## The Development and Validation of Specific Ligand Binding Methods for the Determination of Parathyroid Hormone (1-34) and (1-84) in Human Plasma for Pharmacokinetic Sample Analysis

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## **PURPOSE:**

- Parathyroid hormone (PTH) is an 84 amino acid polypeptide produced in the parathyroid gland and plays a major role in regulating serum calcium concentrations. PTH (1-34) is a polypeptide consisting of the 34 N-terminal amino acids of the intact PTH (1-84) which shows similar effects on serum calcium regulation.
- Methods for determining PTH (1-84) in human plasma need to account for endogenous concentrations of the intact peptide when preparing controls and calibrators in plasma. Methods for determining PTH (1-34) in human plasma also need to account for antibody cross-reactivity with endogenous PTH (1-84).
- Commercially available PTH (1-84) kits and PTH (1-34) kits were adapted and validated to meet FDA guidelines for validation of bioanalytical methods.

## **METHODS:**

- **PTH (1-84):** Unknown samples and calibration samples prepared in human plasma (treated with an antibody against the C-terminal portion of PTH (1-84) attached to a solid substrate to remove endogenous PTH (1-84)) were added to test tubes containing a fixed antibody (C-terminal specific) and a radioactively labeled antibody (N-terminal specific). After washing, the tubes were counted in a gamma counter. The counts detected were directly proportional to the concentration of PTH (1-84) present in the sample (Figure 1).
- PTH (1-34): Unknown samples and calibration samples in human plasma were pre-treated with an antibody against the C-terminal portion of PTH (1-84) attached to a solid substrate to remove PTH (1-84). The pre-treated samples and calibration samples were pipetted into microtiter plates coated with the appropriate capture antibodies. The wells were washed to remove unbound material and enzyme-labeled antibody was added. Unbound labeled antibody was removed and a chromogenic substrate was added to the bound labeled antibody. The development of the colored reaction product was directly proportional to the concentration of PTH (1-34) present in the sample and was detected using a spectrophotometric plate reader (Figure 2).

## **RESULTS:**

- The PTH (1-84) method uses a 4-parameter logistic regression weighted 1/y over the range of 10.0 – 1500 pg/mL. The PTH (1-34) method uses a 4-parameter logistic regression weighted 1/y<sup>2</sup> over the range of 6.00 - 150 pg/mL.
- The concentrations of PTH (1-84) and PTH (1-34) calibration standards were back-calculated from the regression equation of the experimental data. The coefficients of variation (C.V.) were less than or equal to 4.4% and 8.5%, respectively.
- Inter-batch precision of PTH (1-84) quality control samples between 10.0 and 2000 pg/mL was less than 11.6% C.V. Interbatch accuracy (% Bias) of the same quality control samples was between -1.3 and +8.8% (Table 1). Inter-batch precision (%CV) of PTH (1-34) quality control samples between 6.00 and 150 pg/mL

was less than 9.7% C.V. Inter-batch accuracy (% Bias) of the same quality controls samples was between -8.3 and +12.2% (Table 2).

- Sample collection and handling stability was established in whole blood for 2 hours at ambient temperature under UV-shielded light for PTH (1-84) and under white light for PTH (1-34) (Tables 3 and 4).
- Short-term stability in plasma was established for 24 hours for PTH (1-84) and 13 hours for PTH (1-34) at ambient temperature under white light. Cumulative short-term stability for PTH (1-84) was established for 39 hours at ambient temperature under white light.
- Freeze and Thaw stability was established for six freeze (-80°C for PTH (1-84) and -20°C for PTH (1-34)) and thaw (ambient temperature) cycles for both PTH (1-84) and PTH (1-34).
- Long term stability of matrix samples was established for 98 days for PTH (1-84) at -80°C, 10 days for PTH (1-84) at -20°C, and for 259 days for PTH (1-34) at -20°C and -80°C.
- The quantitative integrity of PTH (1-84) and PTH (1-34) samples prepared in multiple lots of non-hemolyzed, non-turbid matrix was verified. The quantitative integrity of PTH (1-84) samples prepared in three lots of hemolyzed matrix and in three lots of turbid matrix was verified. Hemolyzed and turbid matrix did have an effect on the measurement of prepared PTH (1-34) samples.
- An evaluation of dilution integrity demonstrated that a dilution factor of 200 can be applied to PTH (1-84) samples and a dilution factor of 150 can be applied to PTH (1-34) samples to dilute them into the quantifiable range without impacting linearity.
- The absence of a high dose hook effect was demonstrated for both PTH (1-84) and PTH (1-34) by assaying a sample with a concentration higher than the ULOQ at a minimum of 3 dilution levels above the ULOQ. All samples back-calculated with concentrations greater than the ULOQ for the respective assays.

