The Development and Validation of an Enzyme Immunoassay for the Determination of Exenatide (Exendin-4) in Human Plasma for Pharmacokinetic Analysis

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PURPOSE:

- Exenatide is a synthetic version of exendin-4, a peptide found in the saliva of the gila monster. Exendin-4 displays biological properties similar to glucagon-like peptide 1 (GLP-1). Exenatide has been developed for the treatment of diabetes mellitus type II.
- Commercial immunoassay kits for exenatide were evaluated and matrix effects in different lots of human plasma were observed. A commercial kit was adapted and validated to meet the FDA guidelines for validation of bioanalytical methods.

METHOD:

- Aprotinin-treated samples and calibration standards were treated with a protein precipitation reagent and the supernatant was evaporated and reconstituted in a buffer solution. Exenatide in the solution was allowed to bind to the binding sites on the exendin-4 antibody prior to aliquoting onto a microtiter plate coated with a secondary binding antibody. Exenatide in the supernatant competed for binding to the exendin-4 antibody with a fixed amount of biotin-labeled exendin-4 added to the plate. The plate was washed to remove any materials not bound to the plate. An enzyme substrate was added, and allowed to bind to the complex attached to the plate, and unbound material was then washed away. A fluorescent substrate was added to the wells and the signal resulting from the cleavage of the substrate was read on a fluorescence plate reader.

DEVELOPMENT:

- Method development started with comparison testing of exendin-4 assay kits from multiple manufacturers, utilizing different detection technologies (EIA, FIA, RIA).A fluorescence immunoassay kit (FIA) was eventually selected for further development, based on the sensitivity and reproducibility of the assay. A concentration bias was seen when back calculating fortified human plasma samples against calibrators prepared in the kit supplied buffer, this bias was resolved by preparing calibrators in human plasma.
- Aprotinin treatment of the samples was included to ensure that exenatide and endogenous peptides did not degrade, and testing spiked samples incubated with and without aprotinin demonstrated that the addition of aprotinin did not have an effect of the quantitation of exenatide. Whole blood samples were fortified with exenatide, incubated at 37° C, then centrifuged and the plasma treated with aprotinin. These samples quantitated similar to whole blood samples that were centrifuged immediately after exenatide fortification and the plasma subsequently treated with aprotinin.
- During the initial qualification of the immunoassay method, matrix effects in multiple lots of human plasma were affecting the quantitation of both naive samples and samples fortified with exenatide. Since exendin-4 is not endogenous, we were not measuring basal levels, but rather some other component in the sample was effecting the results. A protein precipitation utilizing acetonitrile, followed by evaporation and reconstitution of the supernate, produced naive sample results below the limit of quantitation and accurate quantitation of fortified samples. This was later modified to precipitation with isopropanol, which gave better recovery of exenatide from the samples and provided more efficient evaporation of the supernate.

RESULTS:

- The exenatide method used 4-parameter logistic regression weighted 1/Y² over the range 20.0 – 800 pg/mL. Inter- and intra-batch precision and accuracy, short-term and long-term stability, matrix effect in normal and type II diabetic plasma, dilution integrity and hook effect met validation acceptance criteria. Additional selectivity was tested against a panel of diabetic drugs and met acceptance criteria.

