Altasciences, 575 Armand-Frappier, Laval, Québec, H7V 4B3, Canada
Altasciences.com | contact@altasciences.com

Quantitation of a PEGylated Human Parathyroid Hormone (1-34) Analog in Rat Plasma Using a Hybrid Immunoaffinity Bottom-Up LC-MS/MS Assay

Jean-Nicholas Mess[1], Jean-Francois Dupuis[1], Kevork Mekhssian[1], Erik Wagner[2], Amy Wang[2], Xin Xu[2], Karim Berrada[3], Max Moore[3] and Anahita Keyhani[1]
[1]Altasciences, Laval, QC, Canada [2]National Center for Advancing Translational Sciences, NIH, Rockville, MD, USA
[3]Frederick National Laboratory for Cancer Research - Leidos Biomedical Research, Frederick, MD, USA

OVERVIEW

Low ng/mL quantitation of a novel PEGylated synthetic peptide (PTH-RM) using commercially available capture antibodies coupled with trypsin digestion and LC-MS/MS analysis.

INTRODUCTION

PEGylation is a widely used modification to improve the physicochemical and pharmacokinetic properties of therapeutic proteins. Quantitative bioanalysis of PEGylated drugs by mass spectrometry offers unique challenges due to the polydispersity of the PEG moiety. Various techniques have been described to circumvent this issue and bottom-up LC-MS approaches have gained in momentum over the past decade.

PTH-RM is a novel PEGylated human parathyroid hormone (hPTH(1-34)) analog in development for the treatment of hypoparathyroidism[1]. Herein, the development of a hybrid immunoaffinity bottom-up LC-MS/MS assay for the quantitation of PTH-RM in rat plasma is presented. The assay was qualified and demonstrated to be specific, linear, precise and accurate for the determination of PTH-RM.

[1] Krishnan V *et al*. British Journal of Pharmacology (2018) 175 262–271.

METHOD

SAMPLE PROCESSING

- 25µL rat plasma spiked with PTH-RM
- Isotopically labelled PTH-RM internal standard
- Add antibody-conjugated streptavidin magnetic beads
- Agitate for 2 hours at room temperature
- Wash beads twice in PBS on KingFisher™ Flex
- Release beads in 100 µL ammonium bicarbonate
- Digest with trypsin for 90 minutes at 37°C
- Acidify the samples
- Analyze by LC-MS/MS

LC-MS/MS DETECTION

The digested peptides were separated on a Waters XBridge Peptide BEH C18 column (50 x 2.1 mm, 3.5 µm) using gradient elution with mobile phases of 0.025% acetic acid in water and acetonitrile.

The surrogate peptide SVSEIQLJHNLGR (m/z 489.3 > 640.4) and internal standard SVSEIQLJHNLGR[$^{13}C_6$,$^{15}N_4$] (m/z 492.6 > 645.4) were monitored by MRM on a SCIEX Triple Quad 5500.

RESULTS

SURROGATE PEPTIDE SELECTION

Due to the homology of PTH-RM with the N-terminal sequence of both human (85% identity) and rat (79% identity) parathyroid hormones, sequence analysis had to be carefully performed to ensure assay specificity (**Figure 1**).

Unique PTH-RM tryptic peptides were identified by *in silico* digestion and searched against protein databases. Candidate peptides, generated using Skyline, were verified using using a PTH-RM tryptic digest and a generic LC-MS/MS assay.

Peptide SVSEIQLJHNLGR (J = Norleucine) was selected as surrogate peptide for PTH-RM quantitation based on sensitivity and other considerations, such as peptide length, amino acid composition and chromatographic behavior.

