The Pathway Towards a Reference Measurement Procedure for 1-84 Parathyroid Hormone.

An intact protein analysis approach by LC-MS/MS.

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ABSTRACT

The accurate measurement of parathyroid hormone (PTH) concentrations is of paramount importance, especially for patients with parathyroid gland disorders and for monitoring bone turnover in individuals with chronic kidney disease and mineral bone disorders. Two generations of PTH immunoassays (IA) are currently present on the market, presenting known cross-reactivity issues and a lack of standardization.

The aim of the present work was to develop an antibody-free LC-MS/MS method for the measurement of intact 1-84 Parathyroid Hormone (PTH), representing a significant step towards the elaboration of a reference measurement procedure (RMP).

The first part of the manuscript concerns the development of proof-of-concept LC-MS/MS method for intact glucagon analysis, based on a Solid-Phase Extraction (SPE) sample preparation.

The main part has its focus on the successful validation of an innovative LC-MS/MS approach for 1-84 PTH, calibrated using the International Standard for PTH (WHO 95/646). The presented method shall be suitable to be presented as a candidate RMP upon the availability of a better characterized higher-order reference material for PTH. Our method demonstrated unprecedented sensitivity and robustness to analyze intact PTH by a higher-order technique such as LC-MS/MS, offering a valuable tool for the long-needed standardization of PTH assays.

RÉSUMÉ

La mesure exacte des concentrations de l'hormone parathyroïdienne (PTH) est d'une importance primordiale, notamment pour les patients atteints de troubles des glandes parathyroïdes ainsi que pour le suivi des patients souffrant de maladie rénale chronique et de troubles minéraux osseux. Actuellement, deux générations de dosages immuno-enzymatiques (IA) de la PTH sont disponibles sur le marché, mais ces essais présentent des problèmes connus de réactivité croisée et un manque de standardisation.

L'objectif de cette étude était de développer une méthode LC-MS/MS sans utilisation d'anticorps pour la mesure de la PTH 1-84 intacte, ce qui représente une avancée significative vers l'établissement d'une procédure de mesure de référence (RMP).

La première partie du manuscrit porte sur le développement d'une méthode LC-MS/MS de preuve de concept pour l'analyse du glucagon intact, reposant sur une préparation d'échantillon par extraction en phase solide (SPE).

La partie principale de l'étude se concentre sur la validation d'une approche innovante par LC-MS/MS pour le dosage de la PTH 1-84, calibrée à l'aide du Standard International pour la PTH (WHO 95/646). La méthode présentée devrait être considérée comme une RMP potentielle dès que des matériaux de référence de plus haut niveau, mieux caractérisés pour la PTH, seront disponibles. Notre méthode a démontré une sensibilité et une robustesse sans précédent dans l'analyse de la PTH intacte par LC-MS/MS, offrant ainsi un outil précieux pour la standardisation tant attendue du dosage de la PTH.

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List of abbreviations

AAA	Amino Acid Analysis
AC	Alternate Current
ADL	Allowable Deviation from Linearity
APS	Analytical Performance Specifications
CaSR	Calcium-Sensing Receptor
CE	Collision Energy
CI	Confidence Interval
CID	Collision-induced dissociation
CKD	Chronic Kidney Disease
CRM	Certified Reference Material
CXP	Cell Exit Potential
DC	Direct Current
DIA	Hemodialyzed
DMSO	Dimethylsulfoxide
DT	Dwell Time
ECF	Extracellular fluid
EFLM	European Federation of Clinical Chemistry and Laboratory Medicine

ELISA	Enzyme-Linked immunosorbent assay
EP	Entrance Potential
EQC	External Quality Control
ESI	Electrospray Ionization
FDA	Food and Drug Administration
FGF-23	Fibroblast Growth Factor 23
GLP-1	Glucagon-Like Peptide-1
GLP-2	Glucagon-Like Peptide-2
GRPP	Glicentin Related Pancreatic Polypeptide
HETP	Height Equivalent to a Theoretical Plate
HPLC	High-Pressure Liquid Chromatography
HRMS	High-Resolution Mass Spectrometry
IA	Immunoassay
IFCC	International Federation of Clinical Chemistry
IGF1	Insulin-Like Growth Factor 1
IS	Internal Standard (or International Standard)
IVD	In-Vitro Diagnostics
JCTLM	Joint Committee for Traceability in Laboratory Medicine
K/DOQI	Kidney Disease Outcomes Quality Initiative

KDIGO	Kidney Disease Improving Global Outcomes
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLOQ	Lower Limit of Quantitation
LOQ	Limit of Quantification
MPGF	Major Proglucagon Fragment
MRM	Multiple Reaction Monitoring
MU	Measurement Uncertainty
OPG	Osteoprotegerin
ox	Oxidized
PTH	Parathyroid Hormone
PTH1R	PTH/PTHrP receptor type 1
PTM	Post-Translational Modification
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
RER	Rough Endoplasmic Reticulum
RIA	Radioimmunoassay
RM	Reference Material
RMP	Reference Measurement Procedure
RT	Retention Time
S/N	Signal-to-Noise Ratio

SD	Standard Deviation
SI	International System of Units
SPE	Solid-Phase Extraction
UHPLC	Ultra-High-Performance Liquid Chromatography
ULN	Upper Limit of Normal
UPLC	Ultra Performance Liquid Chromatography
VMR	Vitamin D Metabolite Ratio
WHO IS	World Health Organization International Standard
XIC	Extracted Ion Chromatogram

Chapter 1. Introduction to PTH

1.1 Parathyroid glands

1.1.1 Historical background

The discovery of the parathyroid glands is a relatively recent event, dating back approximately 150 years. When the first Indian rhinoceros (*Rhinoceros unicornis*) owned by the Zoological Society of London died in 1849, its carcass was offered to Sir Richard Owen (1804-1892), Professor and Conservator of the Museum of the Royal College of Surgeons of England. Owen undertook a thorough dissection and study of the animal over the next two years. In 1862 Owen published his account of the work (1), mentioning that he had observed "a small compact yellow glandular body attached to the thyroid gland at the point where the vein emerges". This was what we now know as the parathyroid gland.

Although Owen's statement is the first bibliographic reference to the parathyroid glands, it was not until 1880 that Ivar Viktor Sandström (1852-1889), a medical student at the University of Uppsala (Sweden), discovered and named the glands. He first reported the discovery of a small structure, barely the size of a hemp seed, in the thyroid gland of a dog, and later in other animals, including humans. He named them *glandulae parathyroidae*(2).

Sandström's discovery was overlooked until 1891 when Eugene Gley (1857-1930) identified the endocrine function of the parathyroid glands (3) and, although their central role in calcium metabolism was not yet appreciated, it was agreed that removal of all the parathyroid glands in cats, dogs or even rodents was associated with death from tetany. It was also shown that preserving small amounts of these glands resulted in higher survival rates and the avoidance of tetany.

In the same year that Gley published these findings, and without any apparent connection, Von Recklinghausen described "osteitis fibrosa cystica" (4), a condition in a patient who had suffered recurrent fractures of several bones with minimal trauma, and who subsequently showed "bending" masses of long bones and replacement of the supporting structures by fibrous tissue and cyst-like brown tumors. However, he was unaware of the link between this condition and excessive production of PTH.

It was not until 1925, after many decades of controversy about the role of the parathyroid glands, that the Nobel Prize winner Bertram Collip (5), using the extraction procedure recently developed by Hanson (6,7), described an analytical method for obtaining an "active extract" (PTH as we know it today) from the glands which could be injected into parathyroidectomised dogs to reverse tetany and even keep them alive for some months. He argued unequivocally that the active principle was trapped in the glands and that previous extractions had failed because the compound was not soluble by simple water extraction (as others had tested). In his seminal paper, he stated that "the normal function of the parathyroid gland appears to be related to [...] direct control of the calcium level in the blood.".

The modern era of parathyroid hormone research had just begun with the attribution of PTH's key role in calcium metabolism.

1.2 Parathyroid Hormone and its role in physiology of calcium and phosphate metabolism

1.2.1 Introduction

Parathyroid hormone (PTH) is a single-chain peptide of 84 amino acids produced by the parathyroid glands. Its main role is to maintain ionized calcium concentrations within a very narrow range and to participate in the urinary excretion of phosphorus, by direct actions on bone and kidney, and by indirect actions on the intestine by stimulating the synthesis of 1,25(OH)2 vitamin D.

Calcium and phosphate metabolism are presented in this section.

1.2.2 Calcium

In an average healthy individual weighing 70 kg there are approximately 25 mol of calcium (1000 g), 99% of which is found in the bones, with the remainder in the extracellular fluid. While calcium is of paramount importance for continuous bone remodeling, many cell and organ functions also depend on its tightly controlled extracellular concentration.

The difference between calcium absorption and excretion in the resting state of the body is called the global calcium balance. In brief, this balance is normally positive in children throughout their skeletal development and negative in the elderly, but should be close to zero in healthy adults, with urinary calcium excretion equal to intestinal absorption.

Approximately 500 mg of calcium are released into the bloodstream during bone remodeling in young adults. Equally, the mineralization of the newly formed bone matrix during the bone formation process also requires about 500 mg of calcium, which means that the calcium balance in bone remains neutral, similar to the general calcium balance. In a healthy adult with an elemental calcium intake of 1000 mg, only about a third is absorbed in the small intestine, both passively and under the influence of $1,25(OH)_2$ vitamin D. The intestinal calcium absorption is around 200 mg. Considering that bone mineral accretion of around 500 mg of calcium (which equals the amount released by bone resorption), a kidney excretion (under hormonal regulation) of around 200 mg, should be expected, rendering a neutral global balance (8)(8), as represented in Figure 1 below.

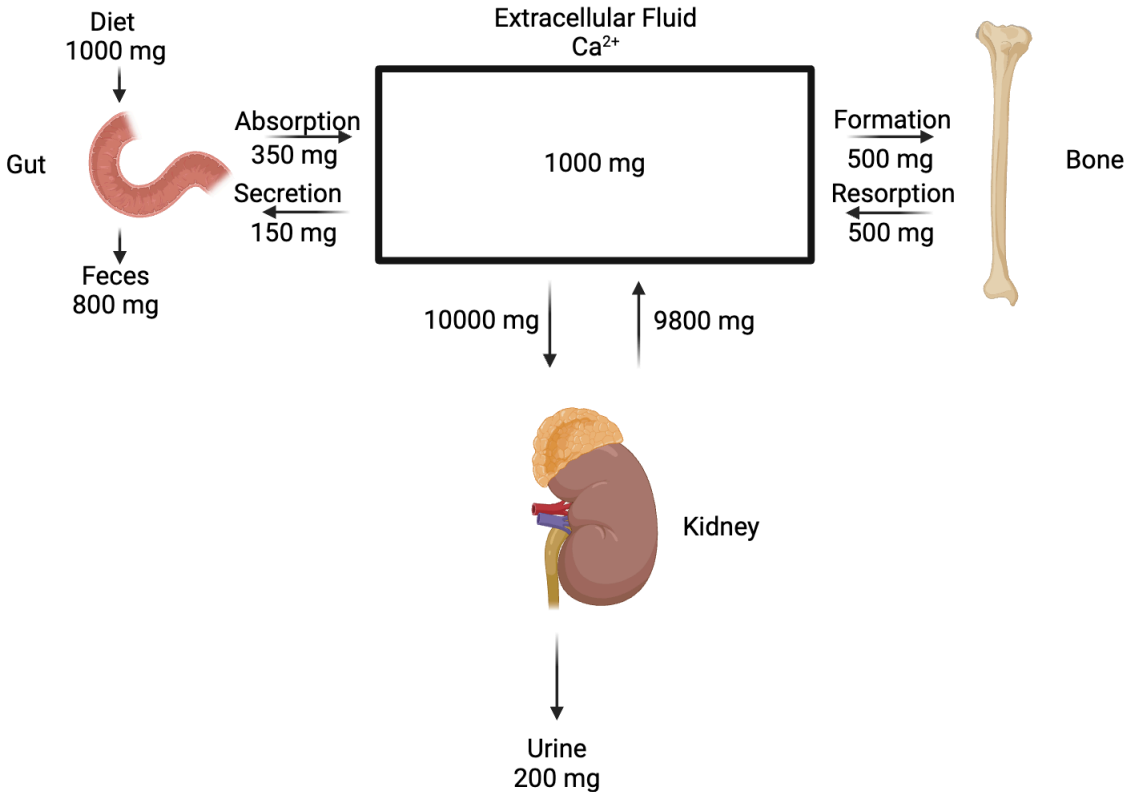

Figure 1. Calcium balance in a normal adult with a recommended intake of calcium.

Bones act as a storage compartment for calcium, which is readily mobilized to the extracellular fluid in its ionized and active form (Ca^{2+}) to carefully maintain blood concentrations between 1.12 – 1.32 mmol/L (9)(9). Since only the ionized calcium is considered "active", total blood calcium levels generally determined in clinical laboratories may not accurately reflect nutritional status, although some formulas have been developed to correct for this deviation with many known limitations (10,11).

1.2.3 Phosphate

Phosphate plays an essential role in signal transmission, cell metabolism, and the preservation of skeletal integrity. It is a required element for several biomolecules, such as ATP, cyclic AMP and the core of one of the most important post-translational modifications (PTMs), protein phosphorylation.

A healthy adult body contains up to 500 to 800 g of phosphate. Most of it (80%) is stored in the bone, the remainder being predominantly found in the intracellular fluid and about only 1% in the extracellular fluid (ECF). The average phosphate intake for an adult, on a Western diet, ranges from 800 to 1600 mg per day, mostly absorbed in the small intestine. Phosphate balance is represented in Figure 2.

While phosphate is a major intracellular anion, it is also present in plasma at concentrations, in adults around 0.81 to 1.45 mmol/L.

Similar to calcium, its balance is generally positive in children, null in adults and negative in elderly.

Ninety percent of the daily phosphate load is excreted by the kidneys, while the remaining ten percent is eliminated through the digestive tract. The glomerulus filters the majority of the phosphorus because it is not strongly bound to albumin. As a result, renal function has a great impact on its regulation, which is hormonally controlled mainly by PTH, 1,25$(OH)_2$ vitamin D, and fibroblast growth factor 23 (FGF-23). The complex interaction between intestinal phosphate absorption, renal phosphate processing, and the transcellular mobilization of phosphate between intracellular fluid and the bone storage pool regulate its serum levels (12,13).

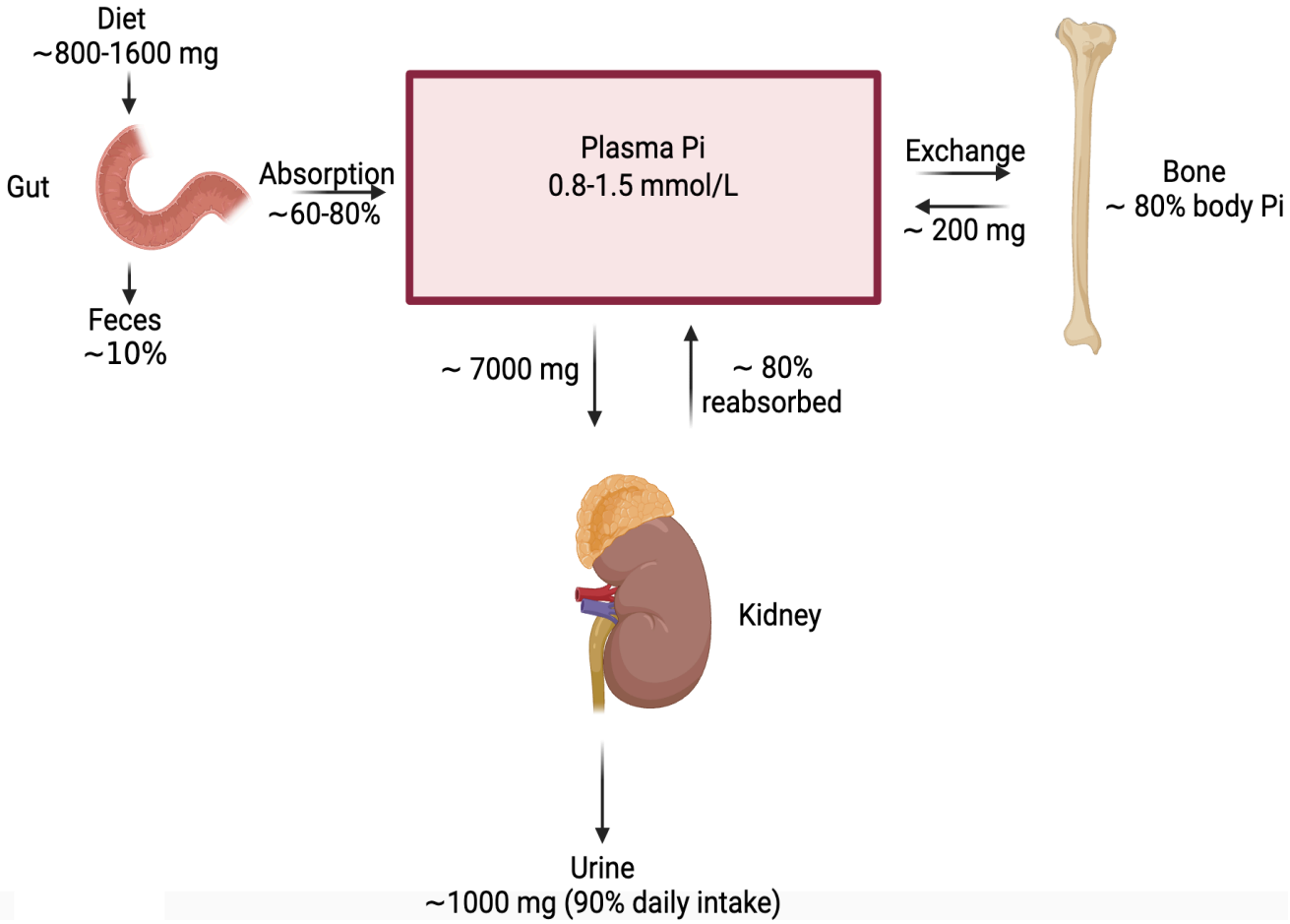

Figure 2. Phosphate balance in a normal adult with a recommended intake of phosphorous.

1.2.4 Calcium and Phosphate regulation

Calcium and phosphate metabolism involves four separate systems: the gastrointestinal system; the endocrine system, characterized by PTH and 1,25$(OH)_2$ vitamin D; the renal system, where both calcium and phosphate are filtered and reabsorbed under the

control of 1,25(OH)2 vitamin D; and the musculoskeletal system, with bone resorption and formation.

Calcemia is a remarkably stable parameter in healthy adults, with an intra-individual variation of <3% according to the EFLM biological variation database(14). Calcium-sensing receptors (CaSRs) in the parathyroid glands monitor changes in ionized calcium concentrations and subsequently vary the release of PTH, which acts to increase plasma calcium. Decreases in plasma calcium promote the production and release of PTH and vice versa.

As introduced above, phosphocalcic absorption occurs throughout the gastrointestinal tract, passively or actively under the stimulation of $1,25(OH)_2$ vitamin D. This active form of vitamin D results from the double hydroxylation of vitamin D (either obtained from the diet or produced after sun exposure). The first of these hydroxylations takes place in the liver by the action of 25-hydroxylase (CYP27B1), while the second occurs mainly in the kidney by 1-α-hydroxylase (CYP24A1), which is on the one hand stimulated by the presence of PTH, low calciemia, low phosphatemia and IGF1. And, on the other hand inhibited by FGF23. Notably, $1,25(OH)_2$ vitamin D also exerts a negative endocrine feedback on the secretion of PTH.

The storage role of the bone is crucial on this regulation. In bone remodeling, the two key roles are sustained by osteoblasts, which mobilize calcium and phosphorous into the bone for its formation, and osteoclasts, which are in charge of resorption, releasing calcium and phosphate from the bone. PTH exhibits both catabolic and anabolic effects on the skeleton. When bound to the receptors (PTH1R) present on osteoblasts, it induces the synthesis of the Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) and

inhibits the expression of its decoy receptor osteoprotegerin (OPG). Osteoclasts are stimulated through their RANK receptor, by the RANKL secreted by osteoblasts, starting their resorption function.

The length and frequency of PTH exposure determines whether an overall catabolic or anabolic effect on bone mass is observed. In vivo studies, show that continuous exposure to PTH increases mRNA encoding for RANKL and decreases mRNA encoding for OPG, leading to an increased RANKL/OPG ratio, which translates to an enhanced osteoclastogenesis with the associated bone resorption.

Intermitent PTH treatment shows an increase in bone turnover, however, a so-called "anabolic window" is observed, where bone formation is initially stimulated without resorption. This is explained, partly, by PTHs ability to downregulate the sclerostin expression in osteocytes, which triggers an anabolic response, a property used in clinical practice for osteoporosis treatment by means of PTH or analogues such as teriparatide. Thus, PTH plays an active role in both catabolic and anabolic pathways (15,16).

The last path of entry or exit for calcium is the kidney. About 99% of the ionized calcium is reabsorbed in the nephron, either passively in the proximal tubules and the ascending loop of Henle or actively in the distal nephron, under the influence of PTH. It is this reabsorption that is responsible for the physiologic regulation of calcium, as well as its dysregulation as observed, for instance, in hyperparathyroidism. On the other hand, phosphate is reabsorbed by the proximal tubule, which is blocked under the presence of PTH. The filtrate's residual calcium and phosphate are eliminated in the urine.

From a general point of view, it can be summarized that PTH increases serum calcium mainly through renal reabsorption, although gastrointestinal absorption and bone

resorption must not be overlooked. On the contrary, PTH acts to decrease serum phosphorous, since the increased renal excretion of phosphorous is more significant than its effects on gastrointestinal absorption and bone resorption, which are more accentuated by 1,25(OH)$_2$ vitamin D. PTH regulation is represented in Figure 3.

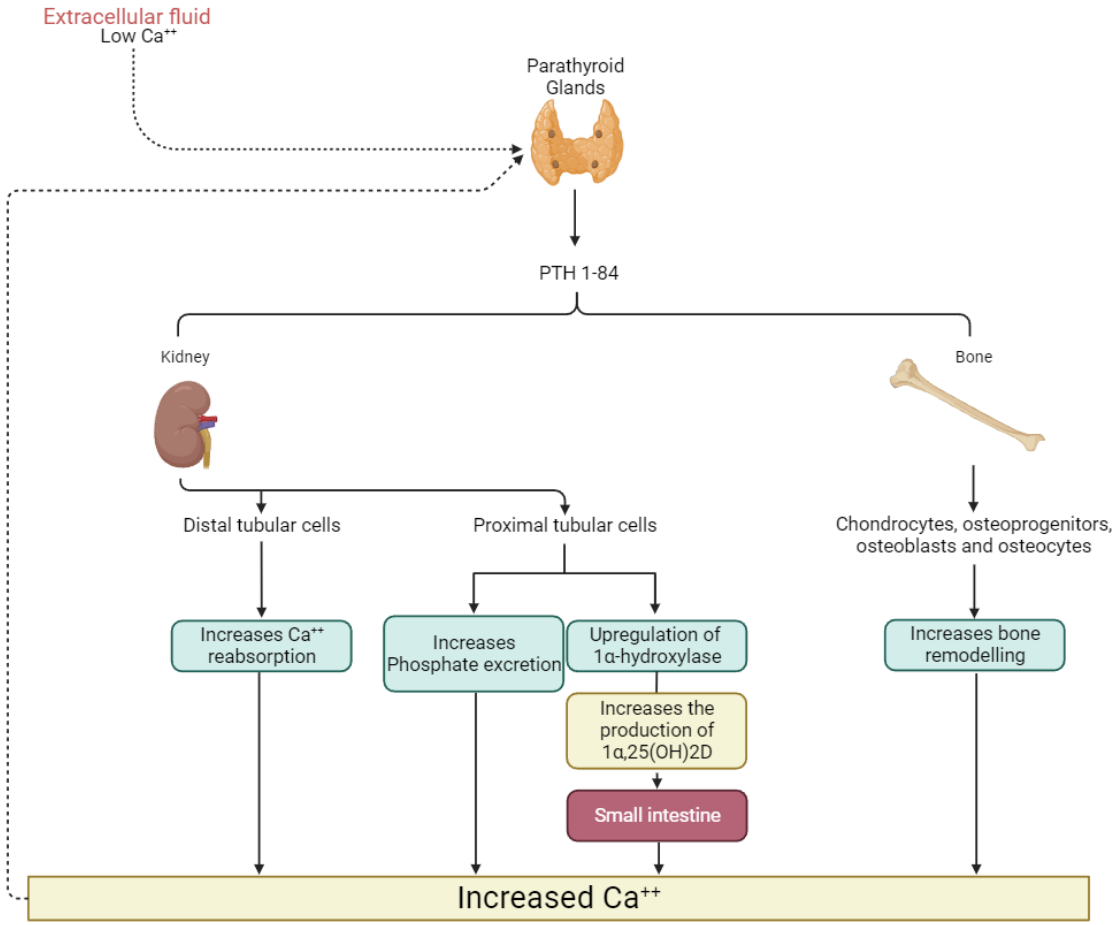

Figure 3. Regulation of the production of PTH mediated by a negative feed-back involving calcium, active vitamin D (calcitriol) and FGF-23. Adapted from: "The path to the standardization of PTH: Is this a realistic possibility? A position paper of the IFCC C-BM", Cavalier, E. 2021(17).

1.2.5 PTH Structure, Secretion and Metabolism

The gene responsible for PTH production in humans is found on chromosome 11's short arm (18). The chief cells in the parathyroid gland are the ones in charge of expressing this gene. The PTH mRNA transcript is translated into prepro-PTH, a 115 amino acid (AA) precursor peptide. Pre-pro PTH contains an amino-terminal, 25 AA pre-sequence (the signal peptide) and a short, subsequent 6 AA pro-sequence that translocate the precursor molecule to the rough endoplasmic reticulum (RER) and the Golgi network. When prepro-PTH enters the ER lumen, the signal peptide is cleaved, leading to Pro-PTH (90 AA), which is then transported to the Golgi network, and finally matured to the bioactive form of PTH (84 AA) (19).

It is of noted importance to highlight that the bioactivity of PTH resides in the NH_2-terminal region of the peptide (20).

The major physiological function of the parathyroid glands is to act as a “calciostat”, which senses the blood ionized calcium and adjusts the PTH secretion accordingly. Very small changes in Ca^{2+}, detected by the calcium-sensing receptors (CaSRs) allow for significant changes in PTH secretion, following a steep sigmoidal relationship. The overall secretory rate is mainly modulated by 6 variables: 1.- changes in the minute-to-minute secretion of preformed PTH; 2.- The rate of PTH degradation; 3.- The level of PTH gene expression; 4.- Parathyroid cellular proliferation; 5.- Parathyroid cellular apoptosis; 6.- Proportion of active and inactive secretory cells in the parathyroid gland(21). In the interest of this work’s clarity, it can be stated that PTH levels are mainly enhanced low Ca^{2+} concentrations and reduced by $1,25(OH)_2$ vitamin D and low serum phosphate levels (22)(21).

Intact PTH 1-84 is expected in circulation at relatively low concentrations ranging from around 5 up to 50 pg/mL (according to Fujirebio's assay) (23) with as relatively short half-life, ranging from 2 to 5 minutes. PTH is rapidly degraded in the liver by the Kupffer cells, leading to various NH_2-terminal truncated PTH fragments in circulation(24), traditionally believed to be 7-84 PTH (24). Post-translationally modified forms (PTMs) are also found in circulation, such as amino PTH (phosphorylated serine in position 17) and oxidized PTH (oxidation of methionines in positions 8 and 18). Similar to 7-84 PTH, oxidized PTH has been described historically although many recent works, including ours, raise serious doubts about its existence (25,26).

PTH 1-84 and its fragments, which present a longer half-life, are cleared through the kidneys, thus they accumulate in the blood of CKD patients (27), having an important impact on the analytical determination of the peptide, as detailed next.

1.3 PTH determination

1.3.1 Immunoassays

Even though the first assay for parathyroid hormone (PTH) was published in the early 1960s, it is still a challenge to measure its active form (PTH 1–84) in a specific way. Since numerous PTH fragments are believed to be present and in highly variable concentration (tightly linked to the renal function), these fragments may accumulate and be recognized to varying degrees by PTH assays in clinical laboratories.

PTH is commonly measured in nowadays clinical chemistry laboratories by fully automated immunoassays (IA) of mainly two generations. Each constituted with antibodies reacting to different epitopes on the peptidic chain. The first radioimmunoassay (RIA) for PTH was conceived in 1963 by Solomon Berson and Rosalyn Yalow at the Bronx Veterans Hospital's Radioisotope Service (28). This test employed polyclonal anti-PTH antibodies obtained from guinea pigs and rabbits that were injected bovine parathyroid glands extract. Patient samples were combined with polyclonal antibodies and radiolabelled bovine PTH.

Although the assay could readily detect endogenous levels of PTH, its cross-reactivity was inadequate, permitting measurement only of elevated PTH levels (e.g., in patients with primary hyperparathyroidism), but not normal or low levels of the hormone. Rosalyn Yalow was granted the Nobel Prize in 1977 for developing this RIA technique for measuring peptide hormones, including this PTH assay.

It was not until 1968, when Reiss and Canterbury developed a much more sensitive assay for human PTH, obtaining the anti-PTH antibodies by injecting bovine PTH to chicken (29). Due to greater antibody cross-reactivity with human PTH, this assay enabled normal

range measurements. In the early 70s, numerous groups worked to implement this currently-called “first generation” assay.

1.3.2 First Generation Assays (disused)

The first generation of PTH assays relied on a ^{125}I labelled peptide (bovine origin). Initially, the samples were incubated for 4 days with the polyclonal anti-PTH antibodies. Then the ^{125}I labelled PTH was added to the mixture which was incubated for 3 more days. Since the number of antigen-binding sites was limited, this subsequent incubation led to a competition for the anti-PTH antibody between the labelled PTH and the native (patient’s) PTH. The formed complexes were precipitated, washed and measured with a gamma-counter. In this assay, the amount of labelled PTH bound to the antibodies is inversely proportional to the amount of “native” PTH, whose concentration could therefore be obtained (After due calibration) (30). Figure 4 schematizes the working principle of a first generation IA.