Figure 1. Exenatide Example Standard Curve

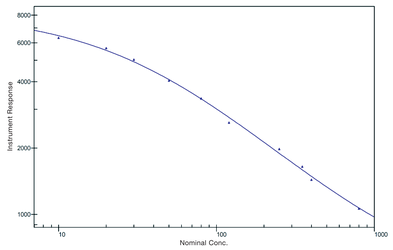

Table 1. Exenatide Validation Summary

Validation Summary	
Analyte	Exenatide (synthetic Exendin-4)
Method Description	Sample pre-treatment with protein precipitation and analysis using enzyme immunoassay (EIA)
Limit of Quantitation (pg/mL)	20.0 pg/mL
Average Recovery of Drug (% Mean)	87.2% at 30.0 pg/mL 94.2% at 300 pg/mL
Standard Curve Concentrations (pg/mL)	10.0 (anchor point), 20.0, 30.0, 50.0, 80.0, 120, 250, 350, 400, and 800 (anchor point) pg/mL
QC Concentrations (pg/mL)	LLOQ QC, 30.0, 100, 300 and ULOQ QC pg/mL
QC Intra-Batch Precision Range (% CV)	0.6 to 17.7%
QC Intra-Batch Accuracy Range (% Bias)	-14.7 to 17.0%
QC Inter-Batch Precision Range (% CV)	5.3 to 11.6%
QC Inter-Batch Accuracy Range (% Bias)	-6.3 to 12.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 29 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 95 days at approximately 250 µg/mL in injection solution in polypropylene tubes at 5°C
Freeze-Thaw Stability (Cycles)	3 freeze (-80°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 79 days in polypropylene tubes at 80°C
Dilution Integrity	Samples diluted up to 50-fold can be quantified
Selectivity	No quantation greater than the LLOQ of exendin-4 was observed from endogenous components in any of the 10 human plasma (heparin) lots screened No quantation greater than the LLOQ of exendin-4 was observed from endogenous components in any of the 10 type 2 diabetic human plasma (heparin) lots screened
Assay Volume Required	0.150 mL
Regression Type	4PL, 1/Y²
Additional Selectivity	No interference was seen when human plasma (unfortified and fortified with exenatide at the low QC and at the high QC level) were fortified with additional selectivity compounds (Table 5).
Matrix Effect [Human Plasma (Heparin)]	No significant matrix effect was observed in any of the 10 human plasma (heparin) lots that were fortified near the concentration of the LLOQ (20.0 pg/mL) or in 9 of the 10 human plasma (heparin) lots that were fortified with exendin-4 near the concentration of the high QC (300 pg/mL) sample
Matrix Effect [Type 2 Diabetic Human Plasma (Heparin)]	No significant matrix effect was observed in any of the 10 human plasma (heparin) lots that were fortified near the concentration of the LLOQ (20.0 pg/mL) or in 9 of the 10 human plasma (heparin) lots that were fortified with exendin-4 near the concentration of the high QC (300 pg/mL) sample
Lipemic Sample Evaluation	No significant interference for exendin-4 was observed in any of the 3 lipemic human plasma (heparin) lots that were fortified at the concentration of the low QC (30.0 pg/mL) or in any of the 3 human plasma (heparin) lots that were fortified at the concentration of the high QC (300 pg/mL) sample
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 250 µg/mL in injection solution in polypropylene tubes at ambient temperature under white light
Stability of Analyte During Sample Collections and Handling	Up to 30 minutes in human whole blood (heparin) in polypropylene tubes in an ice water bath under white light
Batch Size	1, 96 well plate

Table 2. Exenatide Standard Inter-Batch Summary

Inter-Batch	AP1 10.0 pg/mL	STD B 20.0 pg/mL	STD C 30.0 pg/mL	STD D 50.0 pg/mL	STD E 80.0 pg/mL	STD F 120 pg/mL	STD G 250 pg/mL	STD H 350 pg/mL	STD I 400 pg/mL	AP2 800 pg/mL
Mean	11.5	19.2	26.9	46.0	84.9	133	245	332	381	844
SD	0.385	0.796	0.941	2.16	2.46	4.97	12.1	10.9	16.7	12.3
% CV	3.3	4.1	3.5	4.7	2.9	3.7	4.9	3.3	4.4	1.5
% Bias	+15.0	-4.0	-10.3	-8.0	+6.1	+10.8	-2.0	-5.1	-4.8	+5.5
n	8	8	8	8	8	8	8	8	8	8

Table 3. Exenatide Quality Control Inter-Batch Summary

Inter-Batch	LLOQ QC 20.0 pg/mL	QC A 30.0 pg/mL	QC B 100 pg/mL	QC C 300 pg/mL	ULOQ 400 pg/mL
Mean	19.8	28.1	112	315	422
SD	2.29	2.13	5.91	17.4	28.6
% CV	11.6	7.6	5.3	5.5	6.8
% Bias	-1.0	-6.3	+12.0	+5.0	+5.5
n	23	24	24	24	23

