Characterization of intact Parathyroid hormone and its fragments by Liquid Chromatography coupled to High-Resolution Mass Spectrometry

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Introduction

- Parathyroid hormone (PTH) is a 84 amino acids peptide synthetized by the parathyroid glands (PTGs). Intact PTH (PTH 1-84) plays an important role in the calcium phosphate metabolism (1).
- PTH 1-84 is routinely quantified for the diagnosis of primary and secondary hyperparathyroidism (2).
- In addition to PTH 1-84, various fragments can be detected in plasma samples and can represent up to 80% of circulating PTH.
- The exact role of these fragments is still unclear but these species are known to be high in abundance in the case of chronic kidney disease (CKD) (3).
- The goal of this study is to develop a LC-MS method using high resolution mass spectrometry (HRMS).

Material and methods

1) Peptides

Table 1: *List of peptides used in this study*

Species	Amino acid sequence	Monoisotopic non protonated mass (Da)
PTH 1-84	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALGAPLAPRDAGSQR PRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ	9418,96
PTH 28-84	LQDVHNFVALGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVL TKAKSQ	6175,25
PTH 34-77	FVALGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNV	4712,46
PTH 34-84	FVALGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ	5468,91
PTH 37-77	LGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNV	4395,29
PTH 37-84	LGAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ	5151,74
PTH 38-77	GAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNV	4282,21
PTH 38-84	GAPLAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ	5038,65
PTH 45-84	DAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ	4376,27

2) LC-MS conditions

- LC-MS experiments were performed using a SYNAPT XS mass spectrometer in positive ion mode coupled with a NanoAcquity UPLC system equiped with a Protein BEH C4 column (300Å, 1.7µm, 300µmx100 mm of Waters corporation). Acquisition was performed in full scan and TOF-MRM modes.
- Gradient conditions:
  *Flow: 15µL/min
  * Injection volume: 5 µL
  * Mobile phases:
  A: H_2O; 0.4% HCOOH; 5% DMSO
  B: Acetonitrile; 0.4% HCOOH; 5% DMSO

Time (min.)	%B
0	5
1	5
12.5	60
13	95
14	95
15	5
16	95
20	5

3) Immunocapture protocol

- On-beads Immunocapture protocol:

1. 0.5 mg beads (Biorad, SureBeads, Protein G) washes: 2x PBS and 1x PBS-Tween.
2. Antibody coupling (PTH N-term and PTH C-term from Abcam company): + 150µL (6.7µg/mL), incubation (10 min., 1200rpm, 25°C) followed by the 3 washing steps (same as above).
3. Sample incubation: +150µL at 5ng/mL sample ; 1x PBS wash and 1 $NaHCO_3$ 50mM washes and 1h incubation (1h; 1200rpm; 25°C).
4. Elution: +200µL (50% ACN; 0.1%FA) followed by 5 minutes incubation (1600rpm; 25°C).
5. Evaporation and reconstitution in 50µL of 10% ACN; 0.1%FA.

LC-HRMS development

- Figure 1 depicts the extracted chomatograms for all species from table 1.
- In all cases, most abundant charge state is considered.