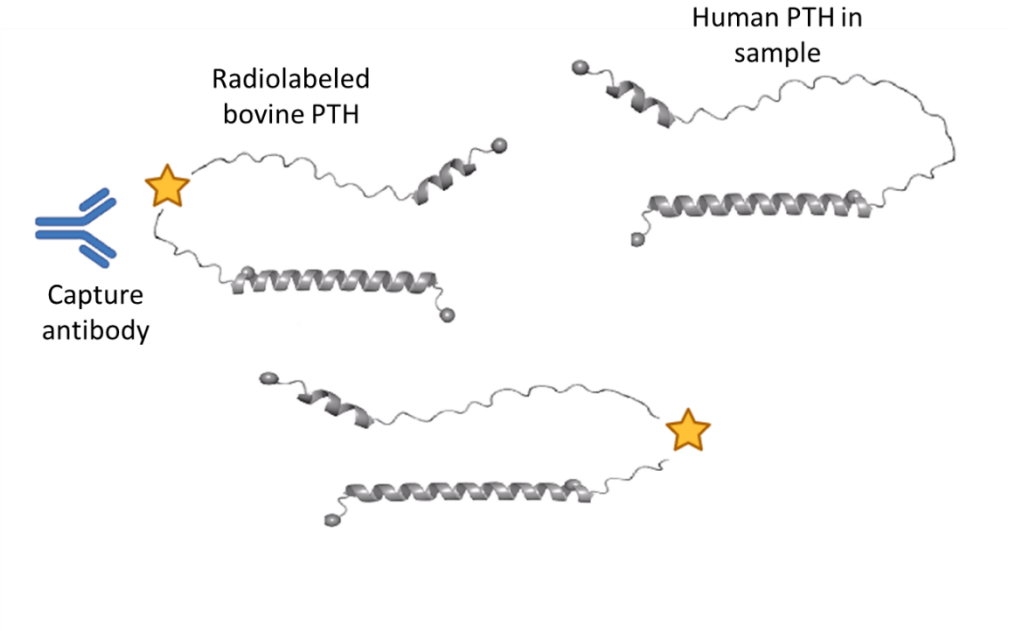

Figure 4. First-generation radioimmunoassay for PTH. Human PTH in the sample competes with and displaces radiolabeled bovine PTH bound to a capture antibody.

These first-generation immunoassays identified not only the biologically active 84–amino acid species, but also a significant number of the hormone's non-functional carboxy-terminal degradation products. This was notably problematic in patients with renal failure, as these carboxy-terminal PTH fragments accumulated at higher levels, thereby confounding the assay results.

1.3.3 Second Generation Assays

Working with Nichols Institute Diagnostics, Samuel Nussbaum and associates released a second-generation test in 1987 (31). This test, which later came to be known as the "intact PTH" assay, addressed many of the issues with the first-generation assays' inadequate sensitivity and specificity. In the test Nussbaum developed, two sets of antibodies (capture antibodies and signal antibodies) that were affinity purified to certain PTH molecule regions were utilized. The capture antibody targeted the hormone's carboxy-terminal portion (amino acids 39-84), while the amino-terminal portion was targeted by the signal antibody (amino acids 1-34). PTH was “sandwiched” between the two antibodies. Only hormone molecules that were "intact" (those that kept both their carboxy- and amino-terminal amino acids) were quantified. This assay provided not only a much better selectivity and sensitivity compared to the first-generation assays, but also a much higher throughput.

Whereas first-generation tests could take up to 7 days to complete, the new generation of assays could be finished in around 24 hours.

In the years that followed, a number of comparable assays, either IRMA or fully automated chemiluminescent assays, hit the market. Some of them, could be completed in less than 20 minutes, making it possible to diagnose patients quickly at nearby health care facilities and even to perform intraoperative PTH monitoring(32).

Some modern second-generation assays signal antibodies, like the Roche Elecsys intact PTH test, recognize a relatively distant epitope in the 26-32 region. Whereas some others, like the Allegro assay, employ an anti-N-terminal antibody targeted towards the proximal (13-24) portion of the hormone.

The shortcomings of the second generation of PTH assays were rapidly identified. According to some studies, the extent of secondary hyperparathyroidism in CKD patients was exaggerated. In fact, some patients presented increased PTH levels while having a low bone turnover from a histological perspective (33,34). This was later understood in 1998, when it was proved that the “intact” PTH assays were varyingly cross-reacting with a PTH molecule distinct from 1-84 PTH. This distinct molecule, which could be chromatographically resolved by HPLC, co-eluted with a synthetic 7-84 PTH fragment (35). This fragment was referred as “7-84 PTH” fragment or more recently as “non-1-84 PTH” (as 7-84 PTH has never been observed to date as detailed later in this work).

To the date, second-generation, or intact PTH tests, are still the most popular assays in use worldwide(17). Figure 5 schematizes the working principle of a second generation IA.

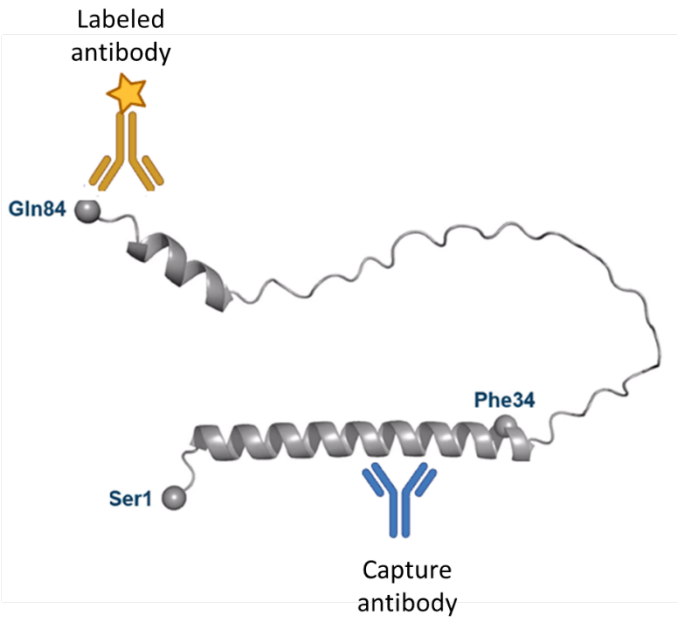

Figure 5. Second generation PTH assay. PTH in the sample analyzed is sandwiched between a capture antibody and a labeled antibody. Both recognizing different epitopes on the PTH molecule (mid region and C-term respectively).

1.3.4 Third Generation Assays

The first "third-generation" immunoradiometric PTH assay was developed by Scantibodies Laboratories in 1999 (36). The assay, known as "Whole PTH assay" or "Bio-intact PTH", employs on the one hand, an antibody targeting the C-terminal region of PTH, similarly to second-generation assays, but on the other hand, employs a second antibody directed against the very first amino acids (1-4) of PTH. Therefore, this IRMA no longer measures the "non-1-84 PTH" fragment nor reacts with N-truncated fragments. This "new" generation allowed a more in-depth understanding of the nature of the circulating PTH, and specially to disregard non biologically active fragments, which

may be of relatively high abundance in patients suffering from CKD(25,37). However, they have not demonstrated any improvement in the diagnoses of bone disease or other secondary hyperparathyroidism. There is no apparent difference in the diagnostic sensitivities between both generations for samples coming from patients with normal renal function (38). Figure 6 schematizes the working principle of a third generation IA.

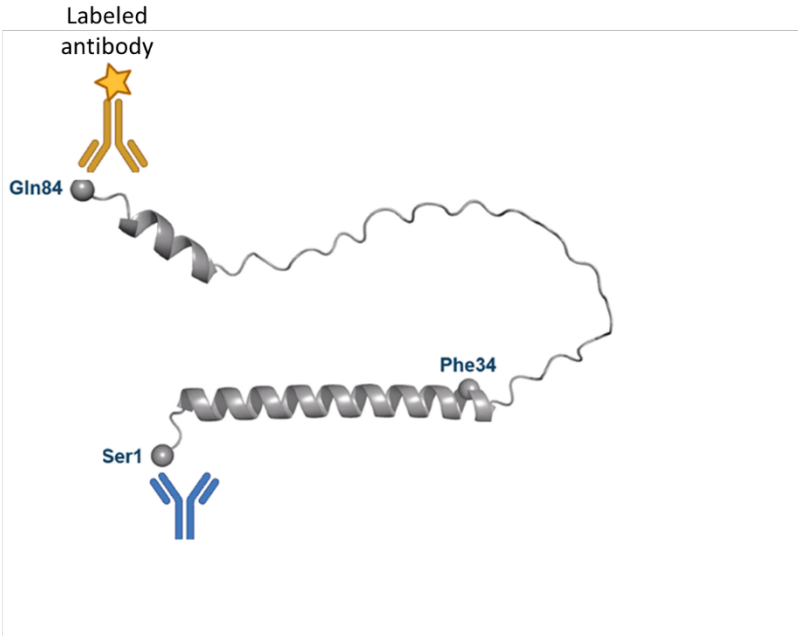

Figure 6. Third generation PTH assay. PTH in the sample analyzed is sandwiched between a capture antibody and a labeled antibody. Both recognizing different epitopes on the PTH molecule (N-term and C-term respectively).

Due to the nature of the immunoassay generations, none of them are able to measure exclusively unmodified 1-84 PTH, as summarized in Table 1 below. The calibrators employed across various immunoassays are often of unknown quality and differ from each other, compounding the issue that they are non-commutable and their concentrations are not established in a traceable manner. Nonetheless, their different cross-reactivities for PTMs may provide a significant clinical insight, specially in patients presenting Amino-PTH species (presumably suffering from a parathyroid carcinoma), where an inversion of the ratio 3rd generation / 2nd generation is observed (39).

Table 1. Cross-reactivities of the different generations of PTH assays.

	1st Generation Assays	2nd Generation Assays	3rd Generation Assays
1-84 PTH	Yes	Yes	Yes
N-truncated PTH	Yes	Yes	No
C-truncated PTH	No	No	No
Amino-PTH	Yes	No	Yes
Oxidized-PTH	Yes	Yes	Yes

1.3.5 Mass spectrometry-based assays

The recent surging of mass spectrometric techniques applied to clinical chemistry has allowed scientists (and clinicians) to have an ever-powerful tool, with unprecedented sensitivity, specificity, and robustness, that provides a novel insight into the characterization and quantitation of biomolecules.

The application of mass spectrometry in the study and analysis of proteins has permitted to address the limitations of the widely and historically used immunoassays, allowing to obtain deeper knowledge into protein sequences, post-translation modifications, protein-protein interactions and the dynamic changes in protein abundance under different physiological conditions. This is mostly possible thanks to the hyphenation of separation techniques to mass spectrometers (such as LC-MS/MS), whose basic principles are introduced hereinafter.

Commercial ultra-high-pressure liquid chromatography systems were developed by Waters Corporation in 2004 under the name UPLC. Nowadays, the term UHPLC is used generically. UHPLC is a term that refers to chromatographic separations carried out using sub-2 μm particles. In contrast to conventional HPLC systems (which are restricted to 400 bars of pressure), current UHPLC instruments may work at a maximum flow rate of 2-5 mL/min and endure backpressures of 1200-1400 bars.

In 1956, Van Deemter released his major works on the basic equation that governs the link between mobile phase linear velocity (u) and height equivalent to a theoretical plate (*HEPT* or *H*).

$$HETP = A + \frac{B}{u} + (C_s + C_m) \cdot u$$

Where: A = Eddy diffusion term

B = Longitudinal diffusion term

C = Resistance to mass transfer term (C_s: Stationary phase; C_m: Mobile phase)

C_m can be expanded to the following expression

$$C_m = \omega \frac{d_p^2}{D_m}$$

Where: d_p = Particle diameter

D_m = Diffusion coefficient of the solute in the mobile phase

ω = Pore size distribution, shape and particle size distribution coefficient

Showing the dependency on the square particle diameter.

Figure 10 illustrates an example of a Van Deemter plot. The lower the HETP, the greater the efficiency of a column. The appearance of sub-2 µm particle columns was a major step toward better resolution and more assay speed. As they virtually suppress the C term of the Van Deemter equation, the resulting slope is significantly flattened.

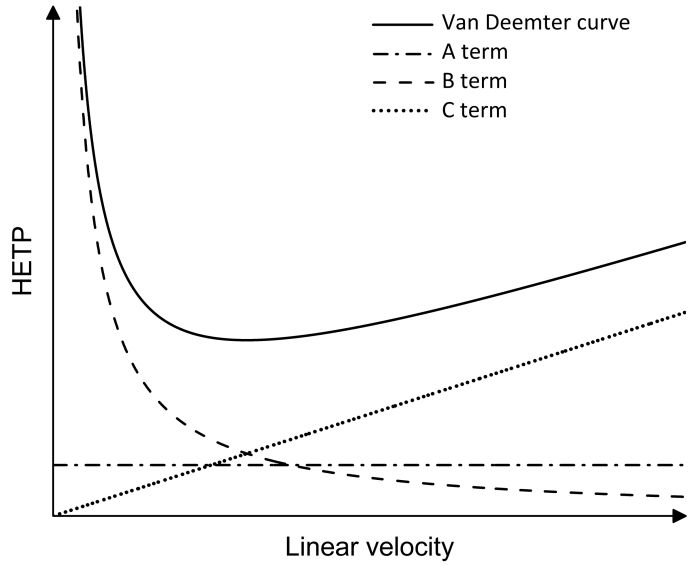

Figure 10. Van Deemter plot.

In the end of the nineteenth century, the principle of mass spectrometry emerged with the observation of canal rays. These rays are beams of positively charged ions generated in an anode ray tube (40,41). Additional investigations by Nobel prize laureate J.J. Thomson revealed that the route of these charged ion canal rays is bent in the presence of a magnetic field proportional to the mass-to-charge ratio (m/z) of the particles (42). This discovery settled the bases of today's mass spectrometry and allowed the continuous growth of mass spectrometry methods and capabilities. Mass spectrometers have substantially increased in sophistication and sensitivity in recent years.

No matter their complexity, form or variant, all mass spectrometers rely on the same principles. They consist of a source of ionization, to produce gas phase ions from a

compound, one (or more) mass analyzers in a vacuum which select the ions according to their m/z and a detector that counts them.

Electrospray ionization (ESI) is a widely used ionization technique in mass spectrometry that allows for the analysis of a wide range of compounds, including large biomolecules such as proteins and peptides. ESI for proteins and peptides allows for the analysis of these compounds in their native form, providing structural information that may not be available with other ionization techniques. By multiple charging, the "soft ionization" plays a key role for the analysis peptides and proteins since very little fragmentation happens during the ionization process and structural features are kept intact.

Mass analyzers are the core of mass spectrometers that allow the separation of the generated ions according to their m/z ratio. There are large variety of mass analyzers available, each with its own set of strengths and weaknesses regarding speed of operation, resolution, and other operating characteristics. For the purposes of the present work, only the triple quadrupole mass spectrometer is detailed.

The quadrupole mass analyzer was first described in 1950 by Nobel prize laureate Wolfgang Paul (43). A quadrupole consists of four parallel rod electrodes arranged in a square configuration, forming a channel through which an ion beam can pass through as represented in Figure 12. The pairs of opposite rods are electrically connected, and when in operation, they hold the same potential, which is composed of a direct current (DC) and an alternate current (AC) component.

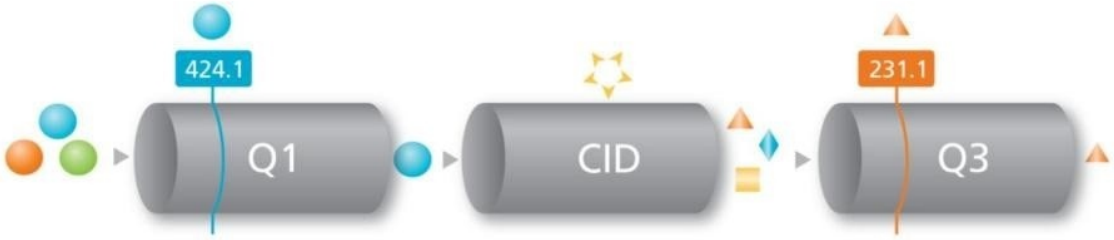

Figure 12. Diagram of a triple quadrupole MS.(Obtained from Sciex (44)). Abbreviations: Q1: First quadrupole; CID: Collision-induced dissociation; Q3: Third Quadrupole.

As an ion approaches the quadrupole assembly in the z-direction, it is attracted to one of the rods whose charge is opposite to that of the ion. Since the voltage and polarity given to the rods are periodic, attraction and repulsion will alternate in time in both the x and y directions. For a given quadrupole setting, only the ions with a certain m/z value can follow a stable trajectory and pass through the quadrupole, the others being filtered out.

Tandem MS is performed by means of mass spectrometers which are equipped with more than one mass analyzer along the instrument (in tandem). The most widespread instrument that falls into this category is known as a triple quadrupole MS (QqQ) which, as its name suggests, consists of three quadrupoles (Q1, q2, and Q3). While Q1 and Q3 are mass analyzers, q2 acts as an ion transfer device and collision cell. A triple quadrupole instrument can function in a variety of modes depending on whether Q1 and Q3 are used as mass scanners or filters. That means if they are fixed for a single m/z to pass through (filter) or if they are allowing the sequential transmission of all m/z

(scan). Regarding the scan function of a quadrupole, it is worth noting that a quadrupole can only be tuned for one specific m/z at a given time, therefore, when scanning, it will sequentially be tuned for all m/z at very high speeds (up to several thousands of m/z per second).

When a MS/MS mode is operated in MRM, Q1 acts as a mass filter, passing ions of interest to q2, where they undergo fragmentation, most commonly by collision-induced dissociation (CID). All fragment ions and remaining precursor ions are then focused and directed into Q3, where they are again filtered by mass. A compound's "transition" is detected rather than its mass alone, which results in an increased degree of confidence of identity and reduced signal-to-noise ratio, as only the ions sharing a given transition will reach the detector. Triple quadrupole mass analyzers are the most frequent type of mass spectrometers due to their high throughput, sensitivity, robustness, and ease of operation.

Mass spectrometers can multiplex analysis, i.e., multiple analytes can be measured in a single run, which is of special interest when potentially clinically relevant species are simultaneously found in circulation. This may allow to explore new windows of knowledge, e.g., elucidate the variable influence of PTH related compounds or, determine whether ratios of PTH related compounds may be of clinical interest. For instance, PTH 1-84/amino-PTH on patients presenting a parathyroid carcinoma; fragment concentrations on CKD patients, etc.

During the last decade, highly sensitive modern LC-MS instruments have been made available, which coupled with appropriate sample preparation techniques, can often reach quite low limits of detection, similar to those obtained with immunoassays. Inevitably, much effort has been put into developing mass spectrometric assays for PTH determination, even if all of the described methods so far are challenged by their sensitivity. Also, due to the large size of the protein, most of them require digestion processes, with the associated loss of structural information.

The two first LC-MS/MS based assays for PTH analysis were published in the same 2010 issue of Clinical Chemistry. Kumar et al., and Lopez et al., (39,40) purified human serum by using one of the commercially available C-terminal antibodies, pulling PTH 1-84 as well as C-terminal fragments which were later digested and analyzed through LC-MS/MS. These innovative studies relied in digestion processes, did not present sufficient sensitivity (LOQ at 39.1 and 31 pg/mL, respectively) and their reproducibility is not well demonstrated; they did however set a solid starting point for newer method building.

More recently, in 2021, Kritmetapak et al., described a novel high resolution mass spectrometry method to measure 1-84 PTH using immunocapture without digestion with an LOQ of around 50 pg/mL (25).

Although these three methods may provide interesting clinical information in patients with hyperparathyroidism, their rather high limits of quantification are too high for the expected PTH levels in “healthy” individuals. Additionally, the fact that they rely on antibodies increases the complexity of the assay and the potential sources of variability.

1.3.6 Standardization of PTH assays

Assay standardization is aimed at achieving reasonable comparability and/or interchangeability of results obtained by different measurement procedures. In practice, the process involves establishing what is known as metrological traceability to internationally agreed measurement standards.

One same sample analyzed in parallel by two different laboratories may provide different results. This issue was recognized early in the history of laboratory medicine. In 1973, Cali published a “call-out” article in Clinical Chemistry which he entitled "An Idea Whose Time Has Come". This idea was trueness and accuracy of measurements, urging scientists to work together to “build the foundations on which meaningful measurement in clinical chemistry can come about” (47). In 1994, Tietz published again in the same journal “Accuracy in clinical chemistry – Does anybody care?” (48). Even after Cali's first call, not much had happened. Still, in 2008, Thienpont published "Accuracy in clinical chemistry - who will kiss Sleeping Beauty awake?", informing the clinical community that all the tools to establish metrological traceability were available, but it was time to use them (49). These calls were driven by the conviction that medical laboratory data should be handled in such a way as to enable the correct diagnosis of diseases, therapies and patient monitoring for which standardization or metrological traceability is required.

The advantage of working with standardized assays is that they meet modern clinical and public health needs, such as that laboratory data should have absolute clinical utility with complete safety for the patient. They should also allow the application of consistent standards of medical care, which require the use of common clinical decision limits, the ability to develop evidence-based clinical practice guidelines, and the ability to aggregate

data from multiple research studies so that it can be translated into patient care, especially in an era where the mobility of the patients is non omittable. Even when seeking second or even a third opinions, the interchangeability of laboratory results is paramount.

PTH assays are well known for their lack of standardization, many of the issues were described in 2006 by Souberbielle et al. at Kidney International (50). By this time, the Kidney Disease Outcom Quality Initiative (K/DOQI) guidelines recommended that a hemodialyzed patient should have a PTH concentration around 150 to 300 pg/mL, regardless of which assay was used (51). Souberbielle et al., in their mentioned work, processed several patient pools at 150 pg/mL, 300 pg/mL and 1000 pg/mL in a multicentric study using different PTH assays. The results from this test revealed serious discrepancies among the different immunoassays, as schematized in Table 2.

Table 2. Equivalent concentrations obtained with the different PTH assays. From Souberbielle et al. 2006, Kidney International. Open Access(50).

Assay	PTH (ng/l)	PTH (ng/l)	PTH (ng/l)	Median bias (%)
Allegro intact PTH	150	300	1000	0
N-tact PTH IRMA	83	160	517	−44.9 (−68.0; −26.2)
PTH IRMA Immunotech	188	369	1216	23.9 (−6.1; 108.3)
ELISA-PTH	149	290	948	−1.6 (−24.3; 47.2)
Total intact PTH IRMA	134	262	857	−14.5 (−41.5; 23.5)
DSL PTH IRMA	323	638	2108	123.0 (53.1; 188.9)
DSL PTH ELISA	264	523	1734	79.6 (−8.0; 180.9)
Elecsys PTH	161	311	1011	7.3 (−13.8; 80.3)
Immulite 2000 intact PTH	212	410	1334	37.8 (3.8; 130.8)
PTH-ACS 180	185	374	1256	18.8 (−9.9; 69.4)
PTH AdviaCentaur	168	342	1154	9.5 (27.6; 55.6)
Intact PTH advantage	174	339	1109	14.6 (−10.4; 72.2)
LIAISON N-tact PTH	111	223	748	−23.4 (−68.2; −1.9)
Ca-PTH IRMA	84	165	543	−44.8 (−65.6; −22.8)
BioIntact PTH advantage	109	214	704	−27.6 (−53.0; 12.5)

Important clinical decisions could be erroneously made depending on which assay was used. It is worth noting that PTH concentrations can present variations accounting up to 4-fold over the different assays. The K/DOQI guidelines were quickly superseded in 2009 by the Kidney Disease Improving Global Outcomes (KDIGO) guidelines which express the expected amounts of PTH in terms of an upper limit relative to the different reference ranges established by IVD manufacturers.

As a matter of fact, many works have pointed out discrepancies between PTH 1-84 results derived from a single sample analyzed on various analytical platforms (52–55), including our own experiments detailed in this work.

Considering the large-scale complexity of PTH analysis, i.e., the coexistence of different assay generations; the different calibration materials used; and the clinical relevance of the assay among CKD patients, deem the standardization of PTH assays critical. Ultimately, for CKD patients, standardization could ensure that clinical decisions be based on reliable and comparable data, leading to better management of their condition without needing to rely on relative quantitation (as recommended by the KDIGO guidelines). Instead, an absolute value could be brought up to a common PTH reference range.

While achieving agreement amongst lab results would be a beneficial goal, standardization goes a step further aiming to develop systems that can achieve unbiased truth in measurements. This is especially crucial for assays with precise cut-points that determine patient care, such is the case of PTH. In 2010, in order to circumvent this unsuitable situation, the IFCC constituted a working group on PTH standardization, nowadays established as the IFCC Committee on Bone Metabolism, whose main

perspectives and priorities were published in 2017 (55). The group has proposed that all assays (2nd or 3rd generation) should be calibrated to the same internationally-recognized standard, which should be traceable to a certified reference material (CRM), which has not been developed yet. That would allow establishing common reference intervals for PTH assays (56). Thus, reference measurement procedures (RMP) and reference materials (RM) are required.

ISO 15193 guidelines for an RMP establish some key elements that must be properly described, including defining the measurand, measurement procedures, and materials such as reference materials and calibrators (57). Once implemented, this reference measurement system provides metrological traceability in routine clinical analyses, linking patient laboratory results to a recognized higher-order standard via an unbroken chain of traceability to a SI unit. Linked to the metrological traceability are national metrology institutes, which furnish primary and secondary reference materials and procedures, accredited reference measurement laboratories offering secondary reference materials and procedures, clinical assay manufacturers providing calibrators and employing value transfer protocols, and clinical diagnostic laboratories (58). An example traceability chain for PTH is displayed in Figure 7.

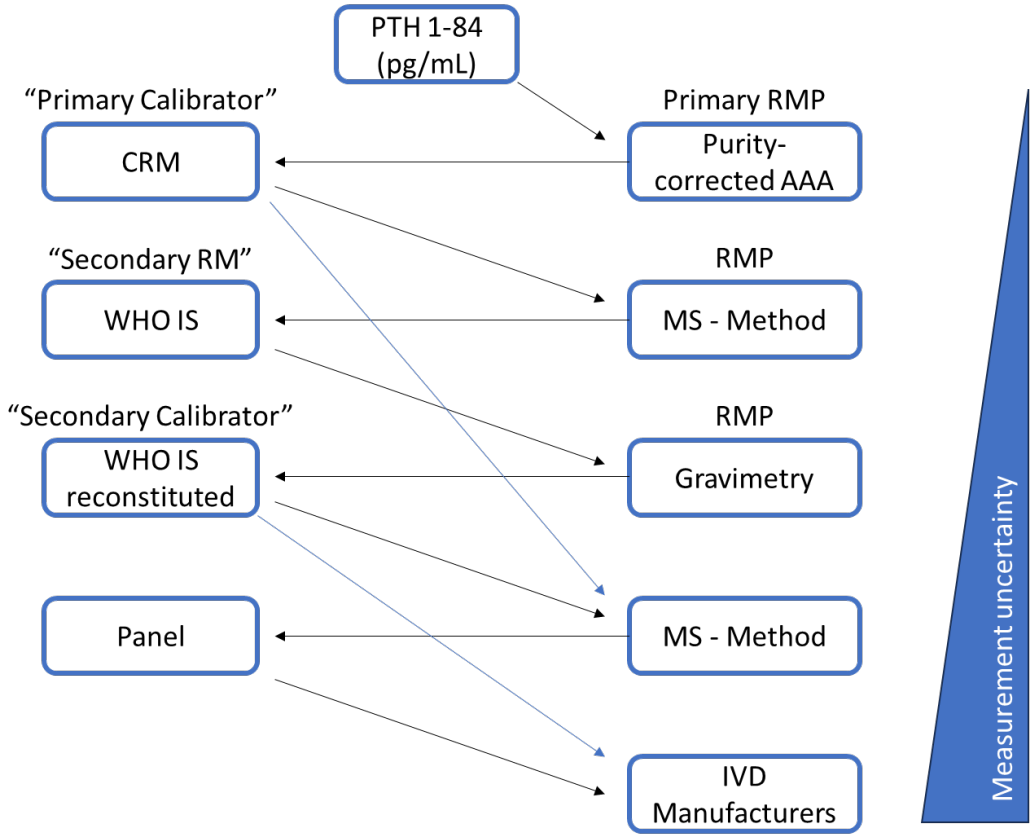

Figure 7. Metrological traceability chain for PTH 1-84. Abbreviations: Certified Reference Material (CRM); Reference Measurement Procedure (RMP); Amino Acid Analysis (AAA); World Health Organization International Standard (WHO IS); In-vitro Diagnostics(IVD).

Within each step of the traceability chain, the measurement uncertainty (MU) and its allowable limits associated must be carefully assessed in order to obtain a final combined MU at the last step that fulfills the required APS (59). Undoubtedly, the higher in the traceability chain, the more stringent APS are demanded for measurement procedures.

Several publications point towards the objectives that a PTH RMP should fulfill (17,55,60,61), namely, a Lower Limit of Quantitation (LLOQ) within the 1-10 pg/mL range, non-discriminatory sample preparation for PTH variants, demonstrated inter-laboratory reproducibility, calibration using a reference material with suitable measurement uncertainty and traceability to the SI, proven commutability of the reference material, ability to identify and quantify PTH variants, and utilization of isotopically labeled Internal Standards (IS).

1.3.7 References

1. Owen R. On the anatomy of the Indian rhinoceros (Rh unicornis, L.). Transactions of the Zoological Society of London. 1852;31–58.

2. Modarai B, Sawyer A, Ellis H. The glands of Owen. J R Soc Med. 2004;97.

3. Gley E. Comptes rendus des séances de la Société de biologie et de ses filiales. 1891;43–551.

4. Von Recklinghausen F. Festschrift für Rudolf Virchow. Berlin; 1891.

5. Collip JB, Clark EP. FURTHER STUDIES ON THE PARATHYROID HORMONE. Journal of Biological Chemistry. 1925;66:133–7.

6. Hanson AM. An elementary chemical study of the parathyroid glands of cattle. Mil Surg. 1923;53:280–4.

7. Hanson A. The hormone of the parathyroid gland. Mil Surgeon. 1924;54:554–60.

8. Negrea LA. Hypocalcemia and hypercalcemia. Nephrology Secrets. Elsevier; 2019. page 526–31.

9. Onifade KU, Mohammad AA, Petersen JR, Okorodudu AO. Ionized calcium: Indications and advantages of its measurement. Laboratoriums Medizin. 2005. page 235–40.

10. Froissart M, Houillier P. Limitations of non-corrected and albumin-corrected total calcium concentrations in CKD patients. Nephrology Dialysis Transplantation. 2009;24:2291–2.

11. Houillier P, Froissart M, Maruani G, Blanchard A. What serum calcium can tell us and what it can't. Nephrology Dialysis Transplantation. 2006;21:29–32.

12. Farrow EG, White KE. Recent advances in renal phosphate handling. Nat Rev Nephrol. 2010;6:207–17.

13. Goyal R, Jialal I. Hyperphosphatemia. 2023.

14. Aarsand AK, Fernandez-Calle P, Webster C, Coskun A, Gonzales-Lao E, Diaz-Garzon J, et al. The EFLM Biological Variation Database. https://biologicalvariation.eu/. 2023 [cited 2023 Apr 1].

15. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster J-Y, et al. Effect of Parathyroid Hormone (1-34) on Fractures and Bone Mineral Density in Postmenopausal Women with Osteoporosis. New England Journal of Medicine. 2001;344:1434–41.

