Measurement of Osteoanabolic Agents PTH (1-34) and PTHrP (1-36) in Therapeutic Studies and Clinical Diagnosis

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Introduction

Teriparatide, a recombinant human PTH (1-34) is an osteoanabolic agent for treatment of osteoporosis. PTH (1-34) can also be used a replacement therapy in hypoparathyroidism and to accelerate fracture healing. Abaloparatide, PTHrP (1-34) analogue is a novel anabolic drug for treatment of osteoporosis. Measurement of plasma PTH (1-34) has also been used to assess response to PTH in conditions such as pseudohypoparathyroidism (PHP) (Ellsworth-Howard test (EHT)).

Aims and Objectives

- To review the use of PTH (1-34) measurements in drug development studies, and in the diagnosis of patients with PHP.
- To highlight the potential use of measurement of PTHrP (1-36) using our LC-MS/MS method for measurement of intact PTHrP (1-36), intact PTH (1-34) and its respective oxidized forms simultaneously.

Study Design and Method

Sample Collection

- PTH (1-34) was analysed in EDTA plasma obtained from human subjects given either single subcutaneous (sc) injection of 20 µg Teriparatide (n=10) or 0.69 mg (n=5), 2.07 mg (n=10) oral PTH (1-34) (EnteraBio).
- Baseline samples were taken immediately before drug administration.
- Post-dose blood samples were collected every 15 minutes for two hours then hourly for three hours (time course 0-300 min).
- Ellsworth-Howard test procedure was carried on a patient with suspected PHP.
- Urine cAMP and PO_4 were analysed on samples voided every 30 min for 3 hours post PTH (1-34).

Sample preparation

200 µL EDTA plasma + (I.S.) + 500 µL 95:5 (v:v) H_2O:NH_4OH In Lo-Bind 1.5 mL tube/2 mL 96-well plate

(Waters Oasis® HLB µElution 96-well plate)

Wash plate with 200 µL 60:40 (v:v) H_2O:MeOH

Elute with 2 x 25 µL 70:30:0.2 (v:v:v) (ACN:H_2O:FA) and collect eluent

LC-MS/MS

Analytes separation and detection

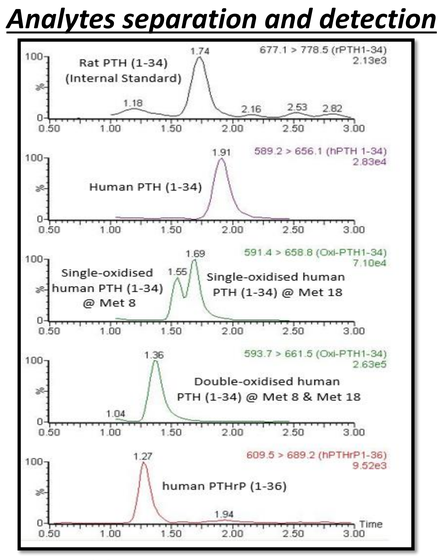

Figure (1). Chromatograms showing the separation of human PTH (1-34) and its respective single- and double-oxidised forms from human PTHrP (1-36) as well as the internal standard rat PTH (1-34) fragment.

PTHrP (1-36) Assay Validation

- Linear calibration curve from 25 to 2000 pg/mL
- Typical linear regression analysis (r^2 = 0.968)
- Lower limit of Quantification (LLoQ): 25 pg/mL
- Lower limit of detection (LLoD): 2.5 pg/mL

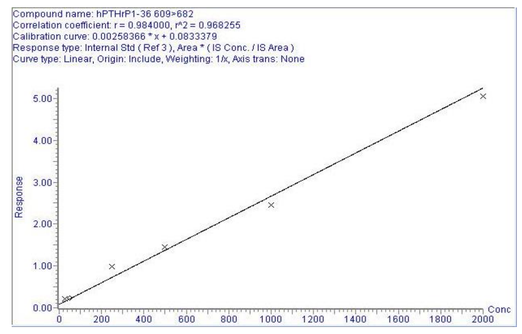

Figure (2). Typical calibration curve for hPTHrP (1-36) spiked into charcoal-stripped human EDTA plasma. R^2 value is 0.968.