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PTH-RM      ------------------------------SVSEIQLJHNLGRHLASJERVEWLRKLLQ 29
PTHY_HUMAN  MIPAKDMAKVMIVMLAICFLTKSDGKSVKKRSVSEIQLMHNLGKHLNSMERVEWLRKKLQ 60
PTHY_RAT    MMSASTMAKVMILMLAVCLLTQADGKPVKKRAVSEIQLMHNLGKHLASVERMQWLRKKLQ 60
                                  :****** ****:** * **::**** **

PTH-RM      DVHNF-------------------------------------------------------  34
PTHY_HUMAN  DVHNFVALGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ      115
PTHY_RAT    DVHNFVSLGVQMAAREGSYQRPTKKEENVLVDGNSKSLGEGDKADVDVLVKAKSQ      115
            *****
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Figure 1. Sequence alignment of PTH-RM, human parathyroid hormone and rat parathyroid hormone. PTH-RM PEGylation site is highlighted in grey. PTH-RM surrogate peptide is underlined (J = Norleucine).

SAMPLE EXTRACTION

Two approaches were evaluated for sample processing: 1) Selective protein precipitation leveraging the improved solubility granted by the PEG moiety, 2) Immunoaffinity purification using anti-PEG or anti-hPTH(1-34) antibodies.

The anti-hPTH(1-34) antibody demonstrated optimal results in terms of recovery (70%) and specificity and was selected for qualification of the assay. The immunoaffinity purification and trypsin digestion steps were characterized and optimized to allow processing of up to two 96-well plates in a day.

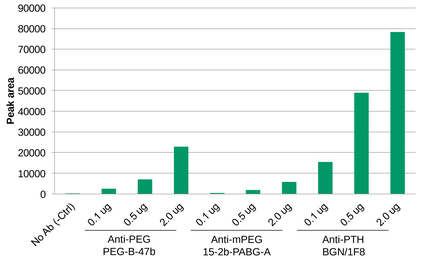

Figure 2. Immunoaffinity purification of PTH-RM in rat plasma using commercially available anti-PEG and anti-PTH capture antibodies.

RESULTS (CONTINUED)

ASSAY QUALIFICATION

For the qualification of this hybrid LBA-LC-MS/MS assay, criteria of accuracy (±20%; ±25% at LLOQ) and precision (≤20%; ≤25% at LLOQ) were used. A corresponding [$^{13}C_6$,$^{15}N_4$]-Arg labeled PTH-RM analog was synthesized and used as internal standard.

The assay is linear (weighted 1/x regression), precise and accurate within an analytical range of 0.200 to 100 ng/mL. Representative calibration curve and inter-day (n = 3) precision and accuracy are shown in **Figure 3** and **Table 1**, respectively. Representative chromatograms of blank and LLOQ samples are shown in **Figure 4**.

Matrix effect was assessed by using 6 independent lots of matrix (including 2% hemolysis) for the preparation of each of the low and high QC samples. Three replicates of the low and high QC samples were assayed for each matrix lot along with a calibration curve. Results are presented in **Table 2** and demonstrate acceptable lot-to-lot variability.

Assay qualification evaluations and PTH-RM stabilities in biological matrices, extracts and solutions are summarized in **Table 3** and **Table 4**.

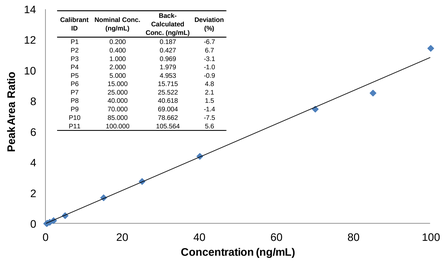

Figure 3. Calibration curve for PTH-RM in rat plasma from 0.200 to 100 ng/mL. Linear 1/x weighted regression with r^2 = 0.9974.

Table 1. Inter-Day (n=3) Precision and Accuracy for PTH-RM in Rat Plasma.