16. Silva BC, Bilezikian JP. Parathyroid hormone: anabolic and catabolic actions on the skeleton. Curr Opin Pharmacol. 2015;22:41–50.

17. Cavalier E, Vasikaran S, Bhattoa HP, Heijboer AC, Makris K, Ulmer CZ. The path to the standardization of PTH: Is this a realistic possibility? a position paper of the IFCC C-BM. Clinica Chimica Acta. Elsevier B.V.; 2021;515:44–51.

18. Naylor SL, Sakaguchi AY, Szoka P, Hendy GN, Kronenberg HM, Rich A, et al. Human parathyroid hormone gene (PTH) is on short arm of chromosome 11. Somatic Cell Genet. 1983;9:609–16.

19. Habener JF, Rosenblatt M, Kemper B, Kronenberg HM, Rich A, Potts JT. Pre-proparathyroid hormone; amino acid sequence, chemical synthesis, and some biological studies of the precursor region. Proceedings of the National Academy of Sciences. 1978;75:2616–20.

20. Marx UC, Adermann K, Bayer P, Meyer M, Forssmann W-G, Rösch P. Structure-Activity Relation of NH2-terminal Human Parathyroid Hormone Fragments. Journal of Biological Chemistry. 1998;273:4308–16.

21. Brown EM. Control of parathyroid hormone secretion by its key physiological regulators. In: J.P Bilezikian, editor. The Parathyroids: Basic and clinical concepts (3rd edition). San Diego: Academic Press; 2015. page 101–18.

22. Kumar R, Thompson JR. The regulation of parathyroid hormone secretion and synthesis. Journal of the American Society of Nephrology. 2011.

23. Cavalier E. Parathyroid hormone results interpretation in the background of variable analytical performance. J Lab Precis Med. AME Publishing Company; 2019;4:1–1.

24. D'Amour P, Lazure C, Labelle F. Metabolism of Radioiodinated Carboxy-Terminal Fragments of Bovine Parathyroid Hormone in Normal and Anephric Rats*. Endocrinology. 1985;117:127–34.

25. Kritmetapak K, Losbanos LA, Hines JM, O'Grady KL, Ulmer CZ, Vesper HW, et al. Chemical Characterization and Quantification of Circulating Intact PTH and PTH Fragments by High-Resolution Mass Spectrometry in Chronic Renal Failure. Clin Chem. 2021;67:843–53.

26. Farré-Segura J, Le Goff C, Lukas P, Cobraiville G, Fillet M, Servais AC, et al. Validation of an LC-MS/MS Method Using Solid-Phase Extraction for the Quantification of 1-84 Parathyroid Hormone: Toward a Candidate Reference Measurement Procedure. Clin Chem. 2022;68.

27. D'Amour P, Brossard J-H. Carboxyl-terminal parathyroid hormone fragments: role in parathyroid hormone physiopathology. Curr Opin Nephrol Hypertens. 2005;14:330–6.

28. Berson SA, Yalow RS, Aurbach GD, Potts JT. IMMUNOASSAY OF BOVINE AND HUMAN PARATHYROID HORMONE. Proceedings of the National Academy of Sciences. Pergamon Press; 1963;49:613–7.

29. Reiss E. Measurement of Parathyroid Hormone. Ann Intern Med. 1968;69:1325.

30. Arnaud CD, Tsao HS, Littledike T. Radioimmunoassay of human parathyroid hormone in serum. Journal of Clinical Investigation. 1971;50:21–34.

31. Nussbaum SR, Zahradnik RJ, Lavigne JR, Brennan GL, Nozawa-Ung K, Kim LY, et al. Highly sensitive two-site immunoradiometric assay of parathyrin, and its clinical utility in evaluating patients with hypercalcemia. Clin Chem. 1987;33:1364–7.

32. Clary BM, Garner SC, Leight GS. Intraoperative parathyroid hormone monitoring during parathyroidectomy for secondary hyperparathyroidism. Surgery. 1997;122:1034–9.

33. Quarles LD, Lobaugh B, Murphy G. Intact parathyroid hormone overestimates the presence and severity of parathyroid-mediated osseous abnormalities in uremia. J Clin Endocrinol Metab. 1992;75:145–50.

34. Wang M, Hercz G, Sherrard DJ, Maloney NA, Segre G V., Pei Y. Relationship between intact 1-84 parathyroid hormone and bone histomorphometric parameters in dialysis patients without aluminum toxicity. American Journal of Kidney Diseases. 1995;26:836–44.

35. Lepage R, Roy L, Brossard JH, Rousseau L, Dorais C, Lazure C, et al. A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples. Clin Chem. 1998;44:805–9.

36. John MR, Goodman WG, Gao P, Cantor TL, Salusky IB, Jüppner H. A Novel Immunoradiometric Assay Detects Full-Length Human PTH but not Amino-Terminally

Truncated Fragments: Implications for PTH Measurements in Renal Failure. J Clin Endocrinol Metab. 1999;84:4287–90.

37. Cavalier E. Determination of parathyroid hormone: from radioimmunoassay to LCMS/MS. Clinical Chemistry and Laboratory Medicine (CCLM). 2023;61:946–53.

38. Brossard JH, Lepage R, Cardinal H, Roy L, Rousseau L, Dorais C, et al. Influence of glomerular filtration rate on non-(1-84) parathyroid hormone (PTH) detected by intact PTH assays. Clin Chem. 2000;46:697–703.

39. Cavalier E, Daly AF, Betea D, Pruteanu-Apetrii PN, Delanaye P, Stubbs P, et al. The ratio of parathyroid hormone as measured by third- and second-generation assays as a marker for parathyroid carcinoma. Journal of Clinical Endocrinology and Metabolism. 2010;95:3745–9.

40. Dempster AJ. The Passage of Slow Canal Rays through Hydrogen. Proceedings of the National Academy of Sciences. 1925;11:552–4.

41. Lawrence EO, Sloan DH. The Production of High Speed Canal Rays without the Use of High Voltages. Proceedings of the National Academy of Sciences. 1931;17:64–70.

42. Thomson JJ. Bakerian Lecture :—Rays of positive electricity. Proceedings of the Royal Society of London Series A, Containing Papers of a Mathematical and Physical Character. 1913;89:1–20.

43. Paul W. Electromagnetic traps for charged and neutral particles. Rev Mod Phys. 1990;62:531–40.

44. Ashley Sage. When is a triple quadrupole not a triple quadrupole? [Internet]. [cited 2024 May 21]. Available from: https://community.sciex.com/2019/11/12/when-is-a-triple-quadrupole-not-a-triple-quadrupole/

45. Kumar V, Barnidge DR, Chen LS, Twentyman JM, Cradic KW, Grebe SK, et al. Quantification of serum 1-84 parathyroid hormone in patients with hyperparathyroidism by immunocapture in situ digestion liquid chromatography-tandem mass spectrometry. Clin Chem. 2010;56:306–13.

46. Lopez MF, Rezai T, Sarracino DA, Prakash A, Krastins B, Athanas M, et al. Selected reaction monitoring-mass spectrometric immunoassay responsive to parathyroid hormone and related variants. Clin Chem. 2010;56:281–90.

47. Cali JP. An Idea Whose Time Has Come. Clin Chem. 1973;19:291–3.

48. Tietz NW. Accuracy in clinical chemistry--does anybody care? Clin Chem. 1994;40:859–61.

49. Thienpont LM. Accuracy in clinical chemistry – who will kiss Sleeping Beauty awake? Clin Chem Lab Med. 2008;46.

50. Souberbielle JC, Boutten A, Carlier MC, Chevenne D, Coumaros G, Lawson-Body E, et al. Inter-method variability in PTH measurement: Implication for the care of CKD patients. Kidney Int. Elsevier Masson SAS; 2006;70:345–50.

51. Eknoyan G, Levin A, Levin NW. Bone metabolism and disease in chronic kidney disease. American Journal of Kidney Diseases. 2003;42:1–201.

52. Garrett G, Sardiwal S, Lamb EJ, Goldsmith DJA. PTH—A Particularly Tricky Hormone. Clinical Journal of the American Society of Nephrology. 2013;8:299–312.

53. Souberbielle JC, Friedlander G, Cormier C. Practical considerations in PTH testing. Clinica Chimica Acta. 2006;366:81–9.

54. Zimmerman MK, Kumar V, Maine GN, Sykes E. Discrepant intact parathyroid hormone result by immunoassay. Clinica Chimica Acta. 2012;413:344–5.

55. Sturgeon CM, Sprague S, Almond A, Cavalier E, Fraser WD, Algeciras-Schimnich A, et al. Perspective and priorities for improvement of parathyroid hormone (PTH) measurement – A view from the IFCC Working Group for PTH. Clinica Chimica Acta. Elsevier B.V.; 2017;467:42–7.

56. Souberbielle J-C, Brazier F, Piketty M-L, Cormier C, Minisola S, Cavalier E. How the reference values for serum parathyroid hormone concentration are (or should be) established? J Endocrinol Invest. Springer International Publishing; 2017;40:241–56.

57. ISO. In vitro diagnostic medical devices. Measurement of quantities in samples of biological origin. Requirements for content and presentation of reference measurement procedures. ISO 15193. ISO London, United Kingdom; 2009.

58. Bunk DM. Reference materials and reference measurement procedures: an overview from a national metrology institute. Clin Biochem Rev. 2007;28:131–7.

59. Braga F, Panteghini M. The utility of measurement uncertainty in medical laboratories. Clin Chem Lab Med. 2020;58:1407–13.

60. Smit MA, Van Kinschot CMJ, Van Der Linden J, Van Noord C, Kos S. Clinical Guidelines and PTH Measurement: Does Assay Generation Matter? Endocr Rev. 2019;40:1468–80.

61. Sturgeon CM, Sprague SM, Metcalfe W. Variation in parathyroid hormone immunoassay results--a critical governance issue in the management of chronic kidney disease. Nephrology Dialysis Transplantation. 2011;26:3440–5.

Chapter 2. Objectives

The main objective of this work will be to demonstrate the possibility of performing the analysis of PTH 1-84, without digestion, by using a higher order technique such as UHPLC coupled to tandem mass spectrometry, in order to set the foundations for a candidate reference measurement procedure for PTH analysis.

The originality of this work will lie in the adaptation of the sample preparation and analysis processes to the power of the instruments available today, breaking away from the classic approaches still used worldwide today, according to which, the quantification of proteins by mass spectrometry is mainly dependent on digestion processes. While this is true, it is also true that today, due to the "youth", and therefore the lack of experience in mass spectrometry applied to clinical analysis, most laboratories throughout the world, base their tests on immunoassays, with the drawbacks they can entail.

This work aims to present the opportunity to use mass spectrometry as a reference procedure in order to be able to standardize and harmonize the quantitation of PTH in its full and intact form.

Initially, as a proof-of-concept, a method will be developed for the intact quantification of glucagon, a protein of 29 amino acids, by liquid chromatography coupled to tandem mass spectrometry. It will be compared with two types of immunoassay (a RadioImmunoAssay (RIA) and an Enzyme-Linked immunosorbent assay (ELISA)), highlighting the added value of the new method developed, which is currently used for routine analysis at the CHU of Liège.

Subsequently, and as the main part of this thesis, a method for the analysis of PTH, in its intact form, will be developed and proposed as a potential candidate reference measurement method, through a SPE sample preparation, allowing to overcome most

of the limitations of the immunoassays and the few described immuno-mass spectrometric assays. On the other hand, assay standardization will be explored by a comparison between our technique and different generations of IAs available on the market, including their successful recalibration with the WHO 95/646 IS for PTH, aligning them to our LC-MS/MS method. It is aimed to evaluate the impact of this recalibration by analyzing a large panel of pooled plasma samples parallelly by LC-MS/MS and IAs.

From a clinical point of view, it will also be demonstrated the practical non-existence of the historically overvalued oxidized PTH, evidencing the importance of exact and precise PTH quantification, especially in cohorts of delicate patients with chronic kidney diseases and on hemodialysis.

Chapter 3. Glucagon as Proof-Of-Concept Intact Protein SPE LC-MS/MS Analysis

Within the field of intact protein analysis by LC-MS/MS, solid-phase extraction (SPE) may become a powerful tool that enables the extraction, purification, and enrichment of intact proteins from complex biological samples without the need of antibodies. The application of SPE to intact protein analysis holds immense potential, but its successful implementation requires a thorough demonstration of its capabilities when coupled to an LC-MS/MS.

For the purpose of our work, glucagon, a peptide hormone of clinical interest, was chosen as a proof-of-concept analyte. It is a single chain peptide hormone with 29 amino acids whose relatively small size, structure, low circulating concentrations and physiological relevance made it an ideal candidate for benchmarking LC-MS/MS intact protein analysis using SPE. Also, due to its peptide nature, its susceptibility to degradation and sensitivity to experimental conditions were intended to mirror some of the potential complexities that could be encountered in larger and more complex proteins such as PTH.

The following chapter is dedicated to glucagon and the development of its SPE based intact protein LC-MS/MS assay.

3.1 Introduction to Glucagon

Glucagon study dates all the way back to the nineteenth century. Paul Langerhans, a medical student in 1869, observed little isolated clusters of cells distributed throughout the exocrine pancreatic tissue but was unaware of their importance (1). In the 1890s, these islets, which comprise 1-2 percent of the pancreas's bulk (average weight 70-100 grams), were called in his honor. Many scientists suspected they were the source of an unknown compound that regulated glucose levels. In 1909, de Meyer, a Belgian physician, suggested the term insulin (the islet hormone) for this unidentified molecule, which was not effectively isolated until 1921 (2).

While trying to prepare insulin from pancreatic extracts, Banting and Best (3) observed that the extracts contained a “toxic fraction” that caused a rise in blood glucose, as opposed to what was desired. The products they studied had both hyperglycemic and hypoglycemic characteristics (4). Two years later, in 1923, Murlin et al. isolated pancreatic secretions with only hyperglycemic action and coined the term glucagon (GLUcose-AGONist) to refer to this potential active compound (5).

In 1948, Nobel-prize awarded Sutherland and De Duve successfully identified and located glucagon in the α-cells of the islets of Langerhans and developed a method to isolate it (6,7). Its amino acid sequence was firstly identified by Bromer et al. in 1956 (8) and it was not until 1959 that Unger et al. developed the first radioimmunoassay for its quantitation (9), which was key to unveil its physiological role. Of note, this radioimmunoassay was the second to ever be developed after its first application on insulin quantitation by Yalow and Berson (10,11), and it was first implemented in Europe at the CHU of Liège (12).

3.1.1 Physiological role of glucagon

Glucagon is a hormone produced by the alpha cells of the pancreas. It plays a crucial role in regulating blood glucose levels in the body. When glycemia levels are low, glucagon is released into the bloodstream to stimulate the liver to convert stored glycogen into glucose, which is then released into the bloodstream. This process, known as glycogenolysis, helps increase blood sugar levels and provides a source of energy for the body.

Glucagon acts opposite to insulin, also produced by the pancreas, which reduces blood glucose levels. While insulin promotes glucose uptake by cells, glucagon promotes glucose release from the liver. Together, insulin and glucagon maintain the balance of glucose in the bloodstream, ensuring that the body's energy needs are met (13).

3.1.2 Impact of glucagon in diabetes physiopathology

In 1975, Unger and Orci presented the bihormonal theory to further explore the physiopathology of diabetes, whether it is type 1 or type 2. They postulated that diabetes mellitus was not only a simple consequence of relative or absolute insulin deficiency by itself, but also a dysregulation of glucagon levels (14). Today, there is a great deal of clinical and experimental evidence to support this theory that glucagon plays a significant role in the progression of the disease. Normal or even elevated glucagon levels have been found in hyperglycemic diabetic patients. Since glucose plays a role of inhibitor of glucagon secretion, it is quite aberrant to return to normal or even increased values of glucagon under these physiological conditions. Insulin also plays a role in inhibiting the secretion of glucagon and is lacking in these patients. In fact, when insulin

is administered to a type 1 diabetes patient, glucagon levels normalize, depicting the important role of insulin in suppressing the secretion of glucagon.

Dysfunctions in the secretion of glucagon have also been observed in situations of hypoglycemia in diabetic patients. The secretion of α-cells in hypoglycemia is impaired in diabetics, especially in those on insulin. This significantly increases the risk of a severe hypoglycaemic episode. This hypoglycemia may be caused on the one hand by the administration of exogenous insulin which induces hypoglycemia and on the other hand by insufficient secretion of glucagon. This is an important factor to consider when treating diabetes with insulin, thus oral antidiabetic treatments are preferred when effective.

The role of glucagon in diabetes, specially type 2, seems to have been underestimated for a long time. The fact that the glucagon level is abnormally high compared to the hyperglycemic situation of uncontrolled diabetes in affected patients appears to have long been overlooked by research. Diabetes is no longer considered as a disease exclusively resulting from a simple lack and / or resistance to insulin, but rather as a metabolic disease involving both a deregulation of insulin metabolism and that of its antagonist: glucagon.

3.1.3 Synthesis

Alpha cells are in charge of producing glucagon, a peptide hormone encoded by GCG gene on chromosome II. Glucagon is first synthesized as a single polypeptide called preproglucagon. Preproglucagon has a short tail called the signal peptide which is cleaved to form proglucagon, a common precursor than can be further cleaved to form

glucagon and other active proteins such as glucagon like peptides-1 and 2 (GLP-1 and GLP-2), oxyntomodulin, glicentin, glicentin related pancreatic polypeptide (GRPP), and major proglucagon fragment (MPGF). The cleaving of proglucagon is dependent on the organ in which it is processed, for instance, in the alpha-cells of the pancreatic islet, prohormone convertase 2 causes glucagon, GRPP, intervening peptide 1 and a major proglucagon fragment to be the most prevalent products. On the other hand, in the intestinal L-cells and specific CNS neurons, prohormone convertase 1 enhances the production of GLP-1, GLP-2, oxyntomodulin, glicentin and intervening peptide 2 (15).

3.1.4 Existing techniques for Glucagon quantitation

The first technique for determining plasma glucagon was based on a radioimmunoassay (RIA) described by Unger et al. in the early 1960s (9). Since then, other methods, such as RIA or enzyme-linked immunosorbent assay (ELISA), have been described. However, these methods suffer from lengthy sample preparation as well as potential cross-reactivity issues inherent in immunoassays, specifically with immature glucagon peptides, degradation metabolites, and other proglucagon-derived peptides.

LC-MS/MS procedures for glucagon have already been described in the literature, some requiring digestion and others depend on multiple sample preparation steps that challenge the feasibility of the assay, especially if aimed for routine analysis (16–18).

Currently, and to the best of our knowledge, we presented the first fast and reliable LC-MS/MS method for glucagon analysis, which is currently being used for routine analysis at the CHU of Liège.

3.1.5 References

1. Langerhans P. Ueber die Nerven der menschlichen Haut. Archiv für Pathologische Anatomie und Physiologie und für Klinische Medicin. 1868;44:325–37.

2. Vecchio I, Tornali C, Bragazzi NL, Martini M. The Discovery of Insulin: An Important Milestone in the History of Medicine. Front Endocrinol (Lausanne). 2018;9.

3. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J. 1922;12:141–6.

4. Fisher NF. I. Preparation of Insulin. American Journal of Physiology-Legacy Content. 1923;67:57–64.

5. Kimball CP, Murlin JR. Aqueous extracts of pancreas. Journal of Biological Chemistry.1923;58:337–46.

6. SUTHERLAND EW, DE DUVE C. Origin and distribution of the hyperglycemic-glycogenolytic factor of the pancreas. J Biol Chem1948 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology.; 1948;175:663–74.

7. SUTHERLAND EW, CORI CF. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J Biol Chem. 1949;180:825–37.

8. Bromer WW, Sinn LG, Staub A, Behrens OK. The Amino Acid Sequence of Glucagon. Diabetes. 1957;6:234–8.

9. Unger RH, Eisentraut AM, McCall MS, Keller S, Lanz HC, Madison LL. Glucagon Antibodies and Their Use for Immunoassay for Glucagon. Exp Biol Med.1959;102:621–3.

10. Unger RH. Radioimmunoassay of glucagon. Metabolism. 1973;22:979–85.

11. Yalow RS, Berson SA. IMMUNOASSAY OF ENDOGENOUS PLASMA INSULIN IN MAN. Journal of Clinical Investigation. 1960;39:1157–75.

12. LEFEBVRE PJ, LUYCKX AS. Glucose and Insulin in the Regulation of Glucagon Release from the Isolated Perfused Dog Stomach*. Endocrinology. 1978;103:1579–82.

13. Taborsky GJ. The Physiology of Glucagon. J Diabetes Sci Technol. 2010;4:1338–44.

14. Unger R, Orci L. The Essential Role of Glucagon in the Pathogenesis of Diabetes Mellitus. The Lancet. 1975;305:14–6.

15. Sandoval DA, D'Alessio DA. Physiology of Proglucagon Peptides: Role of Glucagon and GLP-1 in Health and Disease. Physiol Rev. 2015;95:513–48.

16. Howard JW, Kay G, Tan T. Development of a high-throughput UHPLC – MS / MS (SRM) method for the quantitation of endogenous glucagon from human plasma. 2014;6:3295–309.

17. Howard JW, Kay G, Jones B, Tan T, Creaser CS. Development of a UHPLC – MS / MS (SRM) method for the quantitation of endogenous glucagon and dosed GLP-1 from human plasma. 2017;9:733–51.

18. Miyachi A, Kobayashi M, Mieno E, Goto M, Furusawa K, Inagaki T, et al. Accurate analytical method for human plasma glucagon levels using liquid chromatography-high resolution mass spectrometry: comparison with commercially available immunoassays. Anal Bioanal Chem. Analytical and Bioanalytical Chemistry; 2017;409:5911–8.

3.2 Development and validation of a fast and reliable method for the quantification of glucagon by liquid chromatography and tandem mass spectrometry

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Development and validation of a fast and reliable method for the quantification of glucagon by liquid chromatography and tandem mass spectrometry

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ABSTRACT

Introduction: The quantitation of glucagon remains a challenging immunoassay, mainly due to cross-reactivity. A sensitive, rapid and specific intact glucagon method is therefore necessary for quality routine analysis. A tandem mass spectrometry method to fulfill this objective is described in this work.
Methods: Glucagon was extracted from plasma employing a mixed-mode anion exchange solid-phase extraction. Sample stability was assessed in K2-EDTA and P800 tubes at different temperatures. We compared our method to two different immunoassays. FDA and EMA guidelines were followed for validation. An external quality control program served for comparison with other laboratories.
Results: Assay imprecision was below 4%. Recoveries were within 95–103%. LoQ was 8.75 pg/mL. Total analytical CV was 2.91%. Samples were found stable at 4 °C for less than 4 h. Diasource® RIA disagreed with our method. Mercodia® ELISA provided a closer agreement, also proven by external quality control samples.
Conclusions: A rapid and specific LC-MS/MS method for glucagon quantitation has been developed, validated and is suitable to routine care. The simplicity and the good performances in terms of time and specificity, could open the possibility to establish a standardized method for glucagon.

1. Introduction

Glucagon is a 29 amino acid peptide with a molecular weight of 3483 Da and is mainly secreted by the pancreas. It is derived from the posttranslational processing of proglucagon precursor, whose codifying gene (Gcg) is expressed in multiple tissues, such as the brain, intestine, and pancreas. The proteolytic cleavage of the precursor is strongly dependent on the organ where it is processed [1]. For instance, proglucagon-derived peptides such as glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), oxyntomodulin and glicentin are peptides predominantly obtained in intestinal L-cells and the brain. In contrast, proglucagon-cleaving enzymes producing glucagon are mainly found in pancreatic α-cells [2].

In the event of hypoglycemia, glucagon acts by promoting glycogen breakdown and stimulating gluconeogenesis in the liver, hence acting as a key hormone in glucose homeostasis. Its regulation mechanism is somewhat opposed to that of insulin. Therefore, it is normally found at low concentrations (picomolar range) in the basal nonfasting state and with increased concentrations in response to low glycemic levels, typically, in fasting states [3].

The first technique for plasma glucagon determination was based on a radioimmunoassay (RIA) described in the early 1960 s by Unger et al. [4]. Other methods, either RIA or enzyme-linked immunosorbent assay (ELISA), have been described ever since. However, these methods suffer from long sample preparation [5,6], as well as possible cross-reactivity issues intrinsic to immunoassays, specifically with immature glucagon peptides, degradation metabolites and other proglucagon-derived peptides as mentioned above [7,8].

Glucagon is a relatively small peptide suitable for intact LC-MS/MS analysis, avoiding complicated proteolytic digestion steps normally used for quantitative proteomics by mass spectrometry [9] for which some LC-MS/MS methods have been described [5,6,10,11]. Although more specific than immunoassays, these methods are time consuming and sometimes not sensitive enough for clinical purposes as glucagon has very low expected concentrations and because of the known stability issues in preanalytical steps [12]. For these reasons, accurate determination of

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Table 1
Specific compound conditions.

Precursor ion	RT (min)	Product ion	DP (V)	SRM transitions native compound	SRM transitions SIL-IS
$[M + 5H]^{+5}$	3.7	$[M + 5H\text{-}NH_3]^{+5}$	96	**697.58 > 694.02*** (EP = 12, CE = 21, CXP = 30)	698.65 > 695.20 (EP = 12. CE = 21, CXP = 30)
$[M + 5H]^{+5}$	3.7	$[b24]^{+3}$	96	697.58 > 940.70 (EP = 12,CE = 31, CXP = 30)	698.65 > 942.80 (EP = 12,CE = 31, CXP = 30)
$[M + 4H]^{+4}$	3.7	$[b26]^{+3}$	96	871.46 > 1040.20 (EP = 12,CE = 37, CXP = 25)	873.20 > 1042.80 (EP = 12,CE = 37, CXP = 25)

RT: Retention Time, SRM: Selected Reaction Monitoring, DP: Declustering Potential. Information in brackets: Entrance Potential (EP), Collision Energy (CE) and Cell Exit Potential (CXP), in volts (V).
* Transition selected for quantification.

glucagon remains very challenging. This work aimed to develop and validate a robust and sensitive LC-MS/MS method for simple and fast determination of plasmatic intact glucagon for routine application purposes. No digestion step was included in the sample preparation as glucagon is a relatively small peptide suitable for intact protein analysis. The performance of the method was compared to existing commercial immunoassays.

2. Materials and methods

2.1. Materials and reagents

LC-MS grade acetonitrile, methanol, and water were purchased from Biosolve BV (Valkenswaard, The Netherlands). LC-MS grade formic acid was purchased from Fisher Chemicals (Loughborough, UK). A 25% solution of NH_4OH from Merck (Merck KGaA, Darmstadt, Germany) was used as a concentrated basic buffer. Additionally, Bovine Serum Albumin (BSA) from Sigma-Aldrich (St. Louis, MO, USA) was used in spiking solutions to reduce nonspecific binding to laboratory consumables. Solid-Phase Extraction (SPE) μElution MAX mixed-mode anion-exchange plates were purchased from Waters (Milford, MA, USA). Protein LoBind Eppendorf vials were used throughout the sample preparation.

European Pharmacopeia Reference Standard (Y0000191) of glucagon (HSQGTFTSDYSKYLDSRRAQDFVQWLMNT) was obtained from Sigma-Aldrich and ^{13}C,^{15}N-stable isotope-labeled internal standard (SIL-IS) peptide (HSQGTFTSDYSKYL[^{13}C,^{15}N]DSRRAQDFVQWLMNT) was custom synthesized by Eurogentec (Liège, Belgium).

A purified EDTA-Plasma MSG 7000 surrogate matrix (not containing glucagon) was purchased from Golden West Biologicals (Temecula, CA, USA).

2.2. LC-MS/MS conditions

Separation and quantification were achieved by using a Nexera X2 UPLC from Shimadzu (Shimadzu Corporation, Kyoto, Japan) coupled to a QT6500 triple quadrupole mass spectrometer from Sciex (AB Sciex, CA, USA) fitted with an IonDrive™Turbo V source. The column used for separation was an ACQUITY UPLC Peptide BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm X 100 mm from Waters maintained at 55 °C. The mobile phases for the chromatographic separation were A (water) and B (acetonitrile) both containing 0.01% of formic acid. The binary gradient profile was set at a total flow of 0.35 mL/min with the B phase percentage changing as follows: 0 min, 20%, 1.0 min, 20%, 6.0 min, 60%, 6.2 min, 95%, 8.5 min, 95% with finally equilibration at 20% for 3.0 min. Samples were kept in an autosampler at 5 °C and the optimum injection volume was found to be 40 μL. Carryover effects were assessed by injecting a blank sample after the most concentrated points in the calibration curve. Positive electrospray (ESI + Turbospray) was chosen as the ionization mode for MS/MS operation in Multiple Reaction Monitoring (MRM). Source conditions were optimized to the following values: Curtain gas at 40 PSI, collision gas at 12 PSI, ion source gasses 1 and 2 at 40 PSI and 70 PSI, respectively, source voltage at 5.5 kV and temperature at 500 °C. Analyst® Software v1.6.2 (Sciex) was used to operate the system and Multiquant® Software v3.0.8664 (Sciex) served for peak review and integration. Optimized MS/MS conditions are displayed in Table 1.

2.3. Standard and quality control samples

Standards for natural and internal standard compounds were reconstituted in 10% acetonitrile with 0.1% of formic acid at a concentration of 1 mg/mL and 0.5 mg/mL, respectively. Aliquots of 20 μL were transferred to 1.5 mL Protein LoBind vials, and then centrifuged and frozen at −80 °C.

Spiking solutions for the calibration curve were freshly prepared at 8 different concentrations (10 ng/mL, 7.5 ng/mL, 5 ng/mL, 2.5 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.25 ng/mL, 0.125 ng/mL) by diluting standard solutions using 10% acetonitrile with 0.1% of formic acid and 0.1% (w/v) BSA.

Calibration curves were prepared by spiking 20 μL of each spiking solution into 380 μL of 10% acetonitrile with 0.1% formic acid and 0.1% BSA to obtain an 8-point calibration curve (6.25 pg/mL, 12.5 pg/mL, 25 pg/mL, 50 pg/mL, 125 pg/mL, 250 pg/mL, 375 pg/mL, 500 pg/mL).

Quality control (QC) samples for validation were prepared in the same way as calibration points at 8.75 pg/mL, 17.5 pg/mL, 26.25 pg/mL, 250 pg/mL, and 437.5 pg/mL.

All solutions, calibrators and QC were freshly prepared for each run.

2.4. Sample preparation

Samples were aliquoted from leftover K2-EDTA tubes. These tubes were immediately centrifuged after blood collection at 1500 g for 10 min. From the obtained plasma fraction, 400 μL was aliquoted to 1.5 mL Protein LoBind vials, and then spun and frozen at −80 °C until the day of the analysis.