Imprecision :

Stock of hPTHrP (1-36) calibrators and controls were prepared in our laboratory by spiking high purity (>98.0%) recombinant hPTHrP (1-36) (Creative BioMart, NY 11967, USA) in charcoal-stripped rat EDTA plasma. Intra-imprecision profile was generated by running all QC samples 10 times within a single run, while inter-imprecision profile was generated by repeated measurements (n=10) of all QCs over a period of a month.

* %Accuracy = $[100 - (\frac{100}{n}\sum\frac{Actual - Measure}{Actual})]$

QC level (pg/mL)	Inter-assay imprecision (n=10)					Intra-assay imprecision (n=10)				
	Mean	SD	SE	%CV	%Accuracy	Mean	SD	SE	%CV	%Accuracy
QC1 (50)	51.9	5.6	0.6	10.8	100	52.5	6.5	0.7	12.4	100
QC2 (100)	97.5	11.5	1.2	11.8	100	101.5	10.0	1.0	9.9	100
QC3 (200)	211.9	16.8	1.7	7.9	100	203.5	15.6	1.6	7.7	100
QC4 (800)	803.9	47.4	4.7	5.9	100	822.5	57.3	5.7	7.0	100

Recovery efficiency:

Endogenous PTHrP (1-36) (pg/mL)	Spiked (pg/mL)	Expected concentration (endogenous + spiked) (pg/mL)	Mean (±SEM) measured PTHrP (1-36) (pg/mL)	%Recovery Mean (%CV)
50	50	100	113.7 (±7.7)	113.7 (±27.1)
50	500	550	567.4 (±5.1)	103.2 (±3.6)
400	50	450	435.3 (±9.8)	96.7 (±9.6)
400	500	900	951.5 (±15.5)	105.7 (±6.5)
800	50	850	877.4 (±22.4)	103.2 (±10.2)
800	500	1300	1297.1 (±20.6)	99.8 (±6.4)

Oxidation of PTH (1-34) and PTHrP (1-36)

- Oxidation of the sulphur atom in methionine residues by peroxides is one of the major degradation pathways of therapeutic peptides. PTH (1-34) contains two methionine groups at position 8 (Met8) and position 18 (Met18).
- Three oxidized PTH (1-34) products were isolated, namely Met8 sulfoxide, Met18 Sulfoxide, and both positions Met Sulfoxide
- Oxidation of the methionine residues causes a change in the secondary structure of PTH (1-34), especially oxidation of Met8. The change in the secondary structure is greater when both methionine residues are oxidised.
- Double oxidized forms of PTH (1-34) possess reduced biological activity, which consequently reflected on the potency of the treatment
- In contrast to human PTH (1-34), human PTHrP (1-36) peptide does not contain methionine residue in its structure. We found that PTHrP (1-36) is not oxidised by hydrogen peroxide (H_2O_2).
- Our data showing that oxidation contributes by (23.9 ± 6.1%) to bias between our LC-MS/MS method for PTH (1-34) and immunoassay results.
- Due to the absence of methionine residues in human PTHrP (1-36) and analogues of hPTHrP (1-34) such as Abaloparatide they are resistant to oxidation, hence this may explain some of the difference in efficacy observed in Abaloparatide preclinical/clinical studies. However, further investigations are required to confirm this possibility.