	Concentration (ng/mL)			
	LOQ QC 0.200 ng/mL	Low QC 0.600 ng/mL	Mid QC 20.0 ng/mL	High QC 75.0 ng/mL
Mean	0.210	0.569	20.7	76.7
S.D.	0.030	0.049	0.774	4.50
N	18	18	18	18
% CV	14.1	8.7	3.7	5.9
% Nominal	104.9	94.8	103.5	102.3

RESULTS (CONTINUED)

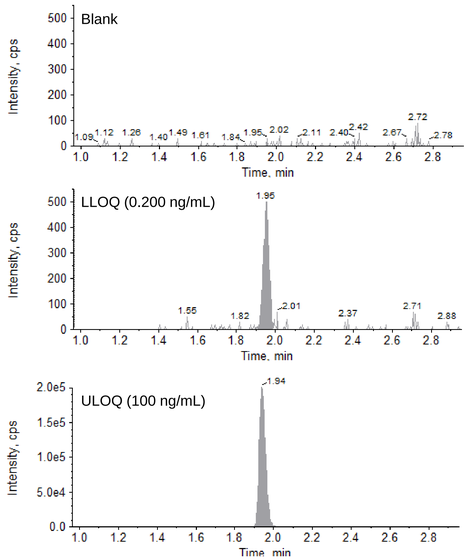

Figure 4. Chromatograms of an extracted blank (top), PTH-RM LLOQ (0.200 ng/mL, middle) and PTH-RM ULOQ (100 ng/mL, bottom). Surrogate peptide SVSEIQLJHNLGR is monitored (m/z 489.3/640.4).

Table 2. Matrix Effect (Lot-to-Lot Recovery) of PTH-RM in Rat Plasma.

Plasma Lot	Low QC (0.600 ng/mL)			High QC (75.0 ng/mL)		
	Calculated (n=3)	%CV	%Dev	Calculated. (n=3)	%CV	%Dev
Lot 1	0.565	10.6%	-5.7	71.2	3.0%	-5.1
Lot 2	0.550	4.2%	-8.4	76.2	2.9%	1.7
Lot 3	0.591	8.1%	-1.6	71.1	1.2%	-5.2
Lot 4	0.598	0.8%	-0.4	72.2	0.6%	-3.8
Lot 5	0.563	12.7%	-6.3	71.3	4.5%	-5.0
2% Hemolyzed	0.590	3.4%	-1.7	74.8	2.2%	-0.2

RESULTS (CONTINUED)

Table 3. PTH-RM Assay Qualification Summary.

Evaluations	Results
Specificity	6 lots including one lot of 2% hemolyzed plasma 100% specificity for analyte and internal standard
Matrix Factor (IS normalized)	6 lots including one lot of 2% hemolyzed plasma Low QC (n =18): 0.9852, %CV 4.6 High QC (n =18): 0.9960, %CV 1.3
Dilution Integrity	2x ULOQ, 5-fold diluted to 40.0 ng/mL Bias -1.2%, %CV 2.4
Percent Extraction Yield	Low QC: 73.3% High QC: 69.1%

Table 4. Stability of PTH-RM in Biological Matrices, Processed Samples and Solutions.

Evaluations	Duration	Results
Whole Blood (wet ice)	2 hours	Low QC: Bias 3.8%, %CV 4.1 High QC: Bias -0.7%, %CV 4.8
Combined Stability (-80°C→wet ice)	8 days 3 F/T cycles 12 hours thawed	Low QC: Bias 13.1%, %CV 7.0 High QC: Bias -0.1%, %CV 8.3
Benchtop (RmT°)	6 hours	Low QC: Bias -9.2%, %CV 1.5 High QC: Bias -4.9%, %CV 6.0
Extracted Sample (4°C)	4 days	Low QC: Bias -11.5%, %CV 3.5 High QC: Bias 5.2%, %CV 1.8
Solution Stability 0.5% BSA in PBS (4°C)	8 days	Low Conc:. Bias -2.5%, %CV 3.3 High Conc.: Bias 2.1%, %CV 3.6

CONCLUSION

A hybrid LBA-LC-MS/MS method for the quantitation of PTH-RM in rat plasma was successfully qualified. The assay was demonstrated to be linear, specific, precise and accurate. Stability evaluations indicate that PTH-RM is stable in biological matrices (whole blood, plasma) and solutions.

The assay performance is acceptable for the analysis of non-GLP/exploratory samples. The assay is currently being applied for the assessment of PTH-RM pharmacokinetic parameters in rats.

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