For each batch, calibrators, QCs and samples were spiked with 20 μL of SIL-IS at 4 ng/mL and diluted with 380 μL of a 5% solution of NH_4OH. Sample clean up and preconcentration was achieved with μElution MAX mixed-mode anion-exchange SPE plates. Wells were conditioned with 200 μL of methanol followed by 200 μL of a 2.5% NH_4OH solution, then 750 μL of diluted samples were loaded and washed first by 200 μL of a 5% solution of NH_4OH and second, by 80% acetonitrile. Finally, samples were eluted twice by 25 μL of 75% acetonitrile, 0.2% formic acid to Eppendorf V-PP 96-well plates. To match the initial chromatographic conditions, each eluate was diluted and vortexed with 60 μL of water.

2.5. Method validation

The method was validated according to the EMA Bioanalytical method validation [13] and the FDA Guidance for Industry – Bioanalytical Method Validation [14] guidelines. Parameters such as measurement uncertainties and sigma performance were also evaluated.

2.5.1. Calibration curve and linearity

The 8-point calibration curve was calculated from the peak area ratio of the native standard to the labeled IS using weighted ($1/x^2$) least squares regression. The **linearity** of the method was assessed following the Emancipator Kroll method, as recommended by CLSI EP6A. Linear,

quadratic and cubic fits, weighted ($1/x^2$), were applied to the calibration curve. Linearity was considered acceptable when the difference between the nonlinear and linear fit was below or equal to 5%.

2.5.2. Recovery and matrix effects

The **extraction recovery and matrix effects** were calculated using the IS, as described by Matuszewski et al. [15] by analyzing three sets of samples in triplicate. The first set of samples (A) did not contain plasma but a surrogate matrix (containing 10% acetonitrile, 0.1% Formic Acid and 0.1% BSA) spiked with glucagon IS. The second set of samples (B) were obtained from a fresh pool of plasma, spiked after the SPE. Finally, the third set of samples (C) were obtained from the same pool spiked before the SPE.

Recovery and matrix effect were calculated as follows:

$$Recovery(\%) = \frac{AUC(C)}{AUC(B)} \times 100$$

$$Matrix\ \ Effect(\%) = \frac{AUC(B)}{AUC(A)} \times 100$$

where AUC are the peak areas for each set.

2.5.3. Limit of quantitation

The **Limit of Quantitation** (LoQ) was determined from the lowest QC that gave a coefficient of variation (CV) below 20% and a signal-to-noise ratio (S/N) above 10.

2.5.4. Precision and accuracy

Intra-assay precision was checked by analyzing five replicates of the five QC concentrations spanning the calibration range in a single run. **Interassay precision and accuracy** were evaluated by analyzing 15 replicates of the five QC concentrations in three separate runs. Accuracy and precision for each QC level were estimated by single-nested ANOVA according to CLSI EP-5A3 [16].

2.5.5. Uncertainty assessment

According to the Joint Committee for Guides in Metrology document JCGM 100:2008 for the expression of uncertainty of measurement [17], four kinds of uncertainties were calculated: the first (**uncertainty of the bias**) is related to the bias of the method; the second (**combined uncertainty**) is related to both the bias and the intermediate precision standard deviation; the third (**expanded uncertainty**) corresponds to the uncertainty multiplied by a coverage factor of k = 2, delimiting an interval within which the true value should appear with a 95% confidence level. Finally, the fourth (**relative uncertainty**) represents the expanded uncertainty divided by the introduced concentration for each level of the validation results and delimits an interval (centered on the introduced value) within which the true value should appear, still with a 95% confidence level.

Desirable uncertainty was established according to the following formula:

$$UNC_{des} = \pm 2\sqrt{(CV_{des}{}^2 + Bias_{des}{}^2)}$$

where;

$$CV_{des} = \frac{CV_i}{2}$$

$$Bias_{des} = 0.25\sqrt{CV_i{}^2 + CV_g{}^2}$$

$\mathbf{CV_i}$ and $\mathbf{CV_g}$ were 19% and 28% respectively, as obtained from the studies performed by Widjaja et al. [18].

2.5.6. Performance evaluation

The σ-performance of the method was calculated as follows:

$$\sigma = \frac{Total\ acceptable\ error\ (TEa) - Bias}{CV_n}$$

where the total acceptable error derived is from the sum of the biological and technical variability [19], according to the following formula:

$$TEa = 1.65CV_{des} + |bias_{des}|$$

2.6. Sample stability

To assess glucagon stability in the whole blood fraction, 5 healthy volunteers donated 14 tubes each, distributed in two types of collection vials: a) K2-EDTA (n = 7) and b) P800 (K2-EDTA + Proprietary cocktail of protease, esterase and DPP-IV inhibitors) (n = 7). Immediately after blood extraction (t = 0), one tube of each kind was centrifuged and frozen at −80 °C. The rest of the specimens were kept at room temperature and 4 °C simultaneously for 1 h, 4 h and 8 h before being centrifuged and frozen at −80 °C no more than one week before analysis.

A biological approach, according to the World Health Organization recommendations, was used to determine the maximum permissible instability for the samples, based on the obtained bias derived from the sum of the biological (CV_i) and analytical variability (CV_a) [19]. To screen the changes due to degradation of the analyte over time and the eventual effect of stabilizers in the collection tube, the Total Change Limit (TCL), as defined by Oddoze et al., [20] was calculated using the following formula:

$$TCL = \sqrt{(1.96x\sqrt{2}xCVa)^2 + (0.5xCVi)^2}$$

where the CV_a was calculated from the validation results.

2.7. Method comparison

2.7.1. Standard samples

Calibration standards from Diasource RIA (ref. RB310) (DIAsource Immunoassays S.A., Louvain-la-Neuve, Belgium) and Mercodia ELISA (ref. 10–1271-01) (Mercodia AB, Uppsala, Sweden) were analyzed by LC-MS/MS. According to the manufacturer, Diasource RIA calibration standards contained 32.75 pg/mL, 65.5 pg/mL, 130.66 pg/mL, 261.32 pg/mL and 522.65 pg/mL and Mercodia ELISA standards contained 10.4 pg/mL, 32.4 pg/mL, 97.3 pg/mL and 423.9 pg/mL. Additionally, the LC-MS/MS calibration standards including blanks were analyzed by the Diasource RIA and Mercodia ELISA.

2.7.2. Patient samples

Patient samples were analyzed using the DIASource RIA kit, the Mercodia ELISA kit and our developed LC-MS/MS method. A total of 63 patient samples (n = 63) were processed independently by the three techniques. Passing-Bablok and Bland-Altman equations were used for method comparison.

2.8. External quality control samples

External quality control samples were supplied by ProBioQual (Lyon, France) to compare our method with other participant labs who used other commercially available techniques. ProBioQual granted consent to use their data from the *Évaluation Externe de la Qualité* program (EEQ) for this purpose.

2.9. Observed values in healthy subjects

To verify glucagon's reference range against previous studies [21,22], a total of 47 samples (n = 47) were processed from a volunteer population of healthy men (n = 20) and women (n = 27). Initially, a Mann-Whitney test was performed to assure there were no significant differences between both genders. Finally, the 95% double-

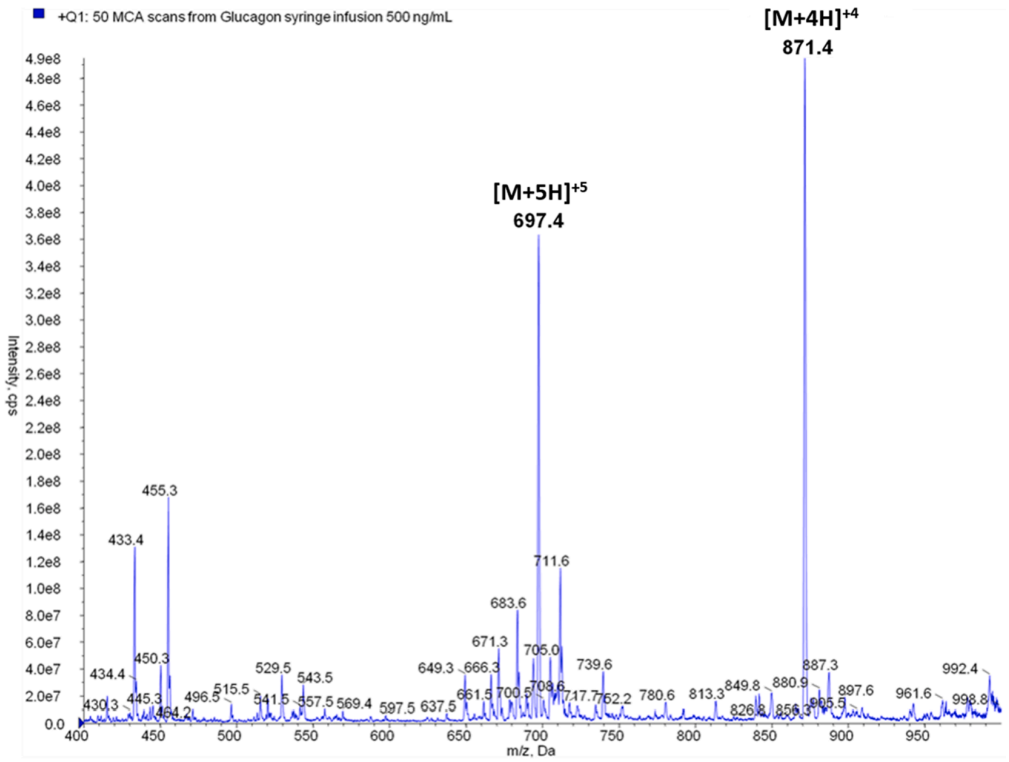

Fig. 1. Q1 Scan of a 500 ng/mL glucagon solution syringe infusion.

sided reference interval was estimated by using the nonparametric percentile method, as defined by CLSI C28-A3 [23].

2.10. Statistical analysis

Statistical analysis for method validation and comparison was performed using MedCalc software version 18.10.2, R version 4.0.2 and MS Excel 2019.

3. Results

3.1. Method development

Glucagon's most intense precursor ions were determined by direct infusion to the mass spectrometer working in Q1 scan mode and corresponded to the $[M + 5H]^{+5}$ and $[M + 4H]^{+4}$ ions at 697.4 *m/z* and 871.4 *m/z*, respectively, as shown in Fig. 1.

These precursors were fragmented in Product ion mode and three masses were observed at *m/z* 694.0 (ammonia loss), *m/z* 940.7 (fragment b24) and *m/z* 1040.2 (fragment b26), as displayed in Fig. 2. The two last product ions (940.7 and 1040.2) were observed by fragmenting any of the two precursors, whereas the 694.0 fragment was exclusively observed for the first precursor (697.4).

MS conditions were optimized using Flow Injection Analysis (FIA) using the developed chromatographic method.

Effective chromatographic separation of glucagon from potential interferences in human plasma was achieved using the developed chromatographic method. Specificity was further confirmed by the ion ratio of the qualitative transitions (displayed in Fig. 3) with a 20% tolerance. No carryover effect was observed as no peaks were observed in blank samples after injection of the high concentration solutions above the calibration curve (1000 pg/mL).

3.2. Method validation

3.2.1. Calibration curve and linearity

Good linearity was obtained along the entire calibration curve with differences between nonlinear models below 0.7%, as shown in Figs. 4 and 5.

3.2.2. Recovery and matrix effects

According to the procedure described above, the SPE recoveries were 83% ±5%. The matrix effect was 101% ±3%.

3.2.3. Limit of quantitation

The lowest QC level (8.75 pg/mL) presented an average S/N of 12.7 with a CV of 4.23%, therefore it was considered as the LoQ. Similar S/N values were obtained in real patient samples, as can be observed in Fig. 6.

3.2.4. Precision and accuracy

Coefficients of Variation (CV) for intra-assay and interassay precision experiments were lower than 4.23% in all samples. CVs for the technique was calculated to be 2.91%. Values for each point in the validation can be consulted in Table 2.

3.2.5. Uncertainty assessment

UNC_{des} was established at ± 25%, considering a CV_{des} of 9.5% and a $Bias_{des}$ of 8.5%. Values for all four types of measurement uncertainty are given in Table 2.

3.2.6. Performance evaluation

A total acceptable error (TEa) of 24.2% was estimated, and, therefore, if considering an average relative bias of 1% and an average CVa of 2.91% (See Table 2), a σ-performance higher than 7 can be reported, which indicates an excellent performance for the method.

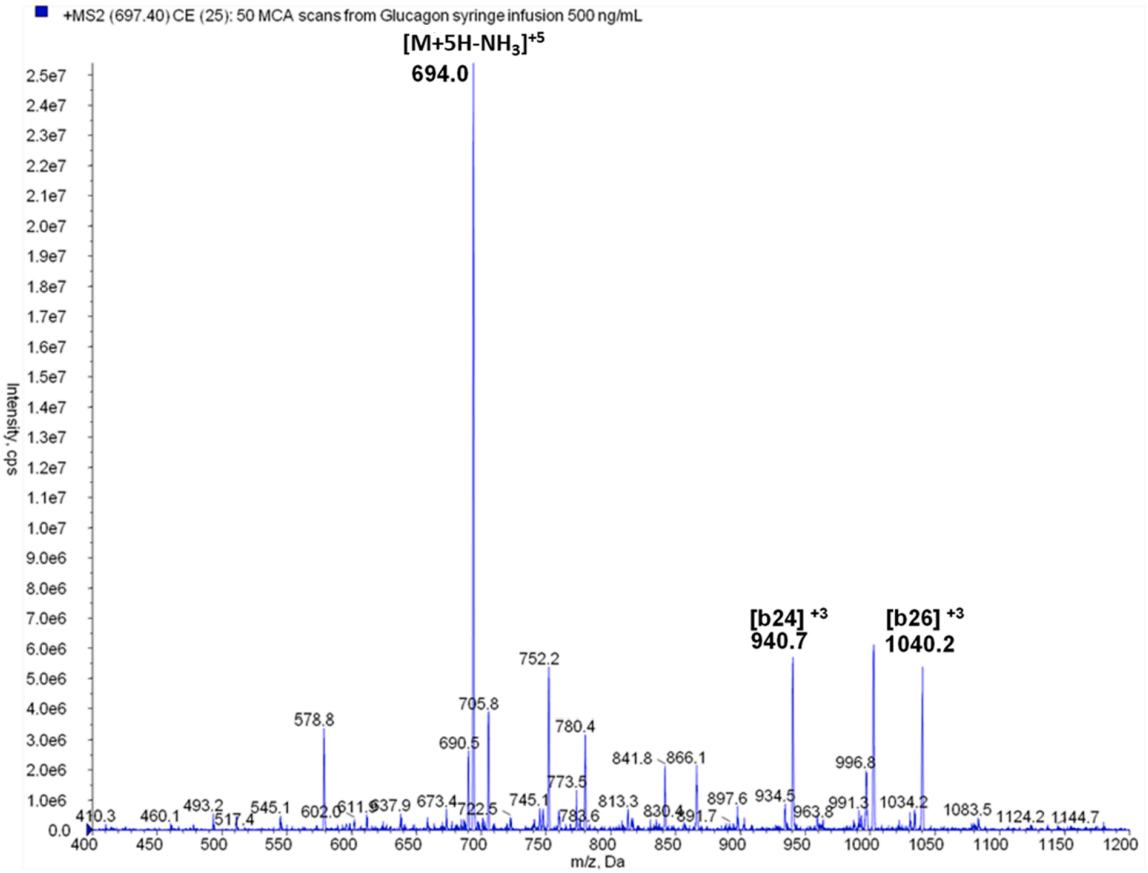

Fig. 2. Product ion scan of 697.4 from a 500 ng/mL glucagon solution syringe infusion.

3.3. Sample stability

A TCL of 13% was fixed for the current method, therefore samples containing > 87% of the initial concentration should be considered as stable. The values given in Table 3 indicate that all specimens kept at 4 °C for less than 4 h, either in P800 or in normal K2-EDTA tubes, are within the limits of acceptance.

3.4. Method comparison

3.4.1. Standard samples

3.4.1.1. Radioimmunoassay (RIA). Six calibrators (including blank) from DIASource RIA were analyzed by LC-MS/MS obtaining satisfactory recoveries ranging from 88% to 92%. In contrast, for the eight LC-MS/MS calibrators (including blank) analyzed by RIA, recovery values were not consistent, ranging from 7% to 119% depending on the concentration. The blank calibrator read a concentration of 175.2 pg/mL. Bland-Altman and Passing-Bablok plots are displayed in Figs. 7 and 8.

3.4.1.2. ELISA. Five Mercodia ELISA calibrators (including blank) processed by LC-MS/MS provided successfully recoveries ranging from 91% to 103%, while LC-MS/MS calibrators analyzed by ELISA gave recoveries ranging from 73% to 112%. Bland-Altman and Passing-Bablok plots are displayed in Figs. 9 and 10.

3.4.2. Patient samples

After analyzing 63 samples by each technique, systematic and proportional differences were calculated according to a Passing-Bablok regression, as shown in Table 4.

3.5. External quality controls

A comparison between the different techniques used in external quality control samples is displayed in Table 5.

For some samples, LC-MS/MS and ELISA found concentrations below their LoQ (expressed as < LoQ) while RIA read high concentrations for the compound.

3.6. Observed values in healthy subjects

According to the Mann-Whitney test, glucagon plasmatic concentrations did not statistically differ between genders (P = 0.097). The observed values ranged from 13 pg/mL to 104 pg/mL, which is very similar to those found in the literature [8,21,22].

4. Discussion

In this project, a rapid method for specific intact glucagon quantitation was developed and validated relying on a simple solid-phase extraction with no requirement for proteolytic digestion step which greatly reduces sample preparation time compared with other techniques. Sample preparation is less than 2.5 h for up to 40 samples. However, the number of samples could be increased without having a significant impact on this time thanks to the 96-well plate support. This contrasts, with RIA and ELISA, which require overnight incubation

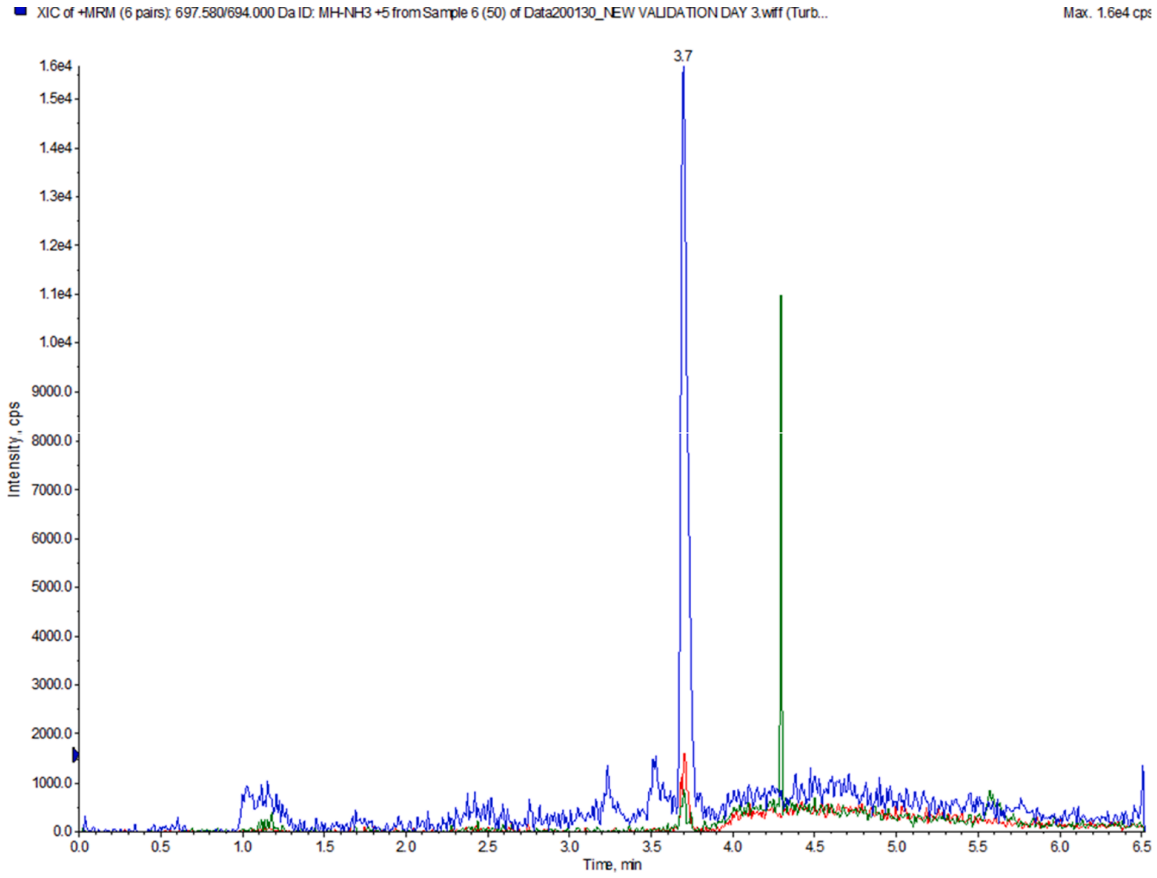

Fig. 3. Chromatogram of a 50 pg/mL glucagon solution. Transitions in order of intensity: [MH + 5H-NH3] + 5 , [b24] + 3, [b26] + 3.

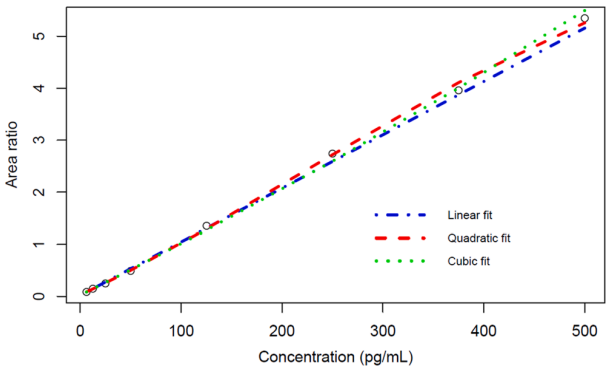

Fig. 4. 8-point calibration curve (6.25, 12.5, 25, 50, 125, 250, 375, 500 pg/mL). Linear, quadratic and fits are plotted respectively in blue, red and green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

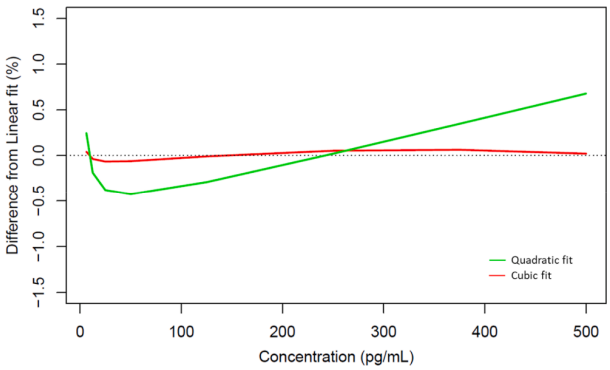

Fig. 5. Linearity difference plot. Quadratic (Green) and Cubic (Red) fits are compared to the linear fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

steps. Other intact LC-MS/MS methods found in the literature are more time demanding since they rely on protein precipitation, solid-phase extraction and evaporation steps, further complicating the sample treatment process, especially when large amounts of samples need to be processed. The method we developed is, to our knowledge, the fastest and most sensitive described in the literature.

The fact that some of the proglucagon-derived peptides have a high similarity with the whole glucagon peptide presumably leads to the evidenced cross-reactivity phenomena. It is nevertheless surprising that in the comparison made with LC-MS/MS calibrators, analyzed by RIA and LC-MS/MS, the blank (in a plasmatic matrix free of glucagon) has high detectable levels of the peptide according to the RIA, but none according to ELISA and LC-MS/MS.

This can be explained presuming that the plasma matrix used for calibrators can contain some glucagon-like molecules derived from the degradation of proglucagon or glucagon that are spuriously detected, as also happens with other immunoassays like B-natriuretic peptide (BNP) [24] and Parathormone (PTH) [25]. This effect is validated and even more pronounced in the comparison with the external quality control

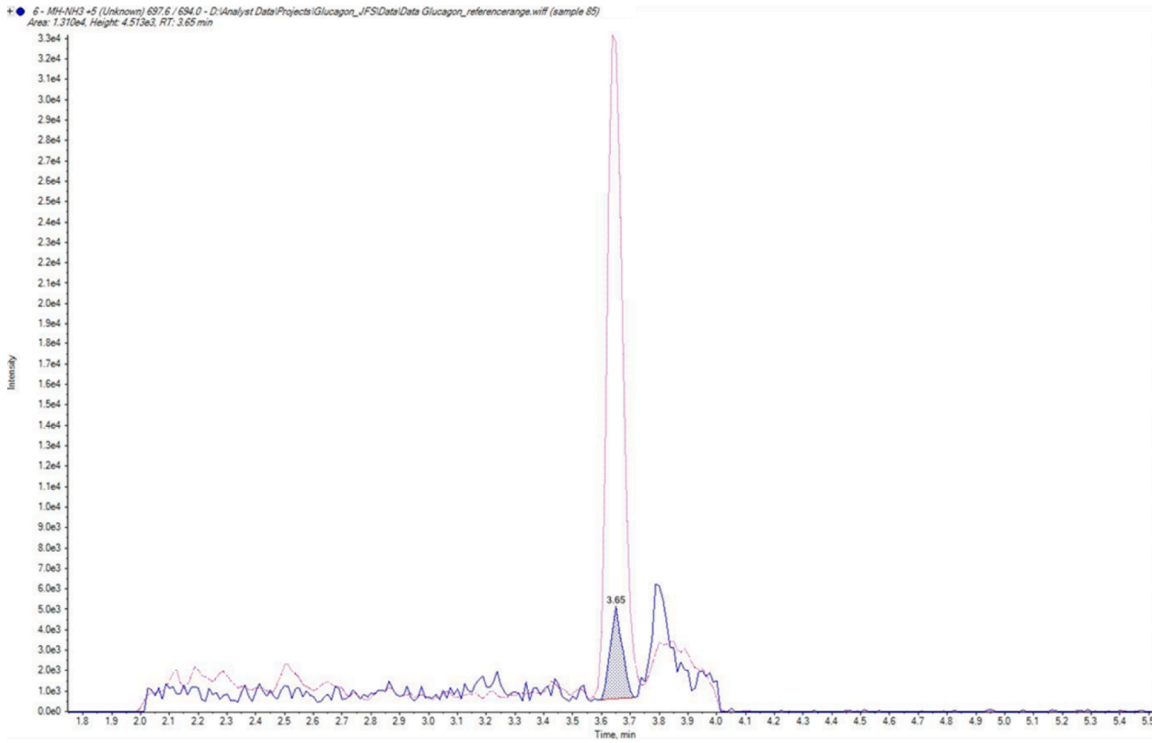

Fig. 6. Chromatogram of a real patient sample at 8.85 pg/mL.

Table 2
Validation data.

Quality control samples concentration [pg/mL]	8.75 (LLOQ)	17.5	26.25	250	437.5
CV_a Intraday	3.91%	2.12%	1.42%	2.80%	3.75%
CV_a Interday	4.23%	3.15%	2.10%	1.91%	3.15%
Total CVa	5.76%	3.80%	2.54%	3.39%	4.90%
Intraday Accuracy	98%	103%	98%	102%	100%
Interday Accuracy	99%	100%	99%	101%	99%
Relative bias	−2%	0%	−1%	−1%	−1%
Uncertainty of the bias	−0.11	−0.03	−0.16	1.41	−2.01
Combined uncertainty	0.14	0.14	0.25	2.17	4.44
Expanded uncertainty	0.28	0.27	0.50	4.34	8.87
Relative uncertainty	3.21%	1.55%	1.90%	1.74%	2.03%

Table 3
Sample stability in P800 and EDTA tubes at 4 °C and room temperature (23 °C).

	P800		EDTA	
Time (h)	4 °C	23 °C	4 °C	23 °C
0	100%	100%	100%	100%
1	90%	81%	87%	77%
4	83%	71%	83%	54%
8	79%	64%	57%	29%

samples. In these samples, RIA determinations are substantially distanced from ELISA and LC-MS/MS. This can be explained as being mainly due to the nature of the techniques, presenting different cross-reactivities with other fragments.

ELISA relies on monoclonal antibodies with enhanced affinity for more than one region of glucagon in opposition to the RIA, which uses a not very specific competitive approach, as observed, despite the manufacturer's provided information.

The use of specific techniques such as LC-MS/MS for the quantitation of diverse compounds in general, and, specifically, glucagon, becomes of great importance due to the big differences found among different methods, with possible implications in clinical practice.

According to stability studies, special attention needs to be paid to preanalytical steps. After blood collection, samples should be kept at 4 °C for less than 4 h before they are centrifuged and frozen. Otherwise, the sample stability cannot be guaranteed.

Regarding the observed values in healthy subjects, a reference range could not be established as we considered that the number of samples analyzed was insufficient. However, the results obtained were closely related to those observed in the literature [8,21,22] and were, therefore, considered as a good estimation.

The simplicity and the good performances in terms of time and specificity of the described LC-MS/MS method can open up the possibility to establish standardization for glucagon analysis.

CRediT authorship contribution statement

Jordi Farré-Segura: Conceptualization, Methodology, Validation,

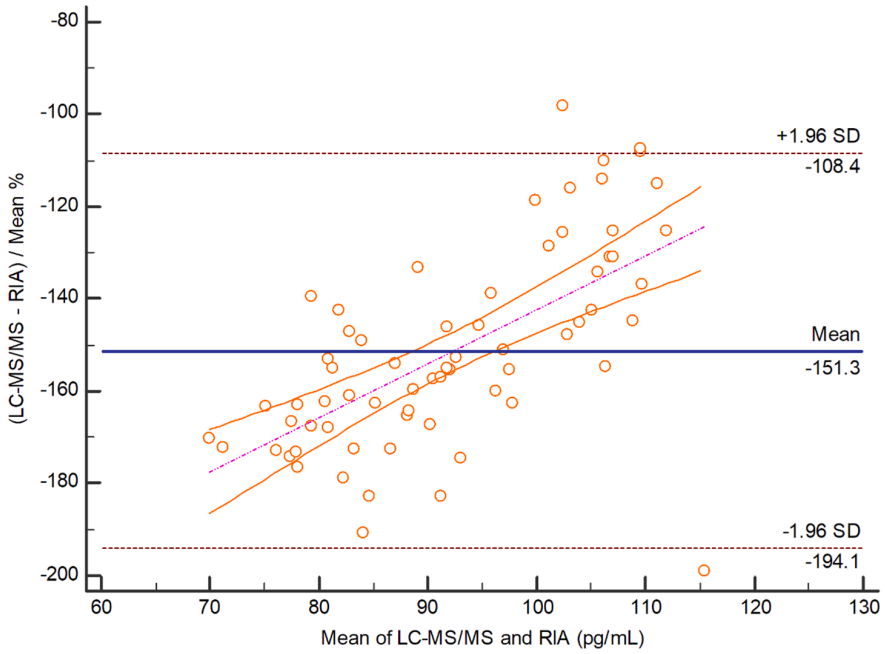

Fig. 7. LC-MS/MS vs RIA Bland-Altman plot.