## **CONCLUSIONS:**

• The validated methods allow for rapid, selective, accurate and reproducible quantitation of PTH (1-84) and PTH (1-34) in human plasma samples for pharmacokinetic evaluation.





PTH (1-34)	LLOQ QC 6.00 pg/mL	QC A 18.0 pg/mL	QC B 75.0 pg/mL	QC C 120 pg/mL	ULOQ QC 150 pg/mL
Inter-Batch Mean	6.73	16.5	77.6	125	162
Inter-Batch SD	0.651	0.774	2.26	4.17	8.04
Inter-Batch % CV	9.7	4.7	2.9	3.3	5.0
Inter-Batch % Bias	12.2	-8.3	3.5	4.2	8.0
n	17	18	18	21	18

#### ability of PTH (1-84) During Sample Collection and Handling from Human Whole Blood (EDTA) at Ambient Temperature Under UV Shielded Light Conditions

	Endogenous		<b>Endogenous + Low Spike</b>		Endogenous + High Spike	
PTH (1-84)	0 minutes	120 minutes	0 minutes	120 minutes	0 minutes	120 minutes
	28.0	30.2	51.7	49.9	938	949
	26.6	29.3	50.6	53.5	983	955
	27.3	29.3	49.7	52.0	909	947
	26.5	29.4	49.9	49.7	921	887
	28.0	29.3	46.5	51.2	923	943
	27.2	26.5	48.9	50.5	943	885
Mean	27.3	29.0	49.6	51.1	936	928
% CV	2.4	4.4	3.6	2.8	2.8	3.5
% of Control		106.2		103.0		99.1
n	6	6	6	6	6	6



L	ENDO 11.7 pg/mL	QC A 29.7 pg/mL	QC B 160 pg/mL	QC C 1510 pg/mL	ULOQ 2000 pg/mL
	11.7	32.3	163	1490	1990
	1.36	2.02	4.41	71.5	84.9
	11.6	6.3	2.7	4.8	4.3
	0.0	8.8	1.9	-1.3	-0.5
	18	18	18	18	18

#### tability of PTH (1-34) During Sample Collection and Handling from Human Whole lood (EDTA) at Ambient Temperature Under White Light Conditions

	Low Spike		High Spike		
PTH (1-34)	0 minutes	120 minutes	0 minutes	120 minutes	
	33.3	46.9	219	212	
	33.7	31.8	219	211	
	34.7	25.9	222	215	
	34.5	27.5	218	216	
	34.8	25.1	223	214	
	34.5	24.2	221	214	
Mean	34.3	30.2	220	214	
% CV	1.8	28.5	0.9	0.9	
% of Control		88.0		97.3	
n	6	6	6	6	

## Table 5. PTH (1-84) Validation Summary

Information Requested	Data
Analyte	PTH (1-84)
Method Description	Direct analysis using immunoradiometric assay (IRMA)
Limit of Quantitation (pg/mL)	10.0 pg/mL
Standard Curve Concentrations (pg/mL)	4.00 (anchor point), 10.0, 25.0, 50.0, 100, 200, 400, 800, 1600, and 2000 pg/mL
QC Concentrations (pg/mL)	LLOQ QC, endogenous (11.7 pg/mL), 29.7, 160, 1510, and ULOQ QC pg/mL
QC Intra-Batch Precision Range (% CV)	1.2 to 18.0%
QC Intra-Batch Accuracy Range (% Bias)	-12.0 to 16.0%
QC Inter-Batch Precision Range (% CV)	2.7 to 11.6%
QC Inter-Batch Accuracy Range (% Bias)	-1.3 to 8.8%
<b>Bench-Top Stability (Hrs)</b>	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light
	Cumulative Short-Term Stability: 39 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): 132 days at approximately 100 µg/mL in phosphate-buffered saline/0.1% bovine serum albumin in polypropylene tubes at -20°C
Freeze-Thaw Stability (Cycles)	6 freeze (-80°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 98 days in polypropylene tubes at -80°C, 10 days in polypropylene tubes at -20°C
Dilution Integrity	Samples diluted up to 200-fold can be quantified
Assay Volume Required	0.200 mL
<b>Regression Type</b>	4PL, 1/Y
Matrix Effect	No significant matrix effect was observed in any of the 10 human plasma (EDTA) lots that were fortified at low and high concentrations
Hemolyzed Sample Integrity	No significant interference for PTH (1-84) was observed in any of the 3 hemolyzed human plasma (EDTA) lots that were fortified near the concentration of the LLOQ (10.0 pg/mL) and near the concentration of the high QC (1500 pg/mL) sample
Turbid Sample Integrity	No significant interference for PTH (1-84) was observed in any of the 3 turbid human plasma (EDTA) lots that were fortified near the concentra tion of the LLOQ (10.0 pg/mL) and near the concentration of the high QC (1500 pg/mL) sample
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 100 µg/mL in phosphate- buffered saline/0.1% bovine serum albumin in polypropylene tubes at ambient temperature under white light
Short-Term Stability for Stock Solutions (Substock)	6 hours at 1.00 µg/mL and 100 ng/mL in phosphate- buffered saline/2% bovine serum albumin in polypropylene tubes at ambient temperature under white light
<b>Stability of Analyte During Sample Collections and Handling</b>	up to 120 minutes in human whole blood (EDTA) in polypropylene tubes at ambient temperature under UV shielded light light
Batch Size	132 runs

