Selective separation and sensitive detection of intact parathyroid hormone and its variants by CESI-MS/MS

Using a CESI 8000 Capillary Electrophoresis System coupled to a QTRAP® 6500 LC-MS/MS System

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Parathyroid hormone (PTH) is an 84 amino acid protein involved in calcium, phosphate and vitamin D metabolism. It is quantified to investigate parathyroid disorders and bone turnover in chronic kidney disease (CKD). Currently, PTH is measured using immunoassays targeting the amino acid sequence 1-34 (first primary sequence region) as PTH is metabolized into fragments with a conserved C-terminal sequence. These C- terminal fragments can have a longer half-life and can accumulate in patients suffering from CKD.[1] The fragments containing N-terminal amino acids can cross-react with immunoassays, leading to bias, particularly in late-stage CKD patients. The relationship between bone turnover and the 1-84 to 7-84 ratio was investigated and the latter might be a better predictor than PTH alone therefore the ability to selectively quantify each form is beneficial.[1,2]

In addition to selectivity issues, PTH immunoassays can suffer from reliability issues. Studies report discrepancies between results from different commercial assays.[3,4] These differences can be partially explained by the influence of matrix and

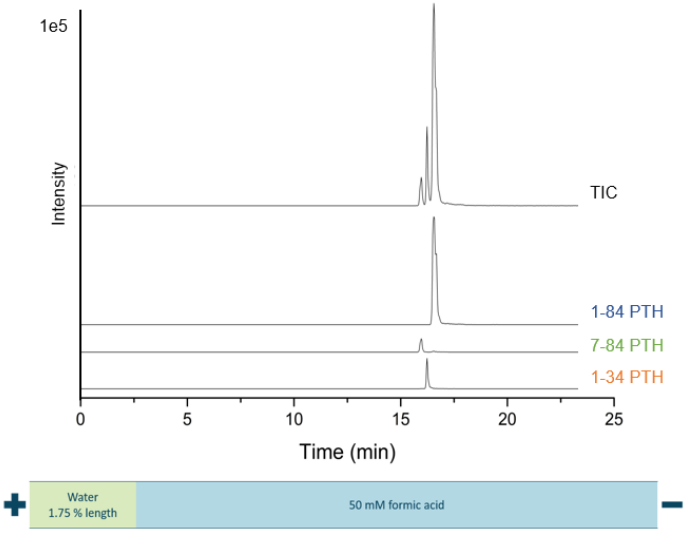

Figure 1. Typical electropherogram separation. Separation of a sample of 1-34 PTH, 7-84 PTH and 1-84 PTH (each at 100 ng/mL) in neutral FASI-CZE-MS/MS is shown.

preanalytical conditions, such as body weight and vitamin D status, and by unrelated compounds such as heterophile antibodies.[5] Finally the immunoassays also seem to react with oxidized PTH. Therefore the development of a more robust quantification method for PTH is needed.

Methods using LC-MS for PTH quantification have been developed but their sensitivity does not allow quantification in the concentration ranges reported in literature for human samples – 5 pg/mL. The LC-MS assays typically use tryptic digestion which results in loss of structural information. Here, the development of a CESI-MS/MS method is described, to detect low levels of intact PTH protein and closely related variants.[5]

Key features of the CESI-MS assay for sensitive detection of PTH variants

- Sheathless CESI-MS assay combines the resolving power and low-flow ESI sensitivity of CE with the specificity of MS analysis for the quantification of PTH in its native conformation in the presence of other related forms
- Intact protein analysis enables the differentiation of variants or the determination of protein modifications
- Separation of 1-84 PTH, 7-84 PTH and 1-34 PTH by CESI-MS was demonstrated at detection levels down to low pg/mL concentration

Methods

Sample preparation: Lyophilized proteins were dissolved using either an aqueous solution that contains 10% (v/v) acetonitrile and 25 mM formic acid, or LC-MS grade water. The stock solutions were aliquoted into Protein LoBind Eppendorf and stored at -80° C until use. Samples were prepared by diluting the stock solutions with 50% (v/v) acetonitrile /water.

Capillary electrophoresis coupled to mass spectrometry: Experiments were conducted on a CESI 8000 ESI-MS-High Performance System coupled to a QTRAP 6500 System through a NanoSpray® III Ion Source modified for use with OptiMS cartridges. Separations were performed on Neutral OptiMS Cartridges ((30 µm ID x 91 cm) at a temperature of 20°C.

The neutral cartridges were initially conditioning steps with 0.1 M HCl (90 psi, 12 min) and water (90 psi,12 min) which were repeated followed by an overnight water rinse (5 psi). At the end of every batch the same procedure was used for cleaning the capillaries. In-between runs, the capillary was rinsed with 0.1 M HCl, water and then background electrolyte [(BGE) 90 psi for 3 minutes]. The BGE made up of 50 mM formic acid was used, giving rise to a current of 3 µA. Samples were introduced by Electrokinetic Injection (EKI) (+10 kV for 100 sec) from a sample in acetonitrile/water 50:50 (v/v) into a previously injected water plug utilizing a Field Amplified Sample Injection (FASI) injection technique:

- 0.4 psi for 48 sec (equivalent to 0.5% of the capillary volume) for experiments using Electrokinetic Supercharging (EKS)
- 1.75% (5 psi for 15 sec) for experiments using Field Amplified Sample Injection (FASI) and no separation pressure

In runs involving electrokinetic supercharging (EKS), a 10% plug of 50 mM NH_4Ac was injected hydrodynamically (4 psi for 96 sec) before the water plug. Separation voltage was set to +30 kV with a 2 min ramp up and 5 min ramp down at the end of the run. A pressure of 3 psi (inlet and outlet) was applied in EKS-CZE runs, and FASI-CZE runs were performed without pressure. A pressure of 90 psi was applied during ramping down to flush the capillary. Table 1 outlines the peptides monitored.