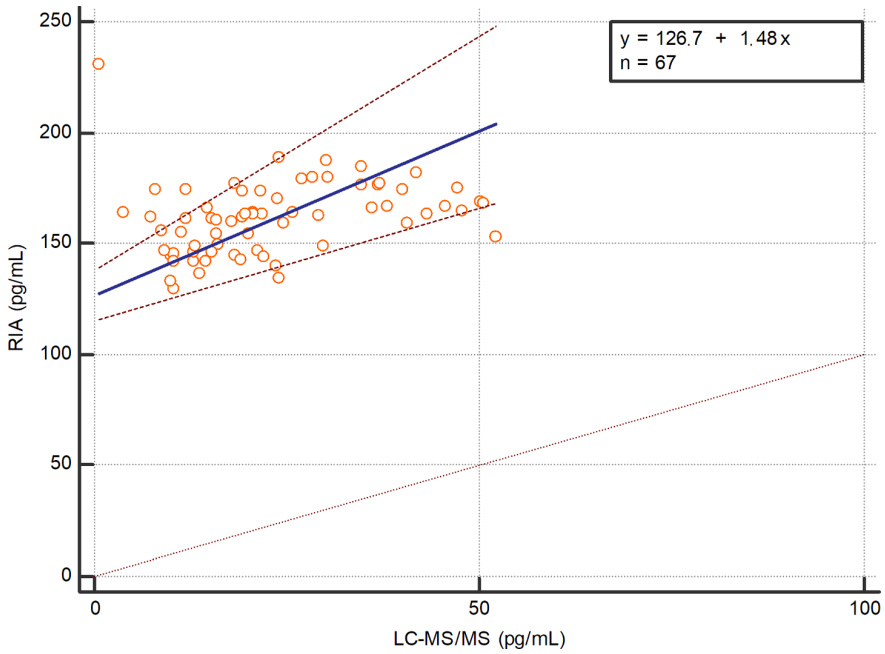

Fig. 8. LC-MS/MS vs RIA Passing-Bablok regression. y-Intercept (Systematic differences): 126.7 (115.39–137.76); Slope (Proportional differences): 1.48 (1.01–2.12). Values in brackets correspond to the 95% Confidence Interval.

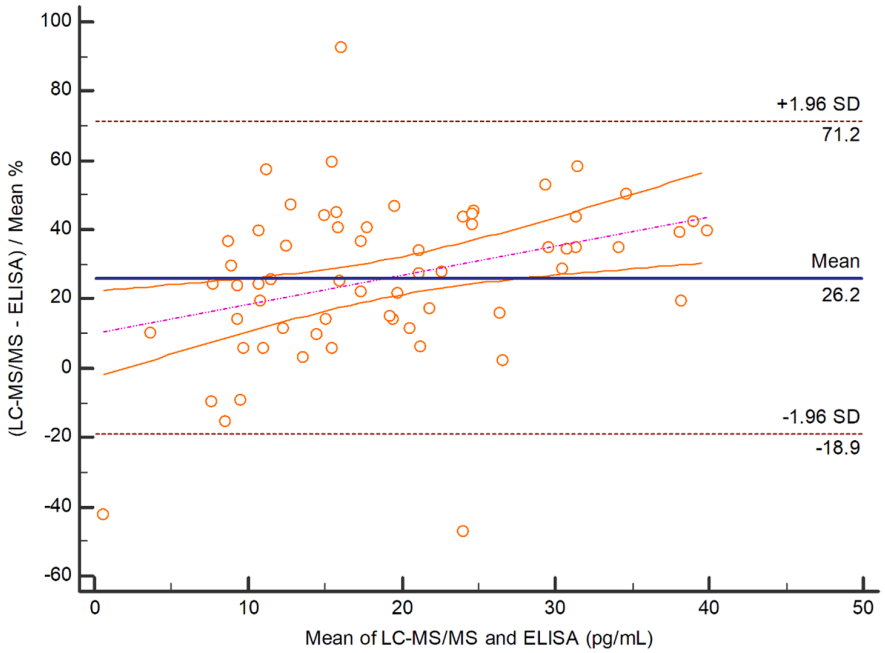

Fig. 9. LC-MS/MS vs ELISA Bland-Altman plot.

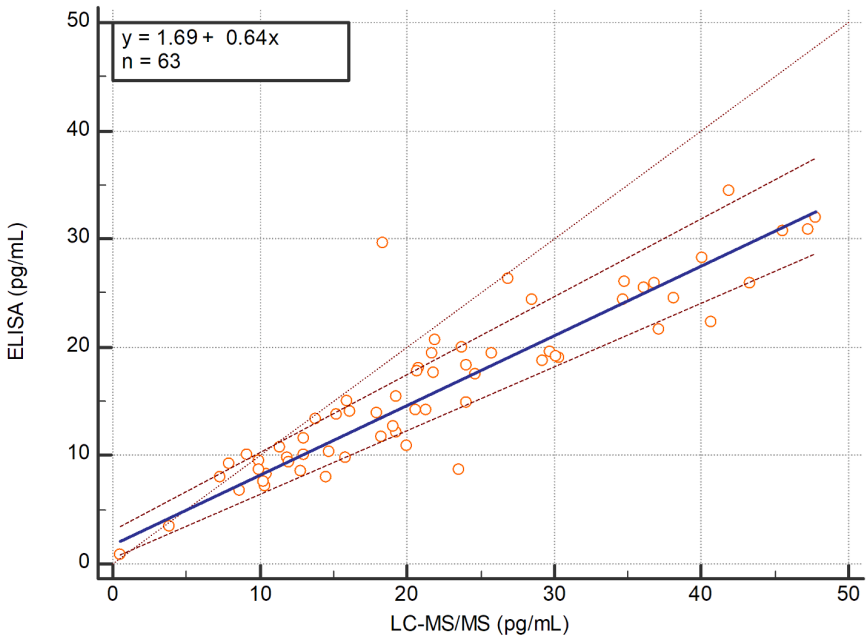

Fig. 10. LC-MS/MS vs ELISA Passing-Bablok regression. y-Intercept (Systematic differences): 1.69 (0.58–3.10); Slope (Proportional differences): 0.64 (0.59–0.72). Values in brackets correspond to the 95% Confidence Interval.

Table 4
RIA and ELISA comparison (Patient samples).

	LC-MS/MS Passing-Bablok regression (CI 95%)	
Technique	Proportional bias	Constant bias (pg/mL)
Diasource RIA	1.48 (1.01–2.12)	126.66 (115.39–137.76)
Mercodia ELISA	0.64 (0.59 – 0.72)	1.69 (0.58 – 3.10)

Formal analysis, Investigation. **Neus Fabregat-Cabello:** Conceptualization, Methodology. **Chiara Calaprice:** Conceptualization, Methodology. **Laurent Nyssen:** Conceptualization, Methodology. **Stéphanie Peeters:** Project administration. **Caroline Le Goff:** Supervision, Project administration, Resources. **Etienne Cavalier:** Supervision, Project administration, Resources, Conceptualization, Formal analysis.

Table 5
External quality control samples. Analyzed by LC-MS/MS, ELISA and RIA (pg/mL). *LC-MS/MS concentration values below LoQ, relative bias is not calculated.

	External Quality Controls (ProBioQual)						
Sample ref.	LC-MS/MS	MercodiaELISA	Rel. Bias % (LC-MS/MS)	DIASource RIA	Rel. Bias % (LC-MS/MS)	Eurodiagnostica RIA	Rel. Bias % (LC-MS/MS)
18MG08	172.42	164.75	−4%	158.15	−8%	162.2	−6%
18MG10	50.44	49.17	−3%			72.5	44%
18MG11	<LoQ	<LoQ	*	233.5	*	144.45	*
18MG12	25	16.24	−35%			37	48%
19MG05	<LoQ	<LoQ	*	181.7	*	188.33	*
19MG06	123.7	151.18	22%	47.8	−61%	170	37%
19MG07	<LoQ	<LoQ	*			86.3	*
19MG08	122.7	154.14	26%			166	*
19MG09	<LoQ	<LoQ	*			191	*
19MG10	34.2	48.26	41%			105	207%
20MG02	47.7	45.3	−5%			63.33	33%
20MG03	145.3	150.62	4%			154.33	6%

Acknowledgments

The authors would like to acknowledge ProBioQual for supplying the external quality control program samples and granting permission to use their data.

References

[1] D.A. Sandoval, D.A. D'Alessio, Physiology of Proglucagon Peptides: Role of Glucagon and GLP-1 in Health and Disease, Physiol. Rev. 95 (2015) 513–548, https://doi.org/10.1152/physrev.00013.2014.
[2] G. Jiang, B.B. Zhang, Glucagon and regulation of glucose metabolism, Am. J. Physiol. - Endocrinol. Metab. 284 (2003) E671–E678, https://doi.org/10.1152/ajpendo.00492.2002.
[3] K.M. Habegger, K.M. Heppner, N. Geary, T.J. Bartness, R. DiMarchi, M.H. Tschöp, The metabolic actions of glucagon revisited, Nat. Rev. Endocrinol. 6 (2010) 689–697, https://doi.org/10.1038/nrendo.2010.187.
[4] B.R.H. Unger, A.M. Eisentraut, M.S. Mccall, L. L, GLUCAGON ANTIBODIES AND AN IMMUNOASSAY FOR GLUCAGON * Despite extensive knowledge of the amino acid composition of beef-pork glucagon , and of the physiologic changes induced by its administration to animals and man , the role of glucagon in the homeostat, (1961) 1280–1289.
[5] A. Miyachi, M. Kobayashi, E. Mieno, M. Goto, K. Furusawa, T. Inagaki, T. Kitamura, Accurate analytical method for human plasma glucagon levels using liquid chromatography-high resolution mass spectrometry: comparison with commercially available immunoassays, Anal. Bioanal. Chem. 409 (2017) 5911–5918, https://doi.org/10.1007/s00216-017-0534-0.
[6] Y.-X. Li, M. Hackman, C.-Y. Wang, Quantitation of polypeptides (glucagon and salmon calcitonin) in plasma samples by 'high resolution' on a triple quadrupole mass spectrometer, Bioanalysis. 4 (2012) 685–691, https://doi.org/10.4155/bio.12.12.
[7] N.J.W. Albrechtsen, R.E. Kuhre, F.K. Knop, The biology of glucagon and the consequences of hyperglucagonemia, 10 (2016) 1141–1151.
[8] M.J. Bak, N.W. Albrechtsen, J. Pedersen, B. Hartmann, M. Christensen, T. Vilsbøll, F.K. Knop, C.F. Deacon, L.O. Dragsted, J.J. Holst, Specificity and sensitivity of commercially available assays for glucagon and oxyntomodulin measurement in humans, Eur. J. Endocrinol. 170 (2014) 529–538, https://doi.org/10.1530/EJE-13-0941.
[9] Y.V. Karpievitch, A.D. Polpitiya, G.A. Anderson, R.D. Smith, A.R. Dabney, Liquid Chromatography Mass Spectrometry-Based Proteomics: Biological and Technological Aspects, Ann. Appl. Stat. 4 (2011) 1797–1823, https://doi.org/10.1214/10-AOAS341.Liquid.
[10] V.N. Lapko, P.S. Miller, G.P. Brown, R. Islam, S.K. Peters, R.L. Sukovaty, P.F. Ruhn, C.J. Kafonek, Sensitive glucagon quantification by immunochemical and LC–MS/MS methods, Bioanalysis. 5 (2013) 2957–2972, https://doi.org/10.4155/bio.13.264.
[11] D.C. Delinsky, K.T. Hill, C.A. White, M.G. Bartlett, Quantitation of the large polypeptide glucagon by protein precipitation and LC/MS, Biomed. Chromatogr. 18 (2004) 700–705, https://doi.org/10.1002/bmc.380.
[12] J.W. Howard, R.G. Kay, T. Tan, J. Minnion, C.S. Creaser, Identification of plasma protease derived metabolites of glucagon and their formation under typical laboratory sample handling conditions, Rapid Commun. Mass Spectrom. 29 (2015) 171–181, https://doi.org/10.1002/rcm.7090.
[13] EMA, Guideline on bioanalytical method validation, EMEA, Comm. Med. Prod. Hum. Use. 44 (2012) 1–23.
[14] U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry Bioanalytical Method Validation Guidance for Industry Bioanalytical Method Validation, Vet. Med. (2001).
[15] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC – MS/MS, Anal. Chem. 75 (2003) 3019 – 3030., Anal. Chem. 75 (2003) 3019–3030.
[16] CLSI, Evaluation of precision of quantitative measurement procedures ; approved guideline—third edition, Wayne CLSI. EP05-A3 (2014) CLSI document EP05-A3.
[17] GUM-2008, Evaluation of measurement data — Guide to the expression of uncertainty in measurement, Int. Organ. Stand. Geneva ISBN. 50 (2008) 134. https://doi.org/10.1373/clinchem.2003.030528.
[18] A. Widjaja, R.J. Morris, J.C. Levy, K.N. Frayn, S.E. Manley, R.C. Turner, Within- and Between-Subject Variation in Commonly Measured Anthropometric and Biochemical Variables, 566 (1999) 561–566.
[19] WHO, Use of anticoagulants in diagnostic laboratory investiga- tions and stability of blood, plasma and serum samples., (2002).
[20] C. Oddoze, E. Lombard, H. Portugal, Stability study of 81 analytes in human whole blood, in serum and in plasma, Clin. Biochem. 45 (2012) 464–469, https://doi.org/10.1016/j.clinbiochem.2012.01.012.
[21] J.H. Sloan, R.W. Siegel, Y.T. Ivanova-Cox, D.E. Watson, M.A. Deeg, R.J. Konrad, A novel high-sensitivity electrochemiluminescence (ECL) sandwich immunoassay for the specific quantitative measurement of plasma glucagon, Clin. Biochem. 45 (2012) 1640–1644, https://doi.org/10.1016/j.clinbiochem.2012.07.111.
[22] J.W. Howard, G. Kay, B. Jones, T. Tan, C.S. Creaser, Development of a UHPLC – MS / MS (SRM) method for the quantitation of endogenous glucagon and dosed GLP-1 from human plasma, 9 (2017) 733–751. https://doi.org/10.4155/bio-2017-0021.
[23] B.J. et al. Horowitz GL, Altaie S, EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition, Clin. Lab. Stand. Inst. (2010). https://doi.org/56238-682-4.
[24] W.L. Miller, M.A. Phelps, C.M. Wood, U. Schellenberger, A. Van Le, R. Perichon, A. S. Jaffe, Comparison of mass spectrometry and clinical assay measurements of circulating fragments of B-Type natriuretic peptide in Patients with chronic heart failure, Circ. Hear. Fail. 4 (2011) 355–360, https://doi.org/10.1161/CIRCHEARTFAILURE.110.960260.
[25] E. Cavalier, M. Plebani, P. Delanaye, J.C. Souberbielle, Considerations in parathyroid hormone testing, Clin. Chem. Lab. Med. 53 (2015) 1913–1919, https://doi.org/10.1515/cclm-2015-0314.

Chapter 4. Validation of an LC–MS/MS method using Solid-Phase Extraction for the quantification of 1-84 Parathyroid hormone: Towards a candidate Reference Measurement Procedure

Jordi Farré-Segura, Caroline Le Goff, Pierre Lukas, Gaël Cobraiville, Marianne Fillet, Anne-Catherine Servais, Pierre Delanaye and Etienne Cavalier.

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Preamble

The accurate and precise measurement of biomarkers is pivotal for effective disease management and therapeutic monitoring. Parathyroid hormone (PTH), a critical regulator of calcium and phosphate homeostasis in the human body, exemplifies such a biomarker, particularly in the context of bone metabolism disorders and chronic kidney disease. The quantification of PTH by traditional immunoassays, while widely used, suffers from significant limitations in specificity and standardization, often leading to diagnostic ambiguities.

Addressing these challenges, this study introduces a robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, employing solid-phase extraction for the quantification of 1-84 PTH. This novel method represents a significant advancement over existing assays by eliminating reliance on antibodies, thus sidestepping the cross-reactivity issues inherent to immunoassays and enhancing the method's specificity for 1-84 PTH. Developed in compliance with rigorous guidelines, including those set by the Clinical Laboratory Standards Institute, our method not only demonstrates superior analytical performance but also establishes a new benchmark for PTH measurement.

This article details the validation of the LC-MS/MS method. The findings underscore the method’s potential to serve as a candidate reference measurement procedure, promising to enhance clinical diagnostics and, ultimately, patient care. This work, therefore, contributes significantly to the field of clinical chemistry and aligns with the ongoing efforts to standardize PTH measurement.

Clinical Chemistry 68:11
1399–1409 (2022)

Validation of an LC-MS/MS Method Using Solid-Phase Extraction for the Quantification of 1-84 Parathyroid Hormone: Toward a Candidate Reference Measurement Procedure

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BACKGROUND: Parathyroid hormone (PTH) measurement is important for patients with disorders of calcium metabolism, including those needing bone-turnover monitoring due to chronic kidney disease–mineral bone disorder. There are currently 2 generations of PTH immunoassays on the market, both having cross-reactivity issues and lacking standardization. Therefore, we developed an LC-MS/MS higher-order method for PTH analysis.

METHODS: The method was calibrated against the international standard for 1-84 PTH (WHO 95/646). Antibody-free sample preparation with the addition of an isotope-labeled internal standard was performed by solid-phase extraction. Extracts were analyzed by LC-MS/MS. EDTA-K_2 plasma was used throughout the development and validation. Bias and uncertainty sources were tested according to ISO 15193. Clinical Laboratory Standards Institute guidelines and reference measurement procedures were consulted for the design of the validation. Patient samples and external quality controls were compared between LC-MS/MS and 2 third-generation immunoassays.

RESULTS: The method was validated for 1-84 PTH from 5.7 to 872.6 pg/mL. The interassay imprecision was between 1.2% and 3.9%, and the accuracy ranged from 96.2% to 103.2%. The measurement uncertainty was <5.6%. The comparison between LC-MS/MS and the immunoassays showed a proportional bias but moderate to substantial correlation between methods.

CONCLUSIONS: This LC-MS/MS method, which is independent of antibodies, is suitable for a wide range of PTH concentrations. The obtained analytical performance specifications demonstrate that development of a reference measurement procedure will be possible once a higher order reference standard is available.

Parathyroid hormone (PTH) is an 84 single-chain amino acid peptide produced by the parathyroid glands and released into the circulation when the plasma-ionized calcium concentration decreases. PTH binds to bone and kidney cells expressing the type 1 PTH/parathyroid hormone–related peptide receptor to exert its biological activity. In the kidney, PTH stimulates the reabsorption of calcium, stimulates the activity of 1-alpha hydroxylase to produce 1,25 dihydroxyvitamin D, and decreases phosphate reabsorption (1). PTH controls bone remodeling and calcium and phosphate homeostasis by acting on chondrocytes, osteoprogenitors, osteoblasts, and osteocytes (2).

Once in circulation, the half-life of 1-84 PTH is 2 to 4 min (3), and the peptide is metabolized in the liver. This pathway leads to the production of truncated forms such as large C-terminal or mid-truncated PTH fragments. Of note, such fragments are also secreted by the parathyroid glands (4–6). These fragments have a longer half-life than 1-84 PTH itself and represent approximately 15% to 30% of the total PTH in healthy individuals. Since these fragments are cleared by the kidneys, they accumulate in the blood of patients suffering from chronic kidney disease (CKD), and can represent up to 70% to 80% of the total PTH (7, 8).

In daily practice, PTH is measured for the diagnosis of primary and secondary hyperparathyroidism (9). It is also measured in patients with CKD and patients

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undergoing hemodialysis (DIA) for the management of CKD–mineral bone disorder, a frequent complication of advanced renal disease leading to high- or low-turnover bone diseases and/or vascular or soft-tissue calcification. This measurement is achieved through immunoassays using a pair of antibodies targeted against the C- and N-terminal parts of the peptide. Two generations of immunoassays are currently present on the market. Intact (or second-generation) PTH assays have been used since 1987 (10). In these assays, an N-terminal antibody is targeted against the 13–24 N-terminal part of the peptide (except the Roche intact PTH assay in which the N-terminal antibody recognizes an epitope located further from the N-terminal). In addition to 1-84 PTH itself, these assays have been shown to recognize a family of large C-terminal fragments referred to as non-(1-84) PTH (11), which explains their overestimation of PTH in the context of secondary hyperparathyroidism (12, 13). In 1999, third-generation immunoassays (also called whole or bioactive PTH assays) appeared on the market. Third-generation assays have the N-terminal antibody directed toward the first 4 amino acids of the peptide and are presented as highly specific for the 1-84 peptide due to their absence of cross-reactivity with the non-(1-84) PTH (14, 15). Both assay generations have also been reported to cross-react with oxidized PTH, a posttranslational modified variant supposedly produced in patients undergoing strong oxidative stress, such as DIA patients (16). Third-generation PTH assays also cross-react with another posttranslational modification variant called amino-PTH, a form of nontruncated PTH (17), which is overproduced in parathyroid carcinoma (18, 19) and severe types of parathyroid hyperplasia in patients undergoing DIA (20). Discrepancies between the different PTH assays can partly be explained by the aforementioned different cross-reactivities, especially in patients in which modified PTH forms tend to accumulate (8, 19).

In addition to immunoassays, higher-order techniques, such as LC-MS/MS, have been described for PTH measurement. Such methods have the potential to become reference measurement procedures (RMPs), as long as they satisfy the required analytical performance specifications (APS) and are able to overcome cross-reactivity issues with fragments or posttranslational modification forms. To date, 2 mass spectrometry-based methods have been described using the proteolytic digestion of the peptide (21, 22). Protein digestion generates many fragments that can lead to a loss of information in the case of modifications on the protein sequence. One other published method measures 1-84 PTH without digestion by high-resolution mass spectrometry (23). Although these 3 methods may provide clinical insight into patients with hyperparathyroidism, they present rather high limits of quantification of >30 pg/mL, which approximately corresponds to higher limit of 1-84 PTH normal range, according to some immunoassays (24, 25). Additionally, these assays rely on an antibody-dependent sample preparation, which increases the assay complexity and potential sources of variability.

We describe here the validation of an LC-MS/MS method with an antibody-free sample preparation, calibrated against an internationally recognized standard (WHO 95/646), for the precise quantification of 1-84 PTH, suitable for a wide range of PTH concentrations.

Materials and Methods

MATERIALS AND REAGENTS

LC-MS grade acetonitrile (ACN), methanol, and water were purchased from Biosolve B.V. DMSO >99.9% purity was purchased from Sigma–Aldrich (MilliporeSigma). LC-MS grade formic acid was purchased from Fisher Chemicals. Bovine serum albumin (Sigma–Aldrich) was used when diluting PTH standard solutions to help reduce nonspecific binding to laboratory consumables. The adsorptive loss assessment is presented in the online Supplemental Material file. Protein LoBind Eppendorf (Eppendorf SE) vials were used throughout the sample preparation.

Fresh horse plasma, used as a surrogate matrix, was donated by the Department of Endocrine Reproduction of the Veterinary Faculty of the University of Liège. For validation, PTH-blank human EDTA plasma (as determined by our method and immunoassays) was pooled from leftover samples from patients presenting with idiopathic hypoparathyroidism. Evolute Express weak cation exchange (10 mg) solid-phase extraction (SPE) 96-well plates were purchased from Biotage. AcroprepAdv 0.2-µm wwPTFE filters from Pall Corporation were used for filtering. WHO PTH 1-84, human, recombinant (First International Standard 95/646), containing 98.52 µg of PTH 1-84, was purchased through the National Institute for Biological Standards and Control. A 1-84 PTH peptide 15N stable isotope label was used as an internal standard (SIL-IS), acquired from Novus Biologicals, LLC. 7-84 PTH was purchased at Bachem AG.

STANDARD, CALIBRATION, AND QUALITY CONTROL SAMPLES

The PTH 1-84 standard and SIL-IS were reconstituted with a 10% ACN, 0.1% formic acid, and 1 mg/mL bovine serum albumin solution at 99.81 µg/g and 10 µg/mL, respectively. Aliquots of 40 µL were transferred to 1.5-mL Eppendorf vials, centrifuged for 30 s at 5000*g*, and frozen at −80°C. Preparation of 9-point calibration curve and quality control (QC) samples is described in Supplemental Material file. The calibration curve was

built from the peak area ratio of the native standard to the IS using weighted (1/x) linear least squares regression.

SAMPLE PREPARATION

One thousand microliters of leftover EDTA-K_2 plasma samples (Becton, Dickinson and Company) were aliquoted into 2.0-mL vials, centrifuged for 30 s at 5000*g*, and frozen at –80°C for less than a week until analysis. For each batch, calibrators, QCs, and samples (thawed at 5°C) were spiked with 20 μL of SIL-IS at 5.0 ng/mL and vortexed for 10 s. After 10 min of benchtop equilibration, they were diluted with 1000 μL of a 0.1 M NaH_2PO4 and 10% ACN solution adjusted to pH 7.0 (buffer), vortexed, and left 5 min for equilibration on the benchtop. Water in all aqueous solutions was LC-MS grade. The SPE procedure is detailed in Fig. 1. A vacuum not exceeding –5 kPa was applied in every step of the SPE until a flow of approximately 1 drop/s was obtained.

ETHICS

Remnant samples only were used in this study. No specific approval was requested to the CHU de Liège Institutional Review Board since a leaflet including the following statement is given to all admitted patients: "According to the law of the December 19, 2008, any left-over of biological material collected from patients for their standard medical management and normally destroyed when all diagnostic analysis have been performed, can be used for validation of methods." The law authorizes such use except if the patient expressed an opposition when still alive (presumed consent). Written informed consent for participation was not required for this study in accordance with the Belgian national legislation and the institutional requirements.

LC-MS/MS ANALYSIS

Separation and quantification were achieved by using a Nexera X2 UPLC from Shimadzu Corporation coupled to a QT6500 mass spectrometer from ABSciex operated at unit resolution for both quadrupoles. The column used was an Acquity® UPLC Protein BEH C4 Column 300 Å, 1.7 μm, 2.1 mm×100 mm from Waters Corporation maintained at 70°C. The mobile phase composition was A (water) and B (ACN) both containing 0.4% formic acid and 5% DMSO. The injection volume was 40 μL. The binary gradient profile is shown in online Supplemental Table 1 and Supplemental Fig. 1. Samples were kept in an autosampler at 5°C. Optimized multiple reaction monitoring and mass spectometry parameters are displayed in Supplemental Tables 2 and 3.

METHOD VALIDATION

The method was thoroughly tested for sources of bias and uncertainty to satisfy the requirements of ISO

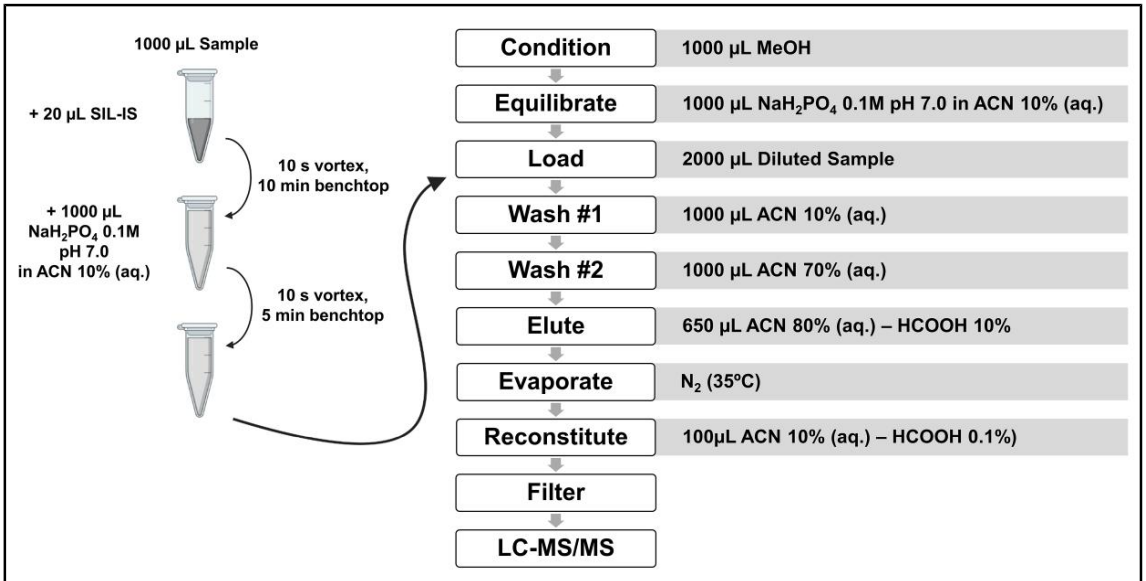

Fig. 1. Sample preparation scheme. SPE was conditioned with 1000 μL of methanol followed by 0.1 M NaH_2PO4 solution. 2000 μL of diluted samples were loaded and washed by 1000 μL of 10 and 70% ACN(aq). Elution required 650 μL of 80% ACN(aq) with 10% HCOOH. After evaporation, samples were reconstituted with 100 μL of 10% ACN(aq) with 0.1% HCOOH and filtered.

15193. CLSI guidelines were consulted for the design of the validation together with the validation approaches of other RMP methods (26–29). QCs from lowest to highest concentration (V1–V5) were prepared and analyzed in quintuplicate during 5 days for the evaluation of the method's precision, accuracy, and measurement uncertainty (MU).

SELECTIVITY

The chromatographic resolution of 1-84 PTH from other modified higher concentration PTH forms, such as oxidized PTH (preparation described in the Supplemental Material file) and 7-84 PTH, was tested. Ion ratios were compared between calibrators, QCs, and bona fide plasma samples (Supplemental Table 4). A difference of <20% was considered acceptable. The contribution of a blank matrix to the analyte and SIL-IS transitions, as well as the contribution of the IS to the analyte transition, are described in the Supplemental Material file.

Additionally, according to CLSI EP07 (30), the method was tested for interferences in samples with various degrees of hemolysis, lipemia, icterus, and hyperalbuminemia (Supplemental Material file).

LINEARITY

The linearity of the method was assessed according to the Study Design A1 described in CLSI guideline EP06 (31). As recommended, a human EDTA pool containing a high concentration of PTH was proportionally mixed with a blank sample (horse EDTA plasma) to constitute 9 relative concentrations at 1, 0.8, 0.5, 0.3, 0.15, 0.08, 0.04, 0.02, and 0.005, as shown in Supplemental Fig. 4, ranging from 5.6 to 1128.1 pg/L. Samples were prepared in quintuplicate and analyzed in the same run. The allowable deviation from linearity was defined at ±5% of the predicted values.