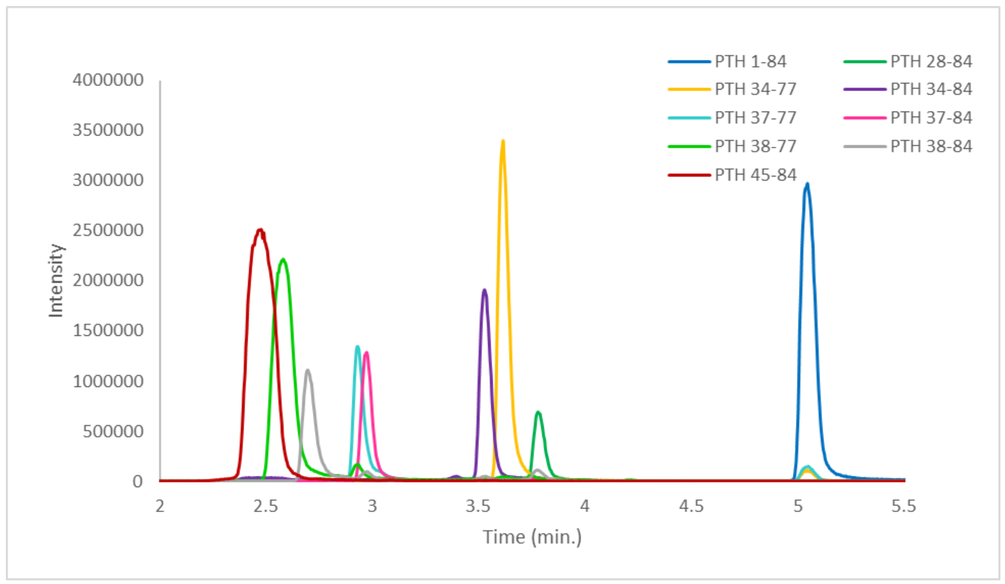

Figure 1: *Chromatograms of all PTH species represented in table 1. Data were obtained in full scan.*

- PTH 1-84 is clearly separated from all other peaks.
- Separation could be improved between PTH 37-77 and PTH 37-84.

TOF-MRM development

Table 2: TOF-MRM parameters developed in this study

Species	Parent ion (m/z)	Daughter ion (m/z)	Fragment identity	Collision Energy
PTH 1-84	725,56	770,43	$y_{82}^{[12+]}$	20 V
PTH 28-84	618,60	671,61	$y_{25}^{[4+]}$	19 V
PTH 34-77	674,18	745,37	$y_{42}^{[6+]}$	17 V
PTH 34-84	684,59	747,08	$y_{49}^{[7+]}$	19 V
PTH 37-77	628,88	714,02	$b_{40}^{[6+]}$	17 V
PTH 37-84	644,94	702,50	$y_{45}^{[7+]}$	20 V
PTH 38-77	714,68	834.00	$b_{39}^{[5+]}$	24 V
PTH 38-84	630,81	688,60	$y_{44}^{[7+]}$	22 V
PTH 45-84	730,35	992,82	$b_{27}^{[3+]}$	28 V

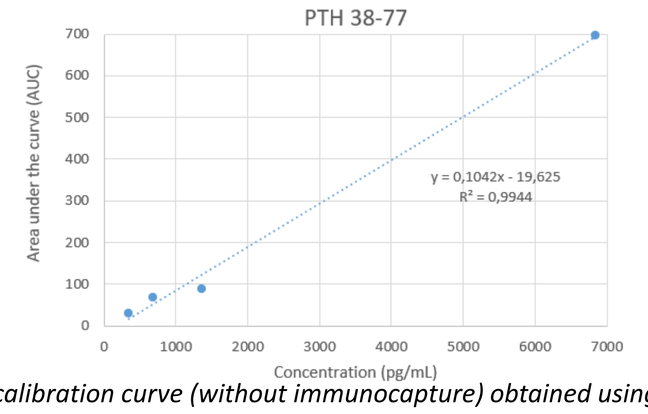

Figure 2: *PTH 38-77 calibration curve (without immunocapture) obtained using TOF-MRM detection.*

- Concentrations of about 5ng/mL (depending on the peptide) were successfully detected using TOF-MRM mode.

Immunocapture results

Table 3: *Fixation % of all species depending on the considered antibody*

Species	% of sample fixed on N-term Ab	% of sample fixed on C-term Ab	Concentration (ng/mL)
PTH 1-84	22,2	49,4	8,77
PTH 28-84	47,3	78,0	7,02
PTH 34-77	43,2	40,3	7,02
PTH 34-84	39,2	40,9	6,93
PTH 37-77	17,5	41,2	6,93
PTH 37-84	16,4	26,4	6,84
PTH 38-77	29,0	21,6	6,84
PTH 38-84	89,1	89,2	6,84
PTH 45-84	62,0	12,4	6,75

- Overall, C-term Ab provides higher fixation yield.
- PTH 38-84 seems the most fixed species while PTH 38-77 gives low results.

Conclusions and prospects

- One LC-HRMS method has been developed for the separation and the quantitation of 9 PTH species.
- Values around 5ng/mL can be detected with and without sample preparation.
- First immunocapture tests show globally better results with the C-term Ab.
- Labelled internal standard will be use to normalize results.
- Combination of TOF-MRM and full-scan mode could be usefull in the future.

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