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### Table 6. PTH (1-34) Validation Summary

Information RequestedDataAnalytePTH (1-34)Method DescriptionSample pre-treatment and direct analysis using enzyme linked immunosorbent assay (ELISA)	
Method DescriptionSample pre-treatment and direct analysis using enzyme linked immunosorbent assay (ELISA)	
enzyme linked immunosorbent assay (ELISA)	
Limit of Quantitation (pg/mL)6.00 pg/mL	
Standard Curve Concentrations (pg/mL)3.00 (anchor point), 6.00, 10.0, 25.0, 40.0, 60.0, 75.0, 100, 130, and 150 pg/mL	
<b>QC Concentrations (pg/mL)</b> LLOQ QC, 18.0, 75.0, 120, and ULOQ QC pg/mL	
<b>QC Intra-Batch Precision Range (% CV)</b> 0.3 to 7.4%	
<b>QC Intra-Batch Accuracy Range (% Bias)</b> -16.1 to 21.7%	
<b>QC Inter-Batch Precision Range (% CV)</b> 2.9 to 9.7%	
<b>QC Inter-Batch Accuracy Range (% Bias)</b> -8.3 to 12.2%	
Bench-Top Stability (Hrs)Short-Term Stability: 13 hours in polypropylene tubes at ambient temperature under white light	
Stock Stability (Days)Long-Term Stability for Stock Solutions (Stock): 131 days at 1.00 mg/mL in water in a polypropylene container at -20°C	
Freeze-Thaw Stability (Cycles)6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light (LLOQ and ULOQ QCs), 5 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light (QC D)	
Long-Term Storage Stability (Days)Long-Term Stability: 284 days (QC A and ULOQ QC) and 259 days (QC D) in polypropylene tubes at -20°C and -80°C	
Dilution IntegritySamples diluted up to 150-fold can be quantified	
SelectivityNo quantitation greater than the LLOQ of PTH (1-34) was observed from endogenous components in any of the 10 human plasma (EDTA) lots screened	
Assay Volume Required 0.600 mL	
Regression Type4PL, 1/Y2	
Known MetabolitePTH 1-84 100, 150, and 500 pg/mL	
Matrix EffectNo significant matrix effect was observed in 8 of the 9 human plasma (EDTA) lots that were fortified near the concentration of the LLOQ (6.00 pg/mL) and in 8 of the 10 human plasma (EDTA) lots that were fortified near the concentration of the high QC (120 pg/mL) sample	
Hemolyzed Sample IntegrityHemolysis has an effect on the measurement of the analyte	
Turbid Sample IntegrityTurbidity has an effect on the measurement of the analyte	
Long-Term Stability for Stock Solutions285 days at 10,000 ng/mL in assay dilution buffer(Substock)285 days at 10,000 ng/mL in assay dilution buffer	
99 days at 5000 ng/mL in human plasma (EDTA) in a polypropylene container at -20°C	
285 days at 5.00 ng/mL in assay dilution buffer in a polypropylene container at -20°C	
Short-Term Stability for Stock Solutions8 hours at 1.00 mg/mL in water in a polypropylene container at ambient temperature under white light	
Short-Term Stability for Stock Solutions (Substock)8 hours at 5.00 ng/mL in assay dilution buffer in a polypropylene container at ambient temperature under white light	
Stability of Analyte During Sample Collections and Handlingup to 120 minutes in human whole blood (EDTA) polypropylene tubes at ambient temperature under white light	
Batch Size1, 96 well plate	

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