SAMPLE PREPARATION RECOVERY AND MATRIX EFFECTS

Absolute recovery and matrix effects were calculated at 25.0 pg/mL, 250.0 pg/mL, and 750.0 pg/mL, following a published procedure (32). PTH 1-84 standard stock solutions were prepared at 3 different concentrations (1.25 ng/mL, 12.5 ng/mL, and 37.5 ng/mL) and spiked in 3 sets of 1 mL samples in triplicate. Set A contained 80 µL of 10% ACN with 0.1% HCOOH. Sets B and C contained 1 mL of freshly pooled horse plasma.

Twenty microliters of stock solution were spiked into sets A and C. Sets B and C underwent sample preparation. After evaporation, Set B was reconstituted with 80 µL (10% ACN, 0.1% HCOOH) and spiked with 20 µL of the respective stock solution before the last filtering step.

Recovery and matrix effects were calculated according to the following equations:

$$Recovery\ (\%) = \frac{\mathrm{AUC(C)}}{\mathrm{AUC(B)}} \times 100$$

$$Matrix\ Effect(\%) = \frac{AUC(B)}{AUC(A)} \times 100$$

where the area under the ROC was the peak area for each set.

Additionally, the ion suppression through different matrices was assessed (Supplemental Material file).

CARRYOVER

Carryover was evaluated by injecting a sample containing 5000.0 pg/mL PTH (5× more concentrated than the highest calibration point) followed by a blank sample. Additionally, during the validation, samples were injected in the concentration order recommended by CLSI C64 section 8.8 (Carryover) (33) (i.e., medium [V3], high [V5], low [V1], medium, medium, low, low, high, high, medium), followed by the remaining validation samples injected in increasing levels of concentrations.

LOWER LIMIT OF THE MEASURING INTERVAL

The lower limit of the measuring interval (LLMI) was determined by the precision obtained from repetitions of the QC at 5.7 pg/mL. A CV < 20% was considered acceptable at the LLMI.

PRECISION AND ACCURACY

Precision and accuracy were evaluated by measuring 5 levels of spiked PTH-blank human EDTA plasma, in quintuplicate, during 5 days. Each day of validation, 5 aliquots per level were thawed and processed following the previously described procedure. Accuracy and precision for each QC level were estimated by single-nested ANOVA according to CLSI EP05 (34). Precision was expressed in terms of intra- and interday CV (%), calculated by single-nested ANOVA of daily means. The accuracy was expressed in terms of mean recoveries (%).

MEASUREMENT UNCERTAINTY ASSESSMENT

The APS defining the total budget for MU (TB_{mu}) were calculated according to the Joint Committee on Traceability in Laboratory Medicine Task Force on Reference Measurement System Implementation (35, 36). APS for reference methods are classified into 3 levels after PTH intrasubject biological variation expressed as CV_I %: minimum: $0.375 \times CV_I$; desirable $0.25 \times CV_I$; and optimal $0.125 \times CV_I$ (37). Following a top-down estimation of the MU or mu_{result} as recommended in ISO technical specification 20914 (38), the

contributions of the different sources of MU were defined along the traceability chain. Since a higher-order reference material does not exist for PTH, the standard value uncertainty ($mu_{value\ assignment}$) was obtained after amino acid analysis for the 1-84 PTH content based on the WHO 95/646 international standard performed by 5 independent laboratories (39). The attributable uncertainty of the calibrator (mu_{cal}) was defined as follows:

$$mu_{cal} = \sqrt{(mu_{value\ assignment})^2 + (mu_{dilution})^2 + (mu_{matrix})^2 + (mu_{IS})^2}$$

where $mu_{dilution}$ represents the uncertainty attributed to the preparation of the spiking solutions, and mu_{matrix} and mu_{IS} represent the uncertainty added through the pipetting of the IS and the matrix where the calibrators were prepared, respectively.

The mu_{result} (MU) was defined as:

$$mu_{result} = \sqrt{(mu_{cal})^2 + (mu_{rw})^2}$$

where mu_{rw} reflected the random variability of the measuring system, derived from the SD of the mean (single-nested ANOVA) obtained during the precision pool replicate studies, for each concentration.

The APS for an RMP was achieved if MU was equal to or less than the defined TB_{mu}. It has been recommended (36) that mu_{cal} should consume $\leq 50\%$ TB_{mu}, leaving the remaining MU available for mu_{rw}.

METHOD COMPARISON

Different cohorts of samples and external QCs (EQC) were used to compare the results obtained with the LC-MS/MS and the third-generation PTH immunoassays from DiaSorin (Liaison XL) and Fujirebio Lumipulse.

The cohorts of samples comprised remnant EDTA plasma samples from 43 subjects presenting with an estimated glomerular filtration rate >60 mL/min/1.73 m^2 (non-CKD), 48 remnant EDTA samples from patients suffering from CKD, and 33 remnant samples obtained from patients on DIA.

The EQC samples (n = 35) from 2019, 2020, and 2021 were kindly supplied by UK NEQAS. These EQCs came as lyophilized tablets obtained from an EDTA pool with or without the addition of exogenous PTH. All samples were analyzed simultaneously with the 3 methods. Concordance correlation coefficient for the different compared groups was assessed and described in the Supplemental Material file.

STATISTICAL ANALYSIS

Passing–Bablok statistical regression and Bland–Altman analysis were used to determine proportional and systematic differences as well as linear deviations. EQCs were treated independently as they may not reflect a true human matrix.

Analyses of the method validation and comparison were performed using MedCalc version 20.009, R version 4.0.2, and MS Excel 2019. Analyst® Software v1.6.2 (Sciex) was used for system operation, and Multiquant® v3.0.8664 (Sciex) served for peak review and integration.

Results

METHOD SELECTIVITY

Three different forms of oxidized PTH (oxidation on methionine 8, methionine 18, and on both) and 7-84 PTH were successfully resolved by our liquid chromatography method (Supplemental Fig. 2). The mean ion ratios between the quantifier and qualifier transitions for native and internal standard were 2.24 ± 0.14.

LINEARITY

All 9 relative concentration levels were within allowable deviation from linearity with deviations ranging from −2.2% to 3.5%. The linearity profile is displayed in Supplemental Fig. 5.

SAMPLE PREPARATION RECOVERY AND MATRIX EFFECTS

Among the 3 tested concentrations, the absolute sample recoveries ranged from 34% to 41%. The calculated matrix effect spanned from 80% to 112%.

CARRYOVER

No deviation from noise was observed following a 5000.0 pg/mL injection (Supplemental Figs. 16 and 17). Carryover was thus considered negligible given the method's LLMI. QC levels during the method validation were not affected by the injection order.

LOWER LIMIT OF THE MEASURING INTERVAL

The QC level at 5.7 pg/mL showed a mean Signal-to-Noise (S/N) of 10:1 with a mean intraday CV of 3.0% and was thus defined as the LLMI. Supplemental Fig. 8 illustrates the limit of quantification response against a blank horse plasma sample.

PRECISION AND ACCURACY

In the validation, the intra- and interday imprecisions ranged from 0.4% to 5.4% and from 1.2% to 3.9%, respectively. Accuracy mean recoveries ranged from 96.2% to 103.2% (Table 1).

MEASUREMENT UNCERTAINTY ASSESSMENT

The European Federation of Clinical Chemistry and Laboratory Medicine has documented a CV_I value for PTH of 15.7% (40); therefore, the minimum, desirable,

Table 1. Precision and accuracy of the LC–MS/MS PTH measurements on plasma.

Sample	Concentration (pg/mL)	Day	Mean (pg/mL)	Recovery (%)	Intraday (CV,%)	Interday (CV,%)
1	872.6	1	864.7	99.1	1.4	3.9
		2	887.3	101.7	1.2	
		3	869.6	99.7	1.8	
		4	858.2	98.3	0.4	
		5	894.5	102.5	0.4	
2	484.8	1	481.2	99.3	1.1	1.7
		2	487.9	100.7	0.5	
		3	478.4	98.7	1.2	
		4	479.3	98.9	0.5	
		5	477.5	98.5	1.4	
3	47.9	1	47.5	99.2	0.8	1.2
		2	48.3	100.7	0.9	
		3	47.6	99.4	1.5	
		4	47.5	99.2	0.8	
		5	48.0	100.2	0.9	
4	9.7	1	9.4	97.4	1.9	1.3
		2	9.8	101.3	1.4	
		3	9.7	100.3	1.1	
		4	9.7	100.3	1.3	
		5	10.0	103.2	2.5	
5	5.7	1	5.5	96.2	5.4	2.1
		2	5.6	97.9	2.1	
		3	5.7	99.6	3.5	
		4	5.5	96.2	2.1	
		5	5.8	101.2	1.9	

and optimal APS for a reference PTH measurement are 5.9%, 3.9%, and 2.0%, respectively. Considering a $mu_{value\ assignment}$, $mu_{dilution}$, mu_{matrix}, and mu_{IS} of 3.6%, 0.4%, 1.6%, and 0.4%, respectively, the mu_{cal} was established at 4.0%. Values for mu_{rw} ranged from 1.2% to 3.9% for the different tested concentrations, which provided a MU that spanned from 4.2% to 5.6%, thus satisfying the TB_{mu} requirements for a RMP. The contribution of mu_{cal} to the TB_{mu} was above the recommended maximum uncertainty budget allowance (≤50%). The calculated measurement uncertainties are shown in Table 2.

METHOD COMPARISON

The previously described samples (n = 157), from normal estimated glomerular filtration rate, CKD, subjects on dialysis, and EQCs, were analyzed by the 3 systems. The obtained data were used to perform Passing–Bablok regressions and a Bland–Altman analysis (Figs. 2 and 3; Supplemental Figs. 9–12). In contrast to Fujirebio Lumipulse, the Liaison showed a higher proportional bias when compared to LC-MS/MS. Samples from patients undergoing DIA showed a seemingly higher degree of scatter by both immunoassays.

Representative patient chromatograms at 32.0 pg/mL and 234.0 pg/mL and an EQC at 87.0 pg/mL are displayed in Supplemental Figs. 13–15.

Discussion

Parathyroid hormone is routinely used for the exploration and management of different diseases, such as primary and secondary hyperparathyroidism or CKD–mineral bone disorder. Unfortunately, this test suffers a lack of standardization, which could lead to erroneous interpretation of the results. The standardization of PTH assays has thus become one of the priorities of the International Osteoporosis Foundation–IFCC for Bone Metabolism, but achieving this ambitious goal

Table 2. Estimation of the measurement uncertainties for LC–MS/MS PTH measurement.

	Level				
	1	2	3	4	5
Mean pool PTH concentration, pg/mL	5.7	9.7	47.9	484.8	872.6
SD of the mean (ANOVA), pg/mL	0.22	0.17	0.56	6.28	18.31
CV (mu_{rw}), %	3.9	1.7	1.2	1.3	2.1
Standard uncertainty ($mu_{value\ assignment}$), %			3.7		
Spiking solutions uncertainty ($mu_{dilution}$), %			0.4		
Calibration solutions uncertainty (mu_{matrix}), %			1.6		
Internal standard uncertainty (mu_{IS}), %			0.4		
Calibrator uncertainty (mu_{cal}), %			3.6		
Measurement uncertainty (mu_{result}), %	5.6	4.4	4.2	4.3	4.6

requires (a) a clear definition of the measurand, (b) a commutable internationally recognized international standard or a panel of samples with assigned values with an RMP, (c) at least one reference method procedure, and (d) international proficiency testing using values assigned with the RMP(s). Regarding the first prerequisite, there is little doubt that the most important analyte, responsible for the biological PTH activity, is the full sequence unmodified PTH form, which is the measurand of interest. Other fragments or forms have been described, but their use in daily practice remains to be clarified. Similar to another study (23), we could not detect any oxidized form of PTH nor 7-84 PTH in the samples we processed by monitoring their respective specific transitions. Regarding the second prerequisite, WHO 95/646 has been proposed as the international reference material, but matrix-based materials enriched with this standard have yet to be proven commutable. For the third prerequisite, so far there is no candidate RMP described for 1-84 PTH measurement. Finally, for the fourth prerequisite, an RMP for PTH, is also mandatory to precisely assign the concentrations of PTH in potential proficiency testing schemes. An RMP for PTH is urgently needed to move forward toward PTH standardization.

The method we describe allows the quantification of the intact 1-84 protein, avoiding tryptic digestion or immunocapture in the sample preparation, and responds to the analytical needs expressed by the IFCC Committee on Bone Metabolism for a higher-order method (41). The high precision, accuracy, and sensitivity achieved allow for the robust coverage of a wide range of PTH concentrations, as those observed in primary and secondary hyperparathyroidism as well as hypoparathyroidism.

The analysis of 1-84 PTH by LC-MS/MS poses a major challenge, given the complexity of liquid chromatography separation and electrospray ionization. An example of the latter was observed during the fragmentation of 1-84 PTH by collision induced fragmentation. For multiple reaction monitoring quantitation, only the y82 fragment provided a sufficiently sensitive transition, illustrating a clear technical limitation that prevented the obtention of independent multiple reaction monitoring transitions. In addition, the high value and the width of the charge state envelope can be sources of lack of analytical selectivity and sensitivity in a tandem quadrupole mass spectrometer. The combination of a rather selective and enriching SPE, with a well-performing liquid chromatography, were critical to overcome these difficulties and to achieve satisfactory APS.

Even if the contribution of the calibrators to the TB_{mu} was above the recommended 50%, it was deemed suitable for the purpose of this work. Unfortunately, the calibration used in this method employed a standard whose concentration was assigned using an amino acid analysis without a well-characterized purity value and lacking a state-of-the-art uncertainty assessment, which are necessary for a primary calibrator in an RMP. A new primary reference material, with better characterized purity and traceable to the SI units, would be necessary to adapt our work to become a candidate RMP.

Interestingly, we compared the results we obtained with the newly developed method and third-generation PTH assays (DiaSorin Liaison and Fujirebio Lumipulse), notably in patients with CKD and patients on DIA. The results showed a moderate to substantial correlation between the 3 methods despite a substantial proportional bias observed with DiaSorin. This is likely attributable to the different standards used to calibrate the methods (WHO 95/646 and Bachem, respectively), whereas Fujirebio Lumipulse was calibrated with the same WHO 95/646 standard. It would be of value to compare the results obtained with different second-generation PTH

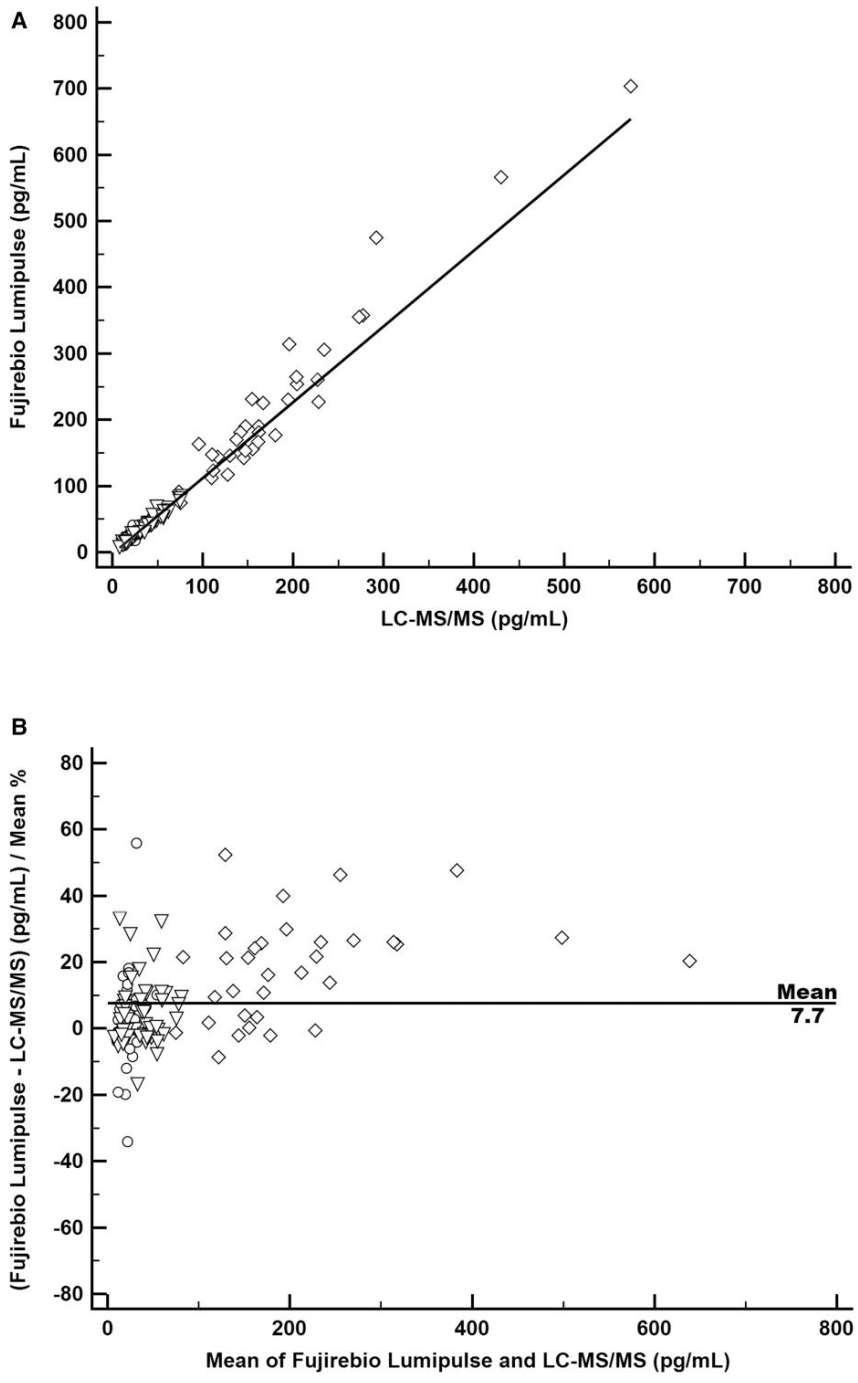

Fig. 2. (A), Fujirebio Passing–Bablok regression and Bland–Altman plots for non-CKD, CKD, and DIA (n = 124). Intercept pg/mL (95% CI): −2.35 (−4.08 to −1.07). Slope (95% CI): 1.14 (1.09 to 1.22). $R^2 = 0.975$; (B), Bland–Altman plot for non-CKD, CKD, and DIA. Mean of the difference from the 2 measurements is represented by the solid line at 7.7%. Circles and inverted triangles represent non-CKD and CKD samples respectively. Diamonds represent DIA samples.

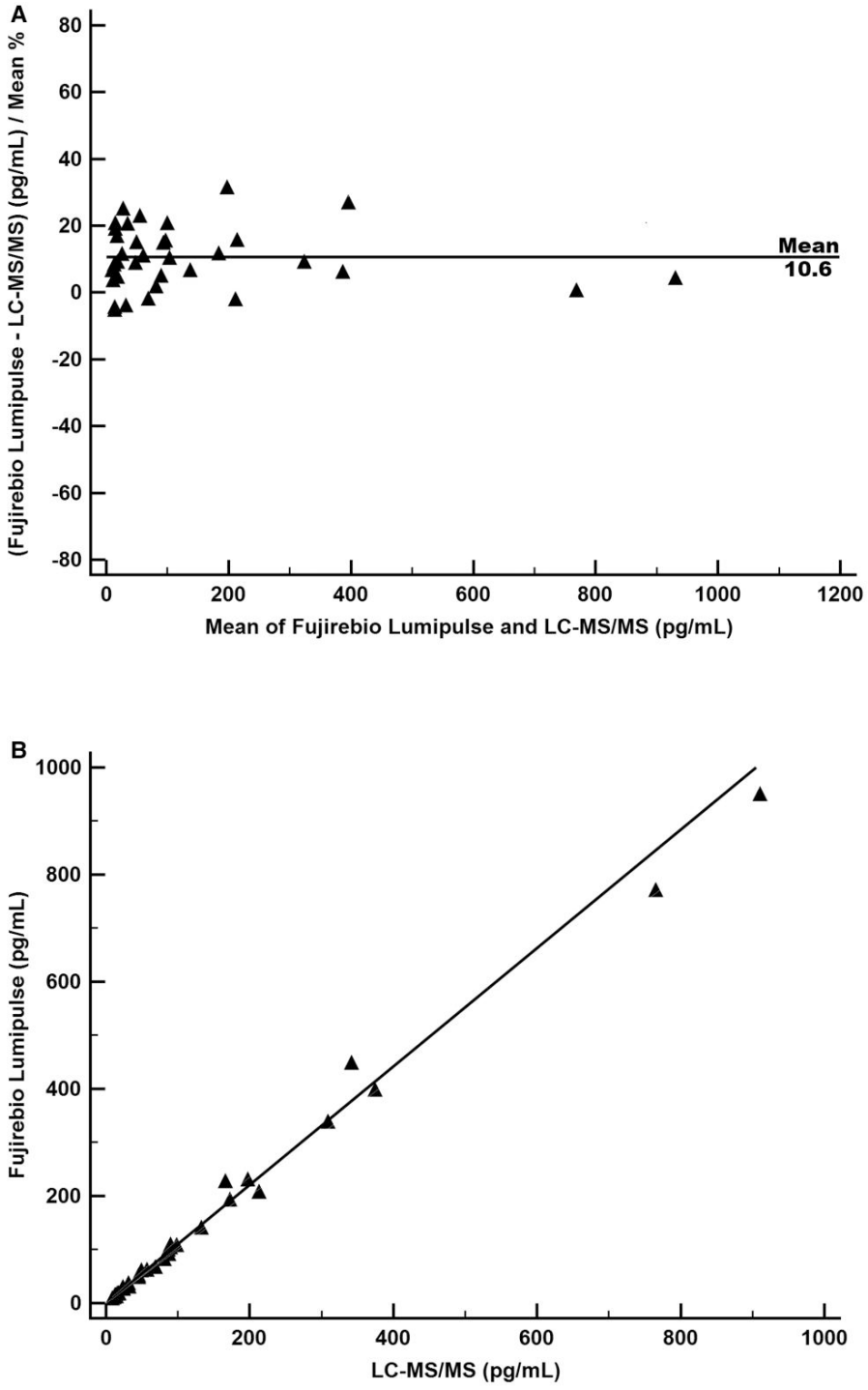

Fig. 3. (A), Fujirebio Passing–Bablok regression for the EQCs (n = 35). Intercept pg/mL (95% CI): −0.15 (−1.80 to 1.95). Slope (95% CI): 1.11 (1.05 to 1.18). $R^2 = 0.996$; (B), Bland–Altman plot for the EQCs. Mean of the difference from the 2 measurements is represented by the solid line at 10.6%.

assays to investigate whether they could be standardized and/or harmonized in patients with and without CKD. Indeed, in patients with CKD and patients on DIA, the accumulation of fragments is known to overestimate PTH concentrations, even if a recent study did not find any interference of the circulating fragments with the Roche second-generation PTH assay (23).

In conclusion, we believe that the presented work is an important but preliminary step to an RMP, standing by for a higher-order reference standard, with state-of-the-art metrological purity assessment and uncertainty evaluation. Additionally, multicenter and multiethnic reference ranges should be established using an appropriate methodology. Harmonization and/or standardization of the different kits available on the market should be achieved and monitored over time. Once all the steps are accomplished, this could improve the situation that clinicians and laboratories face today regarding the lack of standardization of PTH assays.

Supplementary Material

Supplementary material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: PTH, parathyroid hormone; CKD, chronic kidney disease; DIA, hemodialysis; RMP, reference measurement procedures; APS, analytical performance specifications; ACN, acetonitrile; SPE, solid-phase extraction; SIL-IS, stable isotope-labeled internal standard; QC, quality control; MU, measurement uncertainty; LLMI, lower limit of the measuring interval; TB_{mu}, total budget for MU; EQC, external quality control.

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References

1. Pfister MF, Ruf I, Stange G, Ziegler U, Lederer E, Biber J, Murer H. Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/Pi cotransporter. Proc Natl Acad Sci U S A 1998;95:1909–14.
2. Fermor B, Skerry TM. PTH/PTHrP receptor expression on osteoblasts and osteocytes but not resorbing bone surfaces in growing rats. J Bone Miner Res 1995;10:1935–43.
3. Leiker AJ, Yen TWF, Eastwood DC, Doffek KM, Szabo A, Evans DB, Wang TS. Factors that influence parathyroid hormone half-life: determining if new intraoperative criteria are needed. JAMA Surg 2013;148:602–6.
4. Segre G V, Perkins AS, Witters LA, Potts JT. Metabolism of parathyroid hormone by isolated rat Kupffer cells and hepatocytes. J Clin Invest 1981;67:449–57.
5. Hanley DA, Ayer LM. Calcium-dependent release of carboxyl-terminal fragments of parathyroid hormone by hyperplastic human parathyroid tissue in vitro. J Clin Endocrinol Metab 1986;63:1075–9.
6. Mayer GP, Keaton JA, Hurst JG, Habener JF. Effects of plasma calcium concentration on the relative proportion of hormone and carboxyl fragments in parathyroid venous blood. Endocrinology 1979;104:1778–84.
7. D'Amour P, Lazure C, France L. Metabolism of radioiodinated carboxy-terminal fragments of bovine parathyroid hormone in normal and anephric rats. Endocrinology 1985;117:127–34.
8. D'Amour P. Circulating PTH molecular forms: what we know and what we don't. Kidney Int 2006;70:S29–33.
9. Costa AG, Cremers S, Dworakowski E, Lazaretti-Castro M, Bilezikian JP. Comparison of two commercially available ELISAs for circulating sclerostin. Osteoporos Int 2014;25:1547–54.
10. Nussbaum SR, Zahradnik RJ, Lavigne JR, Brennan GL, Nozawa-Ung K, Kim LY, et al. Highly sensitive two-site immunoradiometric assay of parathyrin, and its clinical utility in evaluating patients with hypercalcemia. Clin Chem 1987;33:1364–7.
11. Lepage R, Roy L, Brossard JH, Rousseau L, Dorais C, Lazure C, D'Amour P. A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples. Clin Chem 1998;44:805–9.
12. Quarles LD, Lobaugh B, Murphy G. Intact parathyroid hormone overestimates the presence and severity of parathyroid-mediated osseous abnormalities in uremia. J Clin Endocrinol Metab 1992;75:145–50.
13. Wang M, Hercz G, Sherrard DJ, Maloney NA, Segre G V, Pei Y. Relationship between intact 1-84 parathyroid hormone and bone histomorphometric parameters in dialysis patients without aluminum toxicity. Am J Kidney Dis 1995;26:836–44.
14. John MR, Goodman WG, Ping G, Cantor T, Salusky IB, Juppner H. A novel immunoradiometric assay detects full-length human PTH but not amino-terminally truncated fragments: implications for PTH measurements in renal failure. J Clin Endocrinol Metab 1999;84:4287–90.
15. Gao P, Scheibel S, D'Amour P, John MR, Rao SD, Schmidt-Gayk H, Cantor TL. Development of a novel immunoradiometric assay exclusively for biologically active whole parathyroid hormone 1-84: implications for improvement of accurate assessment of parathyroid function. J Bone Miner Res 2001;16:605–14.
16. Ursem SR, Heijboer AC, D'Haese PC, Behets GJ, Cavalier E, Vervloet MG, Evenepoel P. Non-oxidized parathyroid

hormone (PTH) measured by current method is not superior to total PTH in assessing bone turnover in chronic kidney disease. Kidney Int. 2021;99:1173–8.
17. D'Amour P, Brossard JH, Rousseau L, Roy L, Gao P, Cantor T. Amino-terminal form of parathyroid hormone (PTH) with immunologic similarities to hPTH(1-84) is overproduced in primary and secondary hyperparathyroidism. Clin Chem 2003;49:2037–44.
18. Cavalier E, Daly AF, Betea D, Pruteanu-Apetrii PN, Delanaye P, Stubbs P, et al. The ratio of parathyroid hormone as measured by third- and second-generation assays as a marker for parathyroid carcinoma. J Clin Endocrinol Metab 2010;95:3745–9.
19. Cavalier E, Betea D, Schleck ML, Gadisseur R, Vroonen L, Delanaye P, et al. The third/second generation PTH assay ratio as a marker for parathyroid carcinoma: evaluation using an automated platform. J Clin Endocrinol Metab 2014;99:453–7.
20. Arakawa T, D'Amour P, Rousseau L, Brossard JH, Sakai M, Kasumoto H, et al. Overproduction and secretion of a novel amino-terminal form of parathyroid hormone from a severe type of parathyroid hyperplasia in uremia. Clin J Am Soc Nephrol 2006;1:525–31.
21. Kumar V, Barnidge DR, Chen LS, Twentyman JM, Cradic KW, Grebe SK, Singh RJ. Quantification of serum 1-84 parathyroid hormone in patients with hyperparathyroidism by immunocapture in situ digestion liquid chromatography-tandem mass spectrometry. Clin Chem 2010;56:306–13.
22. Lopez MF, Rezai T, Sarracino DA, Prakash A, Krastins B, Athanas M, et al. Selected reaction monitoring-mass spectrometric immunoassay responsive to parathyroid hormone and related variants. Clin Chem 2010;56:281–90.
23. Kritmetapak K, Losbanos LA, Hines JM, O'Grady KL, Ulmer CZ, Vesper HW, et al. Chemical characterization and quantification of circulating intact PTH and PTH fragments by high-resolution mass spectrometry in chronic renal failure. Clin Chem 2021;67:843–53.
24. Cavalier E, Delanaye P, Vranken L, Bekaert AC, Carlisi A, Chapelle JP, Souberbielle J-C. Interpretation of serum PTH concentrations with different kits in dialysis patients according to the KDIGO guidelines: importance of the reference (normal) values. Nephrol Dial Transplant 2012;27:1950–6.
25. Cavalier E, Salsé M, Dupuy AM, Bargnoux AS, Watar F, Souberbielle JC, et al. Establishment of reference values in a healthy population and interpretation of serum PTH concentrations in hemodialyzed patients according to the KDIGO guidelines using the lumipulse® G whole PTH (3rd generation) assay. Clin Biochem 2018;54:119–22.
26. Tai SSC, Bedner M, Phinney KW. Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D 2 in human serum using isotope-dilution liquid chromatography tandem mass spectrometry. Anal Chem 2010;82:1942–8.
27. Tai SSC, Nelson MA. Candidate reference measurement procedure for the determination of (24 R),25-dihydroxyvitamin D3 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem 2015;87:7964–70.
28. Leinenbach A, Pannee J, Dülffer T, Huber A, Bittner T, Andreasson U, et al. Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-β in cerebrospinal fluid. Clin Chem 2014;60:987–94.
29. Marcovina SM, Clouet-Foraison N, Koschinsky ML, Lowenthal MS, Orquillas A, Boffa MB, et al. Development of an LC-MS/MS proposed candidate reference method for the standardization of analytical methods to measure lipoprotein(a). Clin Chem 2021;67:490–9.
30. CLSI. Clinical and Laboratory Standards Institute guideline EP07: interference testing in clinical chemistry, 3rd Ed. Wayne (PA): CLSI; 2021.
31. CLSI. Clinical and Laboratory Standards Institute guideline EP06: evaluation of linearity of quantitative measurement procedures. Wayne (PA): CLSI; 2020.
32. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. Anal Chem 2003;75:3019–30.
33. Clinical and Laboratory Standards Institute (CLSI) guideline C64: quantitative measurement of proteins and peptides by mass spectrometry, 1st ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2021.
34. Durham AP, Goldford MD, Kondratoovich M V, Middle JG, Vaks JE. CLSI EP05-A3: 2014 evaluation of precision of quantitative measurement procedures; approved guideline, 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
35. Panteghini M, Braga F. Implementation of metrological traceability in laboratory medicine: where we are and what is missing. Clin Chem Lab Med 2020;58:1200–4.
36. Braga F, Panteghini M. The utility of measurement uncertainty in medical laboratories. Clin Chem Lab Med 2020;58:1407–13.
37. Hawley JM, Owen LJ, Mackenzie F, Mussell C, Cowen S, Keevil BG. Candidate reference measurement procedure for the quantification of total serum cortisol with LC-MS/MS. Clin Chem 2016;62:262–9.
38. ISO/TS. 20914:2019 Medical laboratories—practical guidance for the estimation of measurement uncertainty. 1st Ed. Geneva (Switzerland): International Organization for Standardization (ISO); 2019.
39. Burns C, Ellis M, Hockley J, Rigsby P; World Health Organization, Biologicals Unit. WHO international collaborative study of the proposed 1st [first] international standard for parathyroid hormone 1-84, human, recombinant. Geneva (Switzerland): WHO; 2009.
40. Aarsand AK, Fernandez-Calle P, Webster C, Coskun A, Gonzales-Lao E, Diaz-Garzon J, et al. The EFLM Biological Variation Database. https://biologicalvariation.eu/ (Accessed October 2021).
41. Sturgeon CM, Sprague S, Almond A, Cavalier E, Fraser WD, Algeciras-Schimnich A, et al. Perspective and priorities for improvement of parathyroid hormone (PTH) measurement—a view from the IFCC working group for PTH. Clin Chim Acta 2017;467:42–7.

