - 6.9%

Figure 7 Mean Plasma Concentrations Versus Time for Low and High Doses of HIP2B



IMPLICATIONS

Peptide ions formed during electrospray ionization are highly solvated. Adduct formation results in an overly complex spectrum and loss of sensitivity for the peptide of interest. In this case, the most abundant peptide adduct also produced a quantifiable interference at the mass transition of the stable-labeled internal standard.

CONCLUSION

An accurate and robust method was developed, validated and employed for the quantitation of HIP2B in clinical samples from a single ascending dose safety and tolerability study.

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