Detection of PTH by MS was performed in multiple reaction monitoring (MRM) mode. Ionization voltage, MRM transitions, MS and source parameters were determined using a separation buffer enriched with the target compounds while the CE separation voltage and pressure were applied to the sample. The MRM transitions and parameters are shown in Table 2. For all experiments, the MS source temperature was set at 50°C, and gases were turned off except for the curtain gas (5 psi). The position of the capillary tip relative to the MS entrance was optimized prior to sample analysis to maintain signal intensity and stability.

Table 1. Molecular weight and theoretical isoelectric point of the compounds of interest.

Compound	Molecular weight (Da)	Theoretical isoelectric point
1 – 84 PTN	*9424.73*	*9.10*
7 – 84 PTH	*8781.03*	*9.46*
1 – 34 PTH	*41177.76*	*8.29*

Table 2. MRM transitions.

Compound	Q1 (m/z)	Q3 (m/z)	CE (V)
1 – 84 PTN	*857.7 [M+11H]+11*	*924.9 y82+10*	*36*
7 – 84 PTH	*799.3 [M+11H]+11*	*872.0 y49+6*	*35*
1 – 34 PTH	*824.4 [M+5H]+5*	*983.9 y23+4*	*39*

Optimization for separation and sensitivity

The neutral coated capillary was chosen to reduce interactions between proteins and the capillary wall. In FASI, a water plug was injected before performing the Electrokinetic Injection (EKI) and the sample medium was made up of a mixture of acetonitrile and water (50:50, v/v). The high acetonitrile percentage in the sample medium was used to decrease PTH wall adsorption but also to perform conductive stacking as the sample solvent was less conductive than the plug in the capillary. Under these conditions, the three compounds, each at 100 ng/mL, could be detected and separated (Figure 1).

In order to further improve the sensitivity, the use of EKS (Electrokinetic supercharging) was also investigated. EKI is performed after the HDI (HydroDynamic Injection) of NH_4Ac and water plugs, allowing t-ITP (transient IsoTachoPhoresis) to occur. To achieve this, a 10% plug of 50 mM NH_4Ac was injected before the water plug. Under these conditions, there was no signal anymore for 7-84 PTH but concentrations as low as 100 pg/mL could be detected for 1-84 PTH and 1-34 PTH, with S/N values equal to 13 and 27, respectively (Figure 2). It is worth noting that the fragmentation pattern of 7-84 PTH is different compared to the two other compounds. The estimated LOQ (calculated considering a S/N ratio of 10) for 1-84 PTH and 1-34 PTH were found to be 77 pg/mL and 37 pg/mL, respectively, while the estimated LOD (calculated considering a S/N ratio of 3) were found to be equal to 25 pg/mL and 10 pg/mL, respectively.

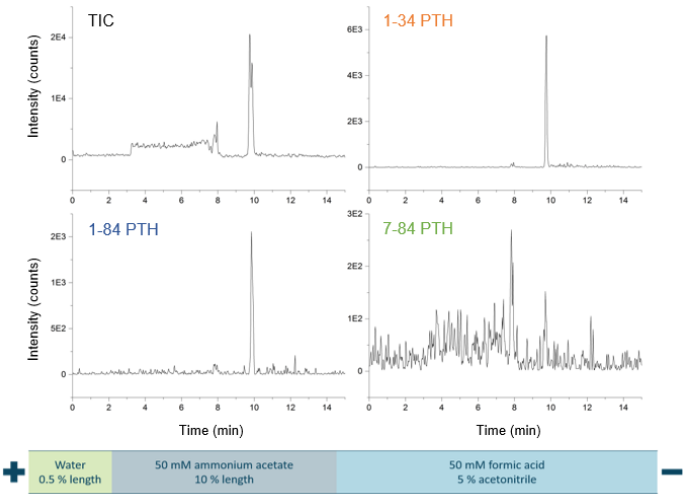

Figure 2. Typical electropherograms of PTH forms using EKS. A sample of 1-34 PTH, 7-84 PTH and 1-84 PTH (each at 100 pg/mL) in neutral EKS-CZE-MS/MS is shown.

Conclusions

- Coupling CE with MS by sheathless CESI-MS is very promising for the quantification of intact PTH and its variants
- By using neutral coated capillary and EKS as preconcentration method, LOQs in the range of 37 to 77 pg/mL were observed for PTH
- MS/MS detection provides method selectivity regarding oxidized PTH and other forms
- This work suggests the potential to reach the low pg/mL range required in biological samples, with further optimization of the sample preparation method

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