Chapter 5. Unveiling a new era with liquid chromatography coupled with mass spectrometry to enhance parathyroid hormone measurement in patients with chronic kidney disease

Etienne Cavalier, Jordi Farré-Segura, Pierre Lukas, Anne-Sophie Gendebien, Stéphanie Peeters, Philippe Massonnet, Caroline Le Goff, Antoine Bouquegneau, Jean-Claude Souberbielle, Vincent Delatour and Pierre Delanaye.

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Preamble

This chapter describes the impact of recalibrating PTH immunoassays using our liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, calibrated against the World Health Organization (WHO) 95/646 International Standard. The primary objective was to harmonize PTH measurements across different immunoassays.

The study was conducted using pooled plasma samples with PTH concentrations determined by the LC-MS/MS method. To ensure representation of reproducibility over time and avoid sample size constraints, the validation was carried out in two separate validation panels. Five commonly used PTH immunoassays, representing both second and third generation assays, were recalibrated. The recalibration process significantly reduced inter-assay variability and aligned the results with the LC-MS/MS reference method, demonstrating the feasibility of standardizing PTH measurements. This harmonization would enable the adoption of common reference ranges, enhancing the reliability of PTH testing and improving patient care.

The findings from this study represent a clear advancement towards the standardization of PTH assays. By addressing the issues of calibration and cross-reactivity with PTH fragments, this research provides a robust framework for achieving consistent and accurate PTH measurements among different IAs.

Unveiling a new era with liquid chromatography coupled with mass spectrometry to enhance parathyroid hormone measurement in patients with chronic kidney disease

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OPEN

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Precise determination of circulating parathyroid hormone (PTH) concentration is crucial to diagnose and manage various disease conditions, including the chronic kidney disease–mineral and bone disorder. However, the lack of standardization in PTH assays is challenging for clinicians, potentially leading to medical errors because the different assays do not provide equivalent results and use different reference ranges. Here, we aimed to evaluate the impact of recalibrating PTH immunoassays by means of a recently developed LC-MS/MS method as the reference. Utilizing a large panel of pooled plasma samples with PTH concentrations determined by the LC-MS/MS method calibrated with the World Health Organization (WHO) 95/646 International Standard, five PTH immunoassays were recalibrated. The robustness of this standardization was evaluated over time using different sets of samples. The recalibration successfully reduced inter-assay variability with harmonization of PTH measurements across different assays. By recalibrating the assays based on the WHO 95/646 International Standard, we demonstrated the feasibility for standardizing PTH measurement results and adopting common reference ranges for PTH assays, facilitating a more consistent interpretation of PTH values. The recalibration process aligns PTH results obtained from various immunoassays with the LC-MS/MS method, providing more consistent and reliable measurements. Thus, establishing true standardization across all PTH assays is crucial to ensure consistent interpretation and clinical decision-making.

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Lay Summary

Accurate diagnosis and treatment of chronic kidney disease–related mineral and bone disorders (CKD-MBDs) hinge on measuring parathyroid hormone (PTH). Unfortunately, current PTH tests often yield inconsistent results, complicating clinical practice. To address this, standardizing PTH measurement methods is essential. Achieving this standardization requires advanced mass spectrometry techniques and understanding whether certain substances in the blood of chronic kidney disease and hemodialyzed patients affect PTH measurements. Recent advances in mass spectrometry revealed that a potentially problematic PTH fragment (7–84) was absent in patients' blood, alleviating concerns. In addition, oxidized PTH was not detected and circulating fragments did not interfere PTH assays. As a result, we explored recalibrating 5 different PTH kits on to a precise liquid chromatography tandem mass spectrometry reference method. The outcomes were promising, aligning PTH results across various immunoassays with the reference method. This calibration process promises more reliable and consistent PTH measurements, ultimately enhancing patient care by reducing result variability.

Beyond its paramount importance in diagnosing and managing various endocrine conditions such as primary and secondary hyperparathyroidism, hypoparathyroidism, and pseudohypoparathyroidism, the measurement of parathyroid hormone (PTH) is routinely conducted in patients with chronic kidney disease (CKD). The Kidney Disease: Improving Global Outcomes 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease–Mineral and Bone Disorder

recommends monitoring PTH levels in CKD patients beginning in CKD G3a and suggests maintaining PTH levels of hemodialyzed (HD) patients at ∼2 to 9 times the upper limit of the normal (ULN) values of the assay used.[1] Yet, a recommendation that treatments should be based on multiples of a ULN value is quite unique and is a circumvolution because of the lack of standardization of PTH assays.[2] Such a lack of standardization is unfortunately a real source of confusion for clinicians in their daily practice, potentially leading to significant medical errors.[3,4]

Several reasons contributed so far to the current lack of standardization in PTH assays. The first one is the presence of circulating PTH fragments alongside the bioactive 1 to 84 PTH peptide.[5] These fragments, which are N-terminal or C-terminal truncated forms of PTH, circulate in the blood owing to liver metabolism of the active peptide or direct secretion by the parathyroid glands.[6–8] These fragments, which are eliminated by the kidney, have a longer half-life than 1 to 84 PTH itself,[9,10] accumulate in the blood of CKD patients,[11–13] and potentially interfere with second generation PTH assays (referred as "intact" PTH assay). Indeed, such assays are supposed to recognize, with various cross-reactivities, a family of large C-terminal fragments referred to as "non-(1 to 84)" PTH.[14] This is not the case of third generation immunoassays (also referred as "whole" or "bioactive" PTH assays) because such assays incorporate an anti–N-terminal antibody directed toward the first 4 amino acids of the peptide, eliminating the issue of cross-reactivity with PTH fragments.[15,16]

The second reason for the lack of standardization in PTH assays is calibration. Indeed, despite the availability of the World Health Organization International Standard PTH 1-84, human, recombinant (NIBSC [National Institute for Biological Standards and Control] code: 95/646) (WHO 95/646 PTH IS), differences in calibration remain, which can be due to noncommutability of the WHO material and/or its incorrect use by assay manufacturers. Consequently, the recovery of the same amount of the WHO 95/646 PTH IS can range from 100% to >250% depending on the assay used.[3]

Finally, the lack of a formally recognized reference measurement procedure, a gold standard method providing true values against which any commercial assay could be calibrated, further contributes to the lack of standardization in PTH assays.

In this study, we aimed at evaluating the impact of a recalibration of 5 PTH immunoassays, representing both second and third PTH generations assays, on the liquid chromatography tandem mass spectrometry (LC-MS/MS) candidate reference method we recently developed. This method is indeed calibrated against the WHO 95/646 PTH IS and has the potential to become a reference measurement procedure.[17]

METHODS

We used the second and third generation PTH assays from Roche on the cobas, the second and third generation assays from DiaSorin on the LIAISON analyzer, and the third generation PTH assay from Fujirebio on the LUMIPULSE instrument. The characteristics of these methods are detailed in Table 1 for reference. As our gold standard, we used the LC-MS/MS method that we recently developed.[17] Notably, this method distinguishes itself by eliminating the use of antibodies during the sample preparation, ensuring complete independence from any cross-reactivity concerns. It is calibrated against the WHO 95/646 PTH IS, providing a robust reference framework. Finally, our LC-MS/MS method has undergone extensive validation for 1 to 84 PTH from 5.7 to 873 ng/l and exhibits a measurement uncertainty of <5.6%.

We prepared a calibration panel constituted of 40 pools of leftover ethylenediamine tetraacetic acid (EDTA) plasma samples by carefully mixing at least 10 different leftover samples indiscriminately originating from our daily routine together to constitute a single pool. The samples were selected on the basis of their assigned nominal value determined through our routine method (DiaSorin LIAISON third generation PTH), and pools were constituted to span the measuring range.

We then prepared 2 validation panels. For validation panel 1, we selected 138 leftover EDTA plasma samples, 58 from CKD patients (19 from G4 [estimated glomerular filtration rate {eGFRcr} between 15 and 29 ml/min per 1.73 m^2], 19 from G3b [eGFRcr between 30 and 44 ml/min per 1.73 m^2], and 20 from G3a [eGFRcr between 45 and 59 ml/min per 1.73 m^2] categories), 37 from HD patients, and 43 from non-CKD persons (eGFRcr >60 ml/min per 1.73 m^2). For validation panel 2, we selected 109 other leftover EDTA plasma samples: 52 from CKD patients (16 from G4, 17 from G3b, and 19 from G3a categories), 37 from HD patients, and 20 from non-CKD patients.

The EDTA plasma samples used for panel preparation were stored at −20 °C for less than a month and had not been previously frozen. After preparation, panels were stored at −80 °C until measurement, which occurred within the same month. For each panel, a fresh WHO International Standard Parathyroid Hormone 1-84, human, recombinant (NIBSC code: 95/646) ampoule was used to establish the calibration curve for the LC-MS/MS method. The measurements of the calibration, validation 1, and validation 2 panels were conducted at intervals of a minimum of 3 months, ensuring the use of different lots for immunoassays. All samples were measured in singlicates using both the 5 immunoassays and the LC-MS/MS method.

On the basis of the results of the calibration panel, we established regression equations for each of the immunoassays versus LC/MS-MS. We then used these equations to "recalibrate" the immunoassays on the LC-MS/MS method, and we verified the robustness of this calibration on the 2 validation panels. To evaluate the clinical impact of recalibration, we investigated the classification of non-CKD persons and CKD patients using both the LC-MS/MS method and the different immunoassays on the basis of a standardized ULN value. Furthermore, we examined the classification of HD patients before and after recalibration by following the Kidney Disease: Improving Global Outcomes guidelines[18] and considering 2 to 9 times multiples of the standardized ULN value.

RESULTS

Of the 40 constituted pools of the calibration panel, 1 was above the measuring range for the DiaSorin second generation assay (>1900 ng/l) and was thus discarded. The spanning range of the 39 remaining pools was 14 to 533, 16 to 1200, 16

Table 1 | Characteristics of second and third generation PTH immunoassays as disclosed by the manufacturers

Method	Intra-assay CV (%)	Interassay CV (%)	Expected values (ng/l)	Generation	N-terminal antibody and target	C-terminal antibody and target	Traceability	Measuring range (ng/l)
Roche Cobas Elecsys PTH	<1.3	<2	15–65	Second	Mouse monoclonal 26–32	Mouse monoclonal 37–42	Commercially available RIA. Claims a mean recovery of 100% ± 4% of the WHO 95/646 PTH IS	1.2–5000
Roche Cobas Elecsys PTH (1–84)	0.9–7.6	1.6–11.4	15–57	Third	Mouse monoclonal N-terminal part	Mouse monoclonal C-terminal part	WHO 95/646 PTH IS	5.5–2300
DiaSorin LIAISON N-TACT PTH Gen II	1.3–5.7	2.8–4.2	15–87	Second	Goat polyclonal 1–34	Goat polyclonal 39–84	Internal preparation of human *recombinant* 1–84 PTH	3–1900
DiaSorin LIAISON 1–84 PTH	3.0–5.9	5.4–9.0	7–39	Third	Polyclonal N-terminal	Polyclonal C-terminal	Internal preparation of *synthetic* human 1–84 PTH	4–1800
Fujirebio LUMIPULSE G whole PTH	1.1–4.1	1.4–4.1	5–36	Third	Goat polyclonal	Goat polyclonal	WHO 95/646 PTH IS	4–5000

CV, coefficient of variation; IS, International Standard; PTH, parathyroid hormone; RIA, radioimmunoassay; WHO, World Health Organization.

to 436, 8 to 355, 6 to 315, and 5 to 251 ng/l for Roche second generation, DiaSorin second generation, Roche third generation, DiaSorin third generation, Fujirebio third generation, and LC-MS/MS method, respectively.

The regression equations of these 39 pools measured with each immunoassay versus LC-MS/MS and the correlations coefficients are presented in Table 2 and Figure 1.

These equations were then applied to the PTH results of the samples of the 2 validation panels. The results obtained from the 2 sets of samples before and after recalibration are presented in Table 3. Before recalibration, the mean PTH concentrations were 138, 258, 104, 89, 77, and 66 ng/l for Roche second generation, Roche third generation, DiaSorin second generation, DiaSorin third generation, Fujirebio third generation, and LC-MS/MS method, respectively, in validation panel 1. After recalibration, the corresponding values were 63, 62, 57, 67, and 66 ng/l for the 5 immunoassays, respectively. Concerning the samples of validation panel 2, the corresponding mean concentrations before calibration were 82, 157, 288, 111, 95, and 86 ng/l for the same methods. After recalibration, the mean values for immunoassays were 71, 68, 60, 71, and 74 ng/l, respectively.

Before recalibration, the average bias between the immunoassays presenting the highest and lowest biases (i.e., Fujirebio third generation and DiaSorin second generation) compared to LC-MS/MS ranged from +9% to +224% in validation panel 1 and from +9% to +223% in validation panel 2. After recalibration, the average bias decreased to −0.8% and −6.8% in validation panel 1 and from −2.5% to −12.3% in validation panel 2 for these 2 immunoassays. The Roche third generation assay's bias against LC-MS/MS also decreased after recalibration (from +71% to −21.5% in validation panel 1 and from +57% to −24.9% in validation panel 2) but to a lesser extent compared to the other assays. The overall mean bias decreased from 86.9% to −7.2% in validation panel 1 and from 81.7% to −11.1% in validation panel 2 after recalibration.

To perform the clinical validation, it was necessary to establish a standardized ULN value. Because a ULN for the LC-MS/MS method had not been established yet, we decided to use the average of the 5 recalibrated ULN values provided for each kit, obtained through the regression equations. The manufacturers' original ULN values were 65, 87, 57, 39, and 36 ng/l for Roche second generation, DiaSorin second generation, Roche third generation, DiaSorin third generation, and Fujirebio third generation, respectively. After recalibration, these values were adjusted to 30, 26, 27, 31, and 31 ng/l, respectively. The average of these recalibrated ULN values was calculated to be 30 ng/l, which was consequently considered as the standardized ULN value to be applied as the reference value for both the LC-MS/MS method and the 5 recalibrated immunoassays. Accordingly, Figures 2 and 3 display the distribution of the results obtained in non-CKD subjects and CKD patients, respectively, before and after recalibration according to the ULN value and Figure 4 displays the results of HD patients according to 2 to 9 times the ULN value before and after

Table 2 | Regression equations and correlation coefficients of the calibration panel's 39 samples for the LC-MS/MS reference method

Y	*X*				
	Roche second generation	DiaSorin second generation	Roche third generation	DiaSorin third generation	Fujirebio third generation
LC/MS-MS =	$0.45X + 0.58$	$0.21X + 7.21$	$0.60X - 7.1$	$0.73X - 1.8$	$0.83X + 2.6$
r^2	0.973	0.988	0.991	0.989	0.983

LC-MS/MS, liquid chromatography tandem mass spectrometry.

recalibration. Regarding these 73 HD patients, using raw values of 60 and 270 ng/l (i.e., 2 × 30 and 9 × 30 ng/l) as targets, 1 patient was misclassified by the Roche second generation assay in the low range (value considered as >60 ng/l whereas it was lower with the LC-MS/MS method) and 5 were misclassified by the Roche third generation assay (values considered as <270 ng/l whereas they were higher). Figure 5 illustrates the representative outcome for a non-CKD subject, a CKD patient, and a HD patient before and after recalibration of the immunoassays using the LC-MS/MS method. Before recalibration, the non-CKD subject exhibited an LC-MS/MS concentration of 27 ng/l. This measurement initially translated to PTH concentrations spanning from 33 ng/l (using the Fujirebio third generation assay) to 77 ng/l (as determined by the DiaSorin second generation assay). However, after recalibration, the same patient's values were refined to a range falling between 22 ng/l (Roche third generation assay) and 30 ng/l (Fujirebio third generation assay). Likewise, in the case of a CKD patient with initial LC-MS/MS PTH concentrations of 81 ng/l and immunoassays PTH concentrations ranging from 99 ng/l (measured using the Fujirebio third generation assay) to 289 ng/l (measured using the DiaSorin second generation assay), the recalibration led to a refined range spanning from 67 ng/l (Roche third generation assay) to 85 ng/l (Fujirebio third generation assay) after recalibration. Finally, an HD patient presenting an LC-MS/MS PTH value of 142 ng/ml initially displayed a PTH concentration range of 181 to 609 ng/mL before recalibration. After recalibration, the PTH concentrations ranged from 117 to 153 ng/l.

DISCUSSION

The key finding of this study suggests that it is now feasible to standardize all PTH assays, regardless of the assay methodology (second or third generation immunoassay). This breakthrough has the potential to bring significant improvements in the management of chronic kidney disease–mineral and bone disorder. In 2006, Souberbielle *et al.* highlighted the misleading nature of the 150 to 300 ng/l National Kidney Foundation Kidney Disease Outcomes Quality Initiative raw values used as targets for PTH in HD patients.[4,19] The authors highlighted that despite high correlation among the assays (r values ranging from 0.975 to 0.994), the 15 assays tested (13 second generation and 2 third generation assays) could produce significantly divergent results. This discrepancy had the potential to lead to significant clinical errors, as individual patients might be classified as within, below, or above the

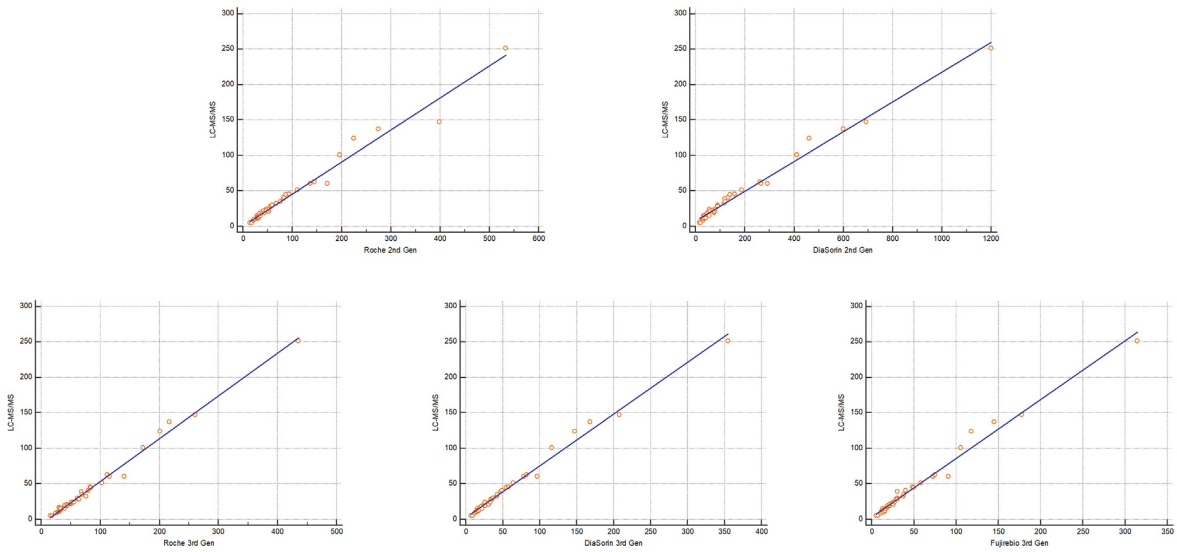


Figure 1 | Scatter diagram depicting the regression line of the calibration panel's 39 samples for each immunoassay compared to the reference liquid chromatography tandem mass spectrometry (LC-MS/MS) method (measurement units are nanograms per liter). 2nd Gen, second generation; 3rd Gen, third generation.

Table 3 | Characteristics of the 2 validation panels before and after recalibration of the samples on the reference LC-MS/MS method

	Validation panel 1						Validation panel 2					
	Before recalibration											
Results	LC-MS/MS	Roche 2nd Gen	DiaSorin 2nd Gen	Roche 3rd Gen	DiaSorin 3rd Gen	Fujirebio 3rd Gen	LC-MS/MS	Roche 2nd Gen	DiaSorin 2nd Gen	Roche 3rd Gen	DiaSorin 3rd Gen	Fujirebio 3rd Gen
Mean (ng/l)	65.7	138	257.5	104.2	89.3	77.0	81.5	156.8	287.5	111.1	95.2	85.8
Mean bias (%)		104	224	71	27	9		102	223	57	17.9	9.2
Overall bias (%)	86.9						81.7					
Overall CV (%)	46.1						59.0					
	After recalibration											
Results	LC-MS/MS	Roche 2nd Gen	DiaSorin 2nd Gen	Roche 3rd Gen	DiaSorin 3rd Gen	Fujirebio 3rd Gen	LC-MS/MS	Roche 2nd Gen	DiaSorin 2nd Gen	Roche 3rd Gen	DiaSorin 3rd Gen	Fujirebio 3rd Gen
Mean (ng/l)	65.7	62.9	61.5	55.6	67.1	66.4	81.5	71.4	67.8	59.8	71.4	73.7
Mean bias (%)		−5.5	−6.8	−21.5	−1.2	−0.8		−7.2	−12.3	−24.9	−8.9	−2.5
Overall bias (%)			−7.2						−11.1			
Overall CV (%)			12.0						20.5			

2nd Gen, second generation; 3rd Gen, third generation; CV, coefficient of variation; LC-MS/MS, liquid chromatography tandem mass spectrometry.

Kidney Disease Outcomes Quality Initiative target range depending on the specific assay used. The alarming implication was that contradictory treatment approaches could have been recommended for a single HD patient on the basis of the choice of PTH assay.

To address this issue, the Kidney Disease: Improving Global Outcomes proposed using multiples of the ULN value established by assay manufacturers as PTH targets for HD patients. Undoubtedly, this approach significantly reduced the disparities in classifying HD patients based on PTH target ranges.[20] However, a potential weakness of this proposition regarding the complexity of establishing PTH reference values has been emphasized.[21] It is now recognized that factors such as vitamin D status, age, GFR, and potential ethnicity of the subjects included in the population used to establish PTH reference values have significant implications for determining the PTH ULN value.[22–24] Many experts agree that the most effective solution to address this problem would be to achieve true standardization of all PTH assays, ensuring that regardless of assay methodology, all assays generate the same or reasonably similar results for a given sample. This approach would also facilitate the adoption of a unified ULN value, irrespective of the specific PTH assay used. Establishing such a single ULN value would necessitate comprehensive analysis

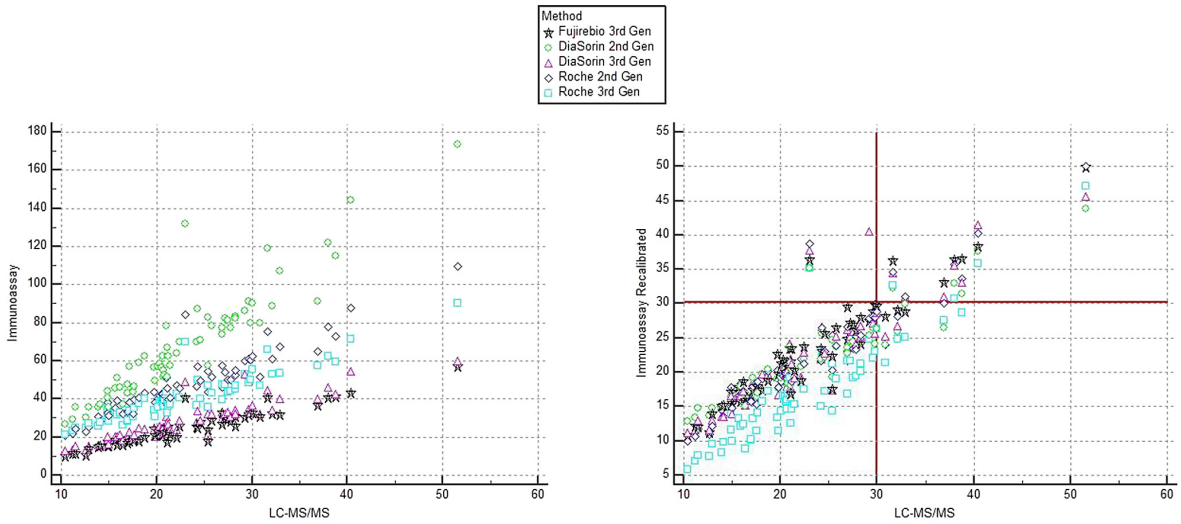


Figure 2 | Comparison of the results obtained in non–chronic kidney disease subjects before (left) and after (right) recalibration. The red solid bars correspond to 30 ng/l, which is the average of the 5 recalibrated upper limit of normal values provided for each kit, obtained through the regression equations (measurement units are nanograms per liter). 2nd Gen, second generation; 3rd Gen, third generation; LC-MS/MS, liquid chromatography tandem mass spectrometry.

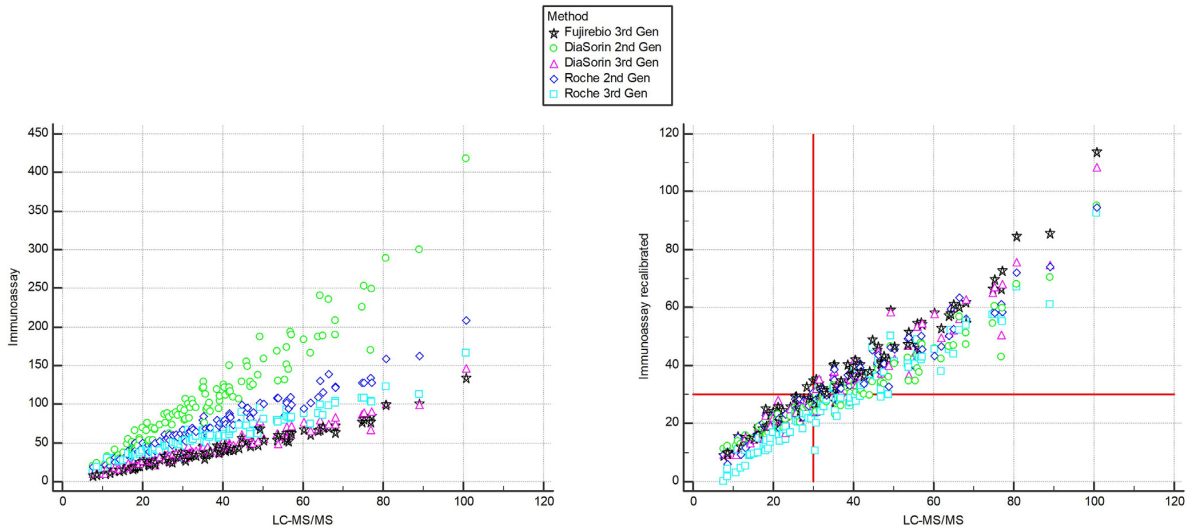


Figure 3| Comparison of the results obtained in chronic kidney disease patients before (left) and after (right) recalibration. The red solid bars correspond to 30 ng/l, which is the average of the 5 recalibrated upper limit of normal values provided for each kit, obtained through the regression equations (measurement units are nanograms per liter). 2nd Gen, second generation; 3rd Gen, third generation; LC-MS/MS, liquid chromatography tandem mass spectrometry.

across diverse, multiethnic cohorts of healthy individuals spanning various ages, all devoid of primary hyperparathyroidism and any instigator of secondary hyperparathyroidism such as vitamin D deficiency, decreased GFR (among many others), and measurement of the samples with the candidate LC-MS/MS reference method.[22–24] Although the necessity for such a study is paramount and falls within the purview of international scientific organizations dedicated to bone metabolism, such as the International Federation of Clinical Chemistry and Laboratory Medicine, Kidney Disease: Improving Global Outcomes, or the International Osteoporosis Foundation, it is noteworthy that such a study is presently lacking and has yet to be realized. As a compensatory measure, we used here a workaround—a standardized ULN value deduced from the regression equations involving PTH assays and LC-MS/MS. It is crucial to note that our intention

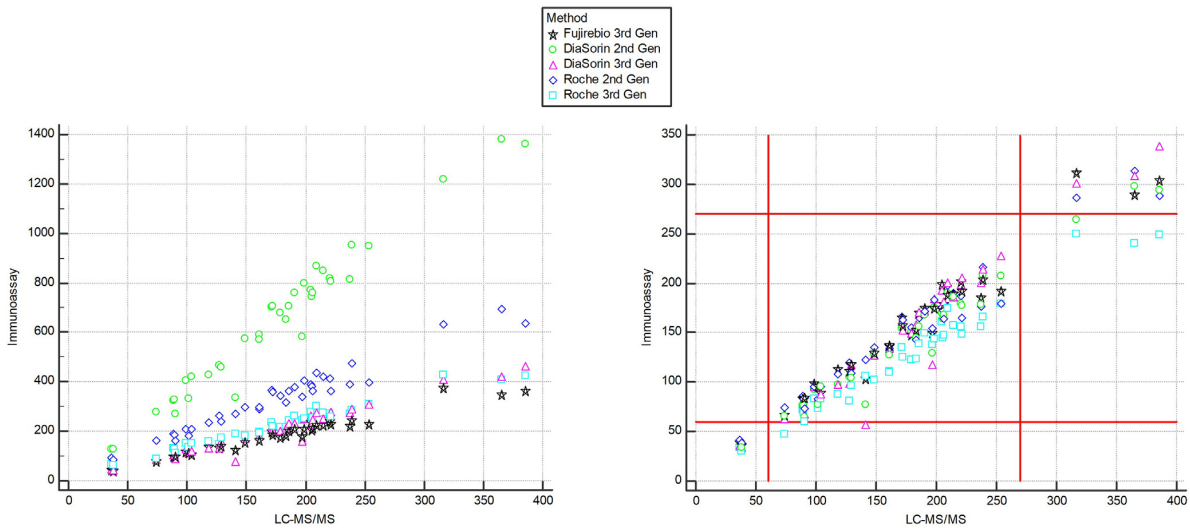


Figure 4| Comparison of the results obtained in hemodialyzed patients before (left) and after (right) recalibration. The red solid bars represent 2 to 9 times the upper limit of normal (ULN) values on the liquid chromatography tandem mass spectrometry (LC-MS/MS) method, determined by the mean of the manufacturers' ULN values obtained after mathematical recalibration (measurement units are nanograms per liter). 2nd Gen, second generation; 3rd Gen, third generation.

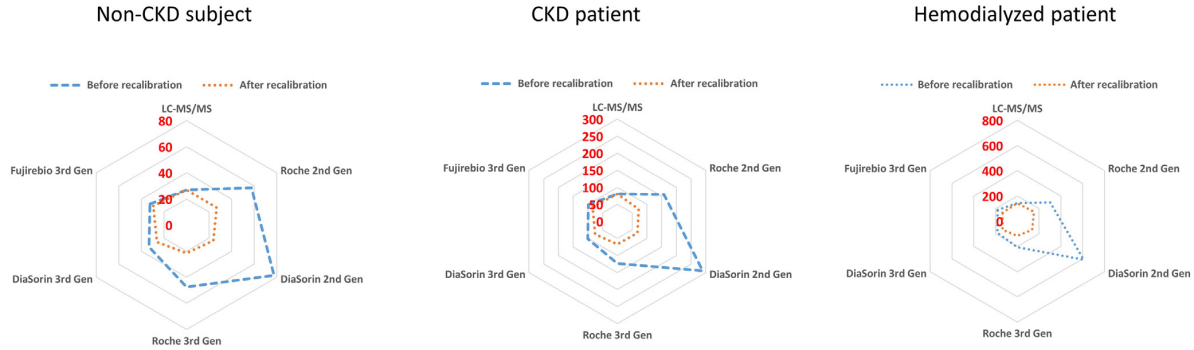


Figure 5 | Radar charts illustrating the variations in parathyroid hormone (PTH) concentrations (measured in nanograms per liter) in 3 different subjects: a non–chronic kidney disease (CKD) subject (left), a CKD patient (middle), and a hemodialyzed patient (right). The data presented include measurements both before (indicated by blue dashed lines) and after (indicated by orange dotted lines) the recalibration of 6 immunoassays using the reference liquid chromatography tandem mass spectrometry (LC-MS/MS) method. The bold and red numbers along the vertical axis represent PTH concentrations (measured in nanograms per liter). 2nd Gen, second generation; 3rd Gen, third generation.

in using this workaround was not to preemptively institute a universal ULN value, given our awareness of potential shortcomings in manufacturer-proposed ULN values. Rather, the objective of using this method was to underscore the positive consequences of recalibrating the assays and their impact on the clinical interpretation of results, should a single ULN value be implemented across all assays. Indeed, our results indicate that a single ULN value can be used for all recalibrated PTH assays and that recalibrated PTH assays can be used interchangeably.

One question that may arise is why this situation has persisted for more than 2 decades. As mentioned above, there are valid and invalid reasons why such differences in calibration persist despite the availability of the WHO 95/646 PTH IS. These reasons can be summarized briefly: the presence of PTH fragments that accumulate in the blood of CKD patients, exhibiting varying cross-reactivity with antibodies used in PTH assays (thus raising doubts about standardization in CKD patients); the definition of the appropriate biomarker to measure (which is nonoxidized 1–84 PTH) and the potential need of a fourth generation assay that would measure only nonoxidized PTH; the lack of higher-order reference methods, namely, an LC-MS/MS method, that offers enhanced specificity and reliability compared to immunoassays, ensuring accurate measurements; and the absence of a robust higher-order commutable reference material, capable of mimicking true human samples, that could facilitate the calibration of all assays.

In the past 2 years, remarkable advancements have been achieved in the standardization of PTH assays, particularly with the introduction of higher-order methods by Kritmetapak *et al.*[13] and Farré-Segura *et al.*[17] These groundbreaking studies have provided crucial insights, revealing that oxidized 1 to 84 PTH, previously believed to be predominantly present in the bloodstream of HD patients according to some authors,[25,26] is actually absent. Moreover, the findings from these articles have corroborated earlier research conducted using mass spectrometry, which demonstrated the absence of the 7 to 84 PTH fragment in human blood.[27,28] This fragment, known to cause cross-reactivity with second generation assays, has been confirmed to be nonexistent in the blood samples of HD patients. Kritmetapak *et al.* also demonstrated that the circulating PTH fragments they identified in the blood of CKD patients did not interfere with the Roche second generation assay, confirming the earlier intuition of Souberbielle *et al.* that a correction factor could be applied to PTH results to improve harmonization.[29] Finally, the methods developed by Kritmetapak *et al.*[13] and Farré-Segura *et al.*[17] are both calibrated against the WHO 95/646 PTH IS and hold promise as candidate reference methods for PTH in the future against which all immunoassays could be calibrated. However, it is worth noting that the former approach, using a high resolution and not a triple quadrupole instrument, still needs some improvement in handling lower 1 to 84 PTH concentrations.

The present study provided further evidence that differences between assays are mostly due to calibration and that standardizing calibration of PTH assays is feasible. By recalibrating 5 PTH immunoassays (2 second generation and 3 third generation assays) using a set of plasma pools with established concentrations using our LC-MS/MS candidate reference measurement procedure, we achieved highly comparable PTH results in 2 different cohorts of CKD: non-CKD and HD patients. Moreover, we demonstrated that the circulating PTH fragments did not hinder this recalibration process.

However, it is important to note that in the case of specific diseases, such as parathyroid carcinoma,[30] where post-translationally modified variants could be expressed, or when PTH monitoring is performed during parathyroid surgical resection,[31] atypical forms and variants may appear

in patients' blood. These unique forms may be detectable using second generation PTH assays but not necessarily with third generation assays, or vice versa. At this point in our understanding, it is crucial to acknowledge that some of these forms might not have been identified through the high resolution mass spectrometry (HR-MS) approach. With the help of previous high-performance liquid chromatography fractionation techniques, Nguyen-Yamamoto *et al.* identified 2 peaks different from 1 to 84 PTH in certain patients with parathyroid adenoma, although not in all cases.[32] This was further corroborated by Yamashita *et al.*,[9] who observed variations in PTH reactivity between the adenoma and the contralateral internal jugular vein in some patients with primary hyperparathyroidism. Exploration of such fragments or modified forms and their potential role in physiopathology will deserve further research.

In this study, we used 2 second generation PTH assays and 3 third generation assays. Yet, our findings warrant further validation through assessment using alternate second generation PTH immunoassays. Indeed, our approach here centered on optimizing sample volumes, with particular emphasis on minimizing inherent dead volume in automated immunoassay analyzers. To this end, we prioritized manufacturers who facilitated the application of second and third generation PTH assays on their platforms, specifically Roche and DiaSorin. Although the Roche second generation assay boasts global utilization, it is worth acknowledging that the remaining assays incorporated in this study might encompass a comparatively narrower distribution volume. As such, a comprehensive evaluation across a spectrum of second generation PTH immunoassays remains a prudent next step to reinforce the robustness of our findings. Although this article represents a significant step toward improving PTH measurement, several aspects still require further work. First, we need to demonstrate that the different candidate methods yield consistent results to build a network of calibration reference laboratories. Second, the current WHO 95/646 PTH IS is expected to be replaced with a newer IS fit-for-purpose uncertainty and IS-traceable certified values determined with a higher-order reference measurement procedure. Finally, nephrologists, laboratory specialists, and also all clinicians involved in bone and mineral metabolism must collaborate to convince manufacturers to recalibrate their assays with the higher-order reference standards. Although this endeavor would undoubtedly require substantial effort, achieving reconciliation between second and third generation assays would be a significant success. The major difference only between the 2 generations of assays would thus become the recognition or not of the amino-PTH, a post-translationally modified form of PTH overproduced in parathyroid carcinoma and in severe forms of secondary hyperparathyroidism observed in some HD patients.[30,33] Even if limited to 5 PTH assays, the successful demonstration of the recalibration results presented here serves as a proof of concept that recalibration of PTH assays is feasible. However, translating this concept into practical implementation will undoubtedly present challenges that can hopefully be overcome through close collaboration among the involved stakeholders.

In conclusion, even if further work is needed, there are good reasons to think that the standardization in PTH measurement is feasible, ultimately benefiting the care of patients.

DISCLOSURE

EC is a consultant to DiaSorin, IDS, Fujirebio, Roche Diagnostics, Snibe, Nittobo, and bioMérieux. PD is a consultant to IDS. CLG is a consultant to Roche Diagnostics. All the other authors declared no competing interests.

DATA STATEMENT

The raw data presented in this study are available on Mendeley Data: https://data.mendeley.com/datasets/skfsbx87b7/1.

REFERENCES

1. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Update Work Group. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl (2011)*. 2017;7:1–59.
2. Cavalier E, Vasikaran S, Bhattoa HP, et al. The path to the standardization of PTH: is this a realistic possibility? A position paper of the IFCC C-BM. *Clin Chim Acta*. 2021;515:44–51.
3. Sturgeon CM, Sprague S, Almond A, et al. Perspective and priorities for improvement of parathyroid hormone (PTH) measurement—a view from the IFCC Working Group for PTH. *Clin Chim Acta*. 2017;467:42–47.
4. Souberbielle J-C, Boutten A, Carlier M-C, et al. Inter-method variability in PTH measurement: implication for the care of CKD patients. *Kidney Int*. 2006;70:345–350.
5. Gardella TJ, Axelrod D, Rubin D, et al. Mutational analysis of the receptor-activating region of human parathyroid hormone. *J Biol Chem*. 1991;266:13141–13146.
6. Segre GV, Perkins AS, Witters L, Potts JT. Metabolism of parathyroid hormone by isolated rat Kupffer cells and hepatocytes. *J Clin Invest*. 1981;67:449–457.
7. Hanley DA, Ayer LM. Calcium-dependent release of carboxyl-terminal fragments of parathyroid hormone by hyperplastic human parathyroid tissue in vitro. *J Clin Endocrinol Metab*. 1986;63:1075–1079.
8. Mayer GP, Keaton JA, Hurst JG, Habener JF. Effects of plasma calcium concentration on the relative proportion of hormone and carboxyl fragments in parathyroid venous blood. *Endocrinology*. 1979;104:1778–1784.
9. Yamashita H, Gao P, Cantor T, et al. Large carboxy-terminal parathyroid hormone (PTH) fragment with a relatively longer half-life than 1-84 PTH is secreted directly from the parathyroid gland in humans. *Eur J Endocrinol*. 2003;149:301–306.
10. Yamashita H, Cantor T, Uchino S, et al. Sequential changes in plasma intact and whole parathyroid hormone levels during parathyroidectomy for secondary hyperparathyroidism. *World J Surg*. 2005;29:169–173.
11. D'amour P, Lazure C, Labelle F. Metabolism of radioiodinated carboxy-terminal fragments of bovine parathyroid hormone in normal and anephric rats. *Endocrinology*. 1985;117:127–134.
12. D'Amour P. Circulating PTH molecular forms: what we know and what we don't. *Kidney Int*. 2006;70(suppl 102):S29–S33.
13. Kritmetapak K, Losbanos LA, Hines JM, et al. Chemical characterization and quantification of circulating intact PTH and PTH fragments by high-resolution mass spectrometry in chronic renal failure. *Clin Chem*. 2021;67:843–853.
14. Lepage R, Roy L, Brossard JH, et al. A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples. *Clin Chem*. 1998;44:805–809.
15. John MR, Goodman WG, Ping G, et al. A novel immunoradiometric assay detects full-length human PTH but not amino-terminally truncated fragments: implications for PTH measurements in renal failure. *J Clin Endocrinol Metab*. 1999;84:4287–4290.
16. Gao P, Scheibel S, D'Amour P, et al. Development of a novel immunoradiometric assay exclusively for biologically active whole

parathyroid hormone 1-84: implications for improvement of accurate assessment of parathyroid function. *J Bone Miner Res*. 2001;16:605–614.
17. Farré-Segura J, Le Goff C, Lukas P, et al. Validation of an LC-MS/MS method using solid-phase extraction for the quantification of 1-84 parathyroid hormone: toward a candidate reference measurement procedure. *Clin Chem*. 2022;3:1399–1409.
18. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease-mineral bone disorder (CKD-MBD). *Kidney Int*. 2009;76(suppl 113): S1–S130.
19. National Kidney Foundation. K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease. *Am J Kidney Dis*. 2003;42(4 suppl 3):S1–S201.
20. Cavalier E, Delanaye P, Vranken L, et al. Interpretation of serum PTH concentrations with different kits in dialysis patients according to the KDIGO guidelines: importance of the reference (normal) values. *Nephrol Dial Transplant*. 2011;27:1950–1956.
21. Souberbielle J-C, Brazier F, Piketty M-L, et al. How the reference values for serum parathyroid hormone concentration are (or should be) established? *J Endocrinal Invest*. 2016;40:241–256.
22. Cavalier E, Lukas P, Bekaert A-C, et al. Analytical and clinical evaluation of the new Fujirebio Lumipulse®G non-competitive assay for 25(OH)-vitamin D and three immunoassays for 25(OH)D in healthy subjects, osteoporotic patients, third trimester pregnant women, healthy African subjects, hemodialyzed and intensive care patients. *Clin Chem Lab Med*. 2016;54:1347–1355.
23. Yalla N, Bobba G, Guo G, et al. Parathyroid hormone reference ranges in healthy individuals classified by vitamin D status. *J Endocrinol Invest*. 2019;42:1353–1360.
24. Delgado JA, Bauça JM, Pastor MI, Barceló A. Use of data mining in the establishment of age-adjusted reference intervals for parathyroid hormone. *Clin Chim Acta*. 2020;508:217–220.
25. Hocher B, Zeng S. Clear the fog around parathyroid hormone assays: what do iPTH assays really measure? *Clin J Am Soc Nephrol*. 2018;13: 524–526.
26. Hocher B, Armbruster FP, Stoeva S, et al. Measuring parathyroid hormone (PTH) in patients with oxidative stress—do we need a fourth generation parathyroid hormone assay? *PLoS One*. 2012;7:e40242.
27. Zhang CX, Weber BV, Thammavong J, et al. Identification of carboxyl-terminal peptide fragments of parathyroid hormone in human plasma at low-picomolar levels by mass spectrometry. *Anal Chem*. 2006;78:1636–1643.
28. Lopez MF, Rezai T, Sarracino DA, et al. Selected reaction monitoring-mass spectrometric immunoassay responsive to parathyroid hormone and related variants. *Clin Chem*. 2010;56:281–290.
29. Souberbielle J-CP, Roth H, Fouque DP. Parathyroid hormone measurement in CKD. *Kidney Int*. 2010;77:93–100.
30. Cavalier E, Daly AF, Betea D, et al. The ratio of parathyroid hormone as measured by third- and second-generation assays as a marker for parathyroid carcinoma. *J Clin Endocrinol Metab*. 2010;95: 3745–3749.
31. Cavalier E, Schleck M-L, Souberbielle J-C. Spurious intraoperative PTH results observed with 2nd, but not with 3rd generation PTH assays. *Clin Chim Acta*. 2018;477:72–73.
32. Nguyen-Yamamoto L, Rousseau L, Brossard JH, et al. Origin of parathyroid hormone (PTH) fragments detected by intact-PTH assays. *Eur J Endocrinol*. 2002;147:123–131.
33. Komaba H, Takeda Y, Shin J, et al. Reversed whole PTH/intact PTH ratio as an indicator of marked parathyroid enlargement: five case studies and a literature review. *NDT Plus*. 2008;1(suppl 3):iii54–iii58.

Chapter 6. Discussion, Conclusions and Perspectives

6.1 Discussion

The aim of our research was to present a higher-order method capable of meeting the requirements to become a reference measurement procedure (RMP) for the determination of parathyroid hormone (PTH), thus paving the way for the accurate determination of PTH to respond to the needs expressed by the International Federation of Clinical Chemistry (IFCC).

Besides its essential importance in the management of primary and secondary hyper- and hypo-parathyroidism, PTH is routinely measured in patients with chronic kidney disease (CKD) according to the Kidney Disease Improving Global Outcomes (KDIGO) 2017 Clinical Practice Guidelines for the Diagnosis, Evaluation, Prevention and Treatment of Chronic Kidney Disease - Mineral Bone Disorders (MBD).

As the biologically active form of PTH (1-84) circulates in the bloodstream along with various fragments and post-translationally modified forms reduces the specificity of currently available immunoassays, inevitably leading to biased results and potentially erroneous clinical decisions. This is even more pronounced in CKD patients, where its accurate determination is of paramount importance since PTH fragments accumulate due to the impaired renal function (1–3).

The fact that PTH assays are not yet standardized (as they do not use a same calibrator or at least one that is traceable to a CRM) makes it even more difficult to follow the recommended guidelines for CKD-MBD monitoring, which aim at maintaining PTH levels of hemodialyzed patients at ≈2 to 9 times the upper limit of the normal (ULN) of the assay being used, which represents the highest value within the established reference range for PTH levels as determined by different IAs. Each IA may have a distinct ULN due

to variations in calibration, antibody specificity and sensitivity to various forms and fragments of PTH, leading to inconsistencies in clinical interpretation across different testing methods. Hence, a common reference range for PTH may not determined when relying in these assays. We believe that the contribution of a higher-order technique that is inherently immune to fragment (and modified forms) cross-reactivity, such as ours, can be an essential tool for this finality.

In this research, after a proof-of-concept demonstration with glucagon, we were able to perform a pioneering analysis of PTH 1-84 by UHPLC-MS/MS without digestion and in its intact form.

The sample preparation procedures used in both methods described in this thesis, which are based on SPE, are normally designed to remove proteins in a complex sample. However, we took advantage of their ionic exchange capabilities combined with their reverse phase properties to retain our compounds of interest while washing out others. Although the pore size of the bead is neither large nor designed for protein analysis, small intact proteins in a favorable conformation interact with the SPE components, making our proposed procedure feasible. This allows the acceleration of otherwise much more complex sample preparation procedures, with some other major advantages. The main difference between SPE and the classical approach to protein analysis is that SPE does not require digestion and does not rely on antibodies. This can be particularly important when antibody availability and cross-reactivities are not well characterized. If PTMs are expected to occur, antibodies may no longer be appropriate and the modified compounds may be lost during sample preparation. Given that the methods described in this study are targeted approaches, this fact may be less relevant, although it can also

be seen as a drawback, since SPE approaches may not be suitable for monitoring species other than those for which the method was designed. In fact, slight variations in the physicochemical properties of a compound may result in a total loss of recovery in an SPE, and thus render it inapplicable. In other words, a given SPE approach may be a very powerful tool for purifying a specific compound, but only suitable for a limited window of species.

The necessity for a more precise PTH determination technique is widely acknowledged, and several attempts have been made to develop one. Although three mass spectrometry methods have been reported to date, each possesses unique attributes and sensitivity problems. For example, two of the methods depend on proteolytic digestion.

Our study employed a cutting-edge triple quadrupole mass spectrometer to fulfill the requirements for a modern RMP. The triple quadrupole's sensitivity is crucial for detecting low-abundance compounds such as PTH in its intact form, with its challenging multiple charge distributions. For this purpose, the use of DMSO was explored as a supercharging agent for ESI, thus reducing the charge distribution on behalf of a 10 fold boost in intensity, permitting us to lower the limit of quantification to 5 pg/mL (versus 235 pg/mL) compared to Kritmetapak's HRMS (3), making it suitable for expected low PTH concentrations in "healthy" population cohorts. Another advantage of triple quadrupole mass spectrometers over HRMS is their robustness and ease of maintenance, making them suitable for larger batch analysis despite an average analysis time of about 24 hours per batch from sample reception until final result. Although their selectivity may not be as good as that of HRMS, a highly selective analysis can still be

achieved by combining the power of both LC and MS/MS, as demonstrated in our study. Similar compounds, like oxidized PTH, could be identified by their m/z ratio and separated chromatographically, which eliminated interferences from other related compounds.

One of the points that deserves additional attention in this work, which at the same time has motivated its realization, is the establishment of a reference measurement procedure (RMP) for the measurement of PTH.

In fact, the method and the main work in this thesis have been published in the article entitled " Validation of an LC-MS/MS Method Using Solid-Phase Extraction for the Quantification of 1-84 Parathyroid Hormone: Toward a Candidate Reference Measurement Procedure", controversial for some of the specialized readers (4,5). Indeed, one of the limitations, external to the method, is the non-existence of a standard for PTH that meets the characteristics expected for a reference material to be used in an RMP.

Currently, one of the factors taken into consideration when categorizing a method as a candidate RMP is the measurement uncertainty (MU), formed by the contribution of the different uncertainties throughout the traceability chain of the analysis to the SI, among others, the measurement uncertainty of the standard (MU_{std}), calibration (MU_{cal}), and random analytical errors (MU_{rw}). Globally it is accepted that for a method to be considered a candidate for RMP (a process that concludes with acceptance by the JCTLM), it must have a measurement uncertainty budget equal to a third of the interindividual coefficient of biological variation for the parameter in question.

In our case, the uncertainties arising from our analytical method would be compatible for a reference measurement procedure. Unfortunately, to date, the best available standard is the World Health Organization international PTH standard, WHO 95/646, which does not have a sufficiently good characterization of its PTH content with a well-studied uncertainty. Faced with this defect, for our purpose, the content uncertainty had to be estimated based on the AAA performed by 8 independent laboratories. Unfortunately, the uncertainty values of this standard became excessively high for it to be a good reference material for an RMP. In fact, the contribution to the total uncertainty leaves virtually no margin in the MU budget for the method to be an RMP. However, it is noteworthy that in the near future a new PTH 1-84 standard will be made available to the market, presumably around 2025, and finally our method, with its due revalidation and compliance with the ISO 15193 guidelines, will be proposable as an RMP.

At this time, we present the first higher-order method sensitive enough for the analysis of intact PTH that provides a powerful tool for the much-pursued standardization of PTH analysis.

Even though our PTH method was not optimized for compounds other than intact PTH 1-84, “in-vitro” oxidized PTH was equally treated by SPE and could be observed in our chromatograms. Observing that the SPE was suitable for PTH samples that were forcedly oxidized with apparent similar recoveries, and never observing oxidized PTH in any of the hundreds of samples analyzed, allowed us to position ourselves in-line with what is actively being discussed, the non-existence of oxidized PTH or at least not in as important amounts as previously believed. It is not to forget that some authors, who used immunoassays, claimed that oxidized PTH could account for up to 80% of the total PTH

in circulation, even with mortality studies involved (6). One of the significant application points of our contribution with mass spectrometry is the possibility of recalibrating the various available and worldwide used immunoassays. In our work published in Kidney International, we aimed to assess the impact of recalibrating five PTH immunoassays using our LC-MS/MS method as a reference, utilizing the World Health Organization (WHO) 95/646 International Standard. Notably, the recalibration process allowed aligning the results obtained from the various immunoassays with the LC-MS/MS method, thus obtaining more consistent and reliable measurements. This latest study once again highlights the pressing and now feasible need to standardize PTH assays to ensure accurate interpretation and subsequent clinical decision-making.

The time required to run a full batch of samples from sample reception until the final result is around 36 hours. In contrast to the existing immunoassays (IAs) which can provide results in a matter of few minutes, our method is not intended to replace IAs but to provide a reference level result instead.

6.2 Conclusions and perspectives

This thesis has advanced the field of PTH analysis by developing and validating a novel LC-MS/MS method for measuring intact 1-84 PTH, offering a potential pathway towards establishing a RMP. The new method demonstrates exceptional sensitivity and specificity without relying on tryptic digestion or antibodies, which could potentially standardize PTH measurements—a longstanding challenge in clinical settings due to the variable results produced by existing IAs. By recalibrating different IAs against the WHO 95/646 International Standard, this method has also shown promise in reducing the

discrepancies across different PTH assays, which is crucial for consistent clinical interpretation and patient management.

Furthermore, the thesis encapsulates the pivotal role of a higher-order technique such as mass spectrometry in overcoming the limitations of current IAs that often suffer from cross-reactivity and lack of specificity due to the presence of PTH fragments in blood samples. The successful implementation of this LC-MS/MS method as a reference for different assays could lead to more reliable and harmonized PTH measurements, ahold of a new, better-characterized PTH standard promises to further refine this method, paving the way for its adoption as a globally recognized RMP.

It would be highly valuable if several facilities equipped with instruments similar to the ones used in this study could join their forces in order to compare results and potentially establish a network of reference laboratories for PTH analysis, especially once a reference standard becomes available, improving the current WHO 95/646. At that time, a revalidation of the method should be performed to assess the compliances defined by ISO 15193 (7) to present the method as a RMP, with new measurement uncertainty values defined. At this step, we would expect different IVD manufacturers to be able to standardize the different existing IAs.

Flexibility and multiplexing capabilities of triple quadrupole instruments, should be considered to incorporate the quantification of PTH fragments found in circulation, whose clinical relevance has never been studied in depth through a sensitive MS/MS method. All of this would likely require adaptation in sample preparation and, of course, the adjustment and integration of new MRM parameters. While it's true that recent studies by Kritmetapak in HRMS (3) demonstrate fragment accumulation in patients

with reduced kidney function, the method's low sensitivity might be hiding relevant data that would be interesting to unveil on large population cohorts. Likewise, studying PTMs such as the amino PTH (PTH phosphorylated at position 17), presumably relevant in parathyroid carcinomas, is a feasible option to understand a path that remains largely unexplored.

6.3 References

1. D'Amour P. Circulating PTH molecular forms: What we know and what we don't. Kidney Int. 2006;70:29–33.

2. D'Amour P, Lazure C, Labelle F. Metabolism of Radioiodinated Carboxy-Terminal Fragments of Bovine Parathyroid Hormone in Normal and Anephric Rats*. Endocrinology. 1985;117:127–34.

3. Kritmetapak K, Losbanos LA, Hines JM, O'Grady KL, Ulmer CZ, Vesper HW, et al. Chemical Characterization and Quantification of Circulating Intact PTH and PTH Fragments by High-Resolution Mass Spectrometry in Chronic Renal Failure. Clin Chem. 2021;67:843–53.

4. Cavalier E, Farré-Segura J. In Reply to A Comment about Analytical Performance Specifications for the Combined Measurement Uncertainty Budget in the Implementation of Metrological Traceability of Parathyroid Hormone. Clin Chem. 2023;

5. Panteghini M. A Comment about Analytical Performance Specifications for the Combined Measurement Uncertainty Budget in the Implementation of Metrological Traceability of Parathyroid Hormone. Clin Chem. 2023;

6. Hocher B, Pasch A. Hope for CKD-MBD Patients: New Diagnostic Approaches for Better Treatment of CKD-MBD. Kidney Diseases. 2017;3:8–14.

7. ISO. In vitro diagnostic medical devices. Measurement of quantities in samples of biological origin. Requirements for content and presentation of reference measurement procedures. ISO 15193. ISO London, United Kingdom; 2009.

Chapter 7. Supplementary data.

7.1 PTH Supplemental Methods

7.1.1 Evaluation of the adsorptive loss during the processing of specimens to the pipette tips and container

To evaluate adsorptive loss to the pipette tips and sample container, we adopted a less favorable scenario to potentially maximize the effect. A first aliquot of 500 µL of a 220.0 pg/mL PTH dilution in water was transferred to the first well of an Eppendorf LoBind 96-well plate, acting as a reference sample. A second 500 µL aliquot of the same solution was transferred to the second well of the plate. Every 5 min, the full content of the second well was transferred to the next five wells. Centrifugation was performed for 30 s at 500 g in order to maximize the volume recovered from each well. Finally, IS was added to the samples, vortexed and analyzed by LC-MS/MS. Each transfer was performed with a new pipette tip. The test was performed in triplicate.

We did not observe adsorptive losses in the experiment performed. The mean concentration of the reference was 221.0 pg/mL, whereas the samples that underwent the transfers were found at 220.2 pg/mL, 99.7% of the reference concentration.

Preparation of calibration curve and quality control samples

From the aliquoted standard, to avoid matrix variations along the different dilutions, a set of spiking solutions were prepared at 9 different concentrations (0.25 ng/mL, 0.50 ng/mL, 1.25 ng/mL, 2.50 ng/mL, 5.0 ng/mL, 12.5 ng/mL, 25 ng/mL, 40 ng/mL and 50 ng/mL), so a constant volume of 20 µL could be spiked (gravimetrically) in all the calibration samples.

The 9-point calibration curve was prepared by weighting 20 µL of each spiking solution into 980 µL of horse plasma (5.0 pg/mL, 10.0 pg/mL, 25.0 pg/mL, 50.0 pg/mL, 100.0 pg/mL, 250.0 pg/mL, 500.0 pg/mL, 750.0 pg/mL and 1000.0 pg/mL).

Quality control (QC) samples for validation were bulk prepared at 5 different concentrations (5.7 pg/mL (V1), 9.7 pg/mL (V2), 47.9 pg/mL (V3), 484.8 pg/mL (V4) and 872.6 pg/mL (V5)), from gravimetrically produced spiking solutions.

Calibrators were freshly prepared every run. Before the validation, each QC was prepared in a PTH-free human EDTA pool (prepared from leftover samples), divided into twenty-five 1 mL aliquots and frozen at -80ºC.

7.1.2 Oxidation of PTH standards

For the partial oxidation of PTH 1-84, 5 µL of H_2O_2 (30%) were added to 1 mL of PTH 1-84 standard at 2.0 µg/mL in 10% ACN, 0.1% bovine serum albumin (BSA) and 0.1% formic acid. The PTH standard was vortexed and placed in an Eppendorf Thermomixer C for