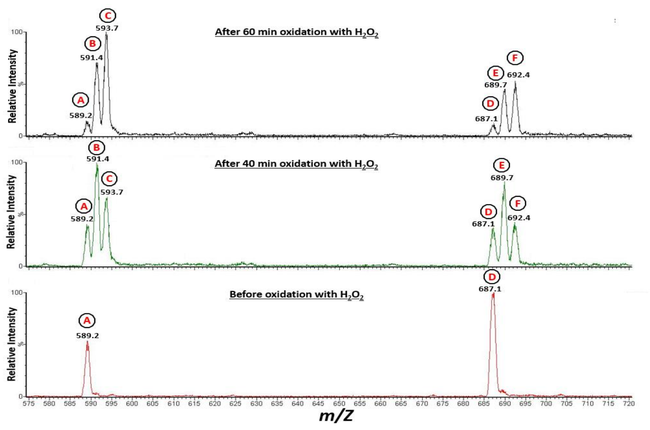

Figure (3). MS spectrum of human PTH (1-34) (MW = 4117.8 D) before and after 40 and 60 min oxidation with H_2O_2. A & D represent +7 and +6 charged state of non-oxidised hPTH (1-34) respectively. B & E represent +6 and +7 charged state of hPTH (1-34) oxidised at Met 8 or Met 18 respectively (single-oxidised form with an increase of 16 mass units). C & F represent +6 and +7 charged state of hPTH (1-34) oxidised at both Met 8 and Met 18 respectively (double-oxidised form with an increase of 32 mass units)

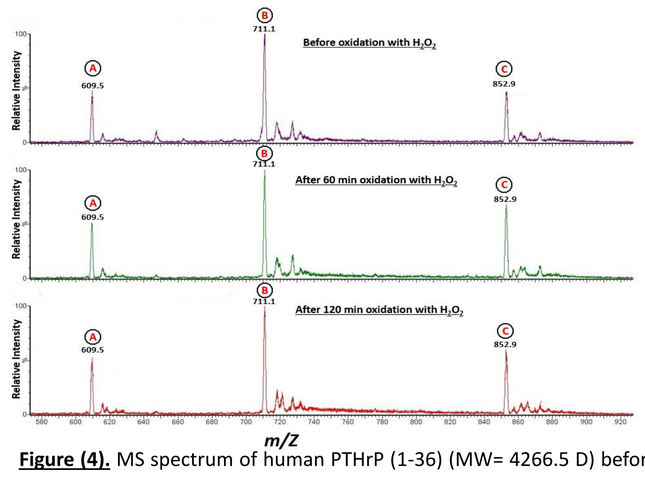

Figure (4). MS spectrum of human PTHrP (1-36) (MW= 4266.5 D) before and after 60 and 120 min oxidation with H_2O_2. A, B and C represents +7, +6, and +5 charged states precursor species of hPTHrP (1-36). No peaks for oxidised forms of PTHrP (1-36) observed even after 2 h oxidation with 0.1 M H_2O_2 due to absence of Methionine residues in its structure.

The Use of PTH (1-34) Measurement in Pharmacokinetics Studies

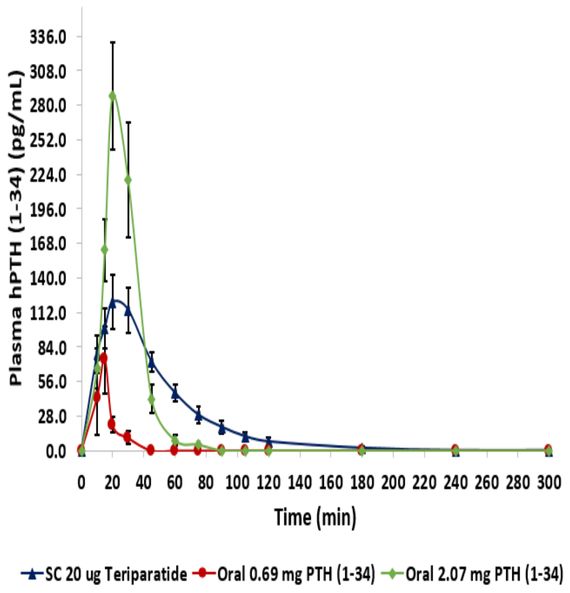

Figure (5). Concentration-time profiles of participants treated with 20 µg teriparatide, and oral PTH (1-34) (0.69 and 2.07 mg). Time course of samples collection was 0-300 minutes. Each point represent (mean±SEM) of plasma PTH (1-34) concentration.

Table: PK parameters for PTH (1-34) of 20 µg subcutaneous Forsteo® injection and oral (0.69 and 2.07 mg) administration.