Table 4. Exenatide Quality Control Total Error

Characteristic	Statistic	LLOQ QC 20.0 pg/mL	QC A 30.0 pg/mL	QC B 100 pg/mL	QC C 300 pg/mL	ULOQ QC 400 pg/mL
Total N	N_obs	23	24	24	24	23
Bias (%RE)	Mean	-0.6	-6.2	12.5	5.0	5.5
	LCL	-8.7	-10.8	9.4	2.2	1.2
	UCL	7.5	-1.6	15.6	7.8	9.8
Precision (%CV)	Intra-batch	8.2	5.7	5.7	5.8	6.3
	Inter-batch	11.7	7.2	5.9	5.8	7.2
|Bias| + Precision	|Mean| + Inter-batch	12.3	13.4	18.4	10.8	12.7
90% Expectation	Lower Limit	-22.1	-19.2	2.0	-5.1	-7.4
Tolerance Inter (%RE)	Upper Limit	20.9	6.8	22.9	15.2	18.4

Table 5. Additional Selectivity

Exendin Concentration	Unspiked 0.0 pg/mL	QC A 30.0 pg/mL	QC C 300 pg/mL
	BLQ	28.7	301
	BLQ	28.8	305
	BLQ	30.2	306
	BLQ	29.2	298
	BLQ	31.1	301
	BLQ	30.9	264
Mean		29.8	296
% CV		3.6	5.4
% Theoretical		99.3	98.7
n	6	6	6

BLQ = Below the limit of quantitation
Additional selectivity quality controls were fortified with the following compounds and concentrations:
Glimepiride (400 ng/mL), Glucagon (50.0 ng/mL), Glyburide (120 ng/mL), Insulin (50.0 ng/mL), Metformin (2000 ng/mL), Repaglinide (60.0 ng/mL), Rosiglitazone (600 ng/mL), Sitagliptin (2000 ng/mL)

Table 6. Selectivity and Matrix Effect for Exenatide in Human Plasma

			LLOQ		High	
	Lot#	Blank	20.0 pg/mL	% Dev.	300 pg/mL	% Dev.
	1	BLQ	20.3	+1.5	285	-5.0
	2	BLQ	20.6	+3.0	327	+9.0
	3	BLQ	21.0	+5.0	312	+4.0
	4	BLQ	20.7	+3.5	344	+14.7
	5	BLQ	19.6	-2.0	313	+4.3
	6	BLQ	19.1	-4.5	295	-1.7
	7	BLQ	20.3	+1.5	301	+0.3
	8	BLQ	21.9	+9.5	345	+15.0
	9	BLQ	21.4	+7.0	361	+20.3
	10	BLQ	20.9	+4.5	346	+15.3
Mean			20.5		320	
% CV			4.2		8.0	
% Theoretical			102.5		106.7	
n			9		9	

BLQ = Below the limit of quantitation

Table 7. Selectivity and Matrix Effect for Exendin-4 in Type 2 Diabetic Human Plasma

			LLOQ		High	
	Lot#	Blank	20.0 pg/mL	% Dev.	300 pg/mL	% Dev.
	1	BLQ	20.3	+1.5	291	-3.0
	2	BLQ	18.4	-8.0	294	-2.0
	3	BLQ	16.6	-17.0	277	-7.7
	4	BLQ	16.0	-20.0	263	-12.3
	5	BLQ	20.6	+3.0	309	+3.0
	6	BLQ	20.6	+3.0	251	-16.3
	7	BLQ	21.1	+5.5	241	-19.7
	8	BLQ	18.2	-9.0	*	N/AP
	9	BLQ	18.0	-10.0	283	-5.7
	10	BLQ	18.9	-5.5	295	-1.7
Mean			18.9		276	
% CV			9.9		8.3	
% Theoretical			94.5		92.0	
n			9		8	

BLQ = Below the limit of quantitation
* = Questionable multiple values

CONCLUSIONS:

- The method allows for rapid, selective, accurate and reproducible quantitation of exenatide in human plasma samples for pharmacokinetic evaluation.

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