Treatment	N	C_{max} (pg/mL) (Geometric mean ± SEM)	$AUC_{0\text{-}last}$ (pg.h/mL) (Geometric mean ± SEM)	T_{max} (min) (Geometric mean ± SEM)	$T_{1/2}$ (h) (Geometric mean ± SEM)
Forsteo® 20µg sc injection	10	124.5 ± 21.3	105.7 ± 9.3	16.0 ± 2.5	37.7 ± 6.8
Oral 0.69 mg hPTH (1-34)	5	56.2 ± 27.4	11.5 ± 7.4	14.7 ± 1.4	11.3 ± 4.8
Oral 2.07 mg hPTH (1-34)	10	267.8 ± 43.0	109.0 ± 19.2	20.8 ± 1.0	12.6 ± 2.6

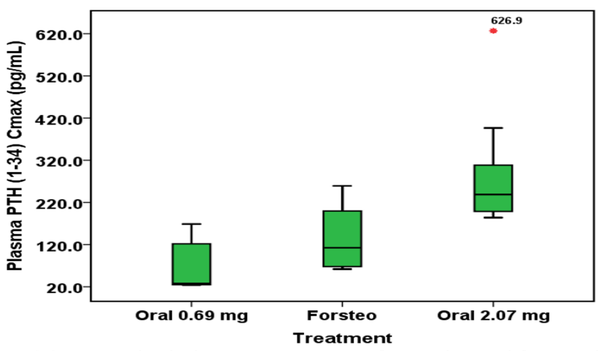

Figure (6). Box and Whisker representation of C_{max} obtained for standard Forsteo® injection and oral (0.69 and 2.07 mg) administration. Asterisk represents outlier (C_{max}= 626.9 pg/mL) recorded for one participant given 2.07 mg oral PTH (1-34) dose. Forsteo's C_{max} is bracketed by the two oral doses C_{max} . The C_{max} of oral treatment is proportional to dosage.

The Use of PTH (1-34) Measurement in the Diagnosis of PHP

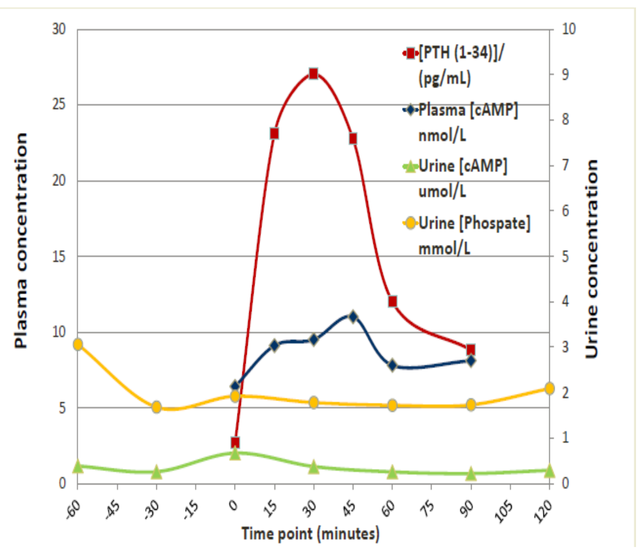

Figure (7). Ellsworth-Howard test results on a patient suspected of PHP. Note the blunted plasma cAMP response and the lack of urine cAMP excretion despite the sharp increase in plasma PTH (1-34) level. Phospateuric response is also deficient.

PHP disorders are characterized by impaired signalling of various hormones (mainly PTH) that activate cAMP-dependent pathways via $G_s\alpha$ protein. Ellsworth-Howard test or PTH loading test has been used traditionally to confirm PHP. Measurement of serum and urinary cAMP concentrations after the injection of exogenous PTH plus PO4 measurement confirmed the diagnosis of PHP type 1 (PHP1), in which a blunted cAMP response is observed, from PHP type 2 (PHP2) in which the cAMP response to PTH is conserved but the phosphaturic response is deficient.

Conclusions

Our method for measurement of non-oxidised and oxidised forms of PTH (1-34) as well as for PTHrP (1-36) may:
1) offer new insights into the physiology and pathophysiology of PTH
2) help investigate the therapeutic use/efficacy of osteoanabolic agents
3) help in development of combination therapy with other anti-resorptive/ anti-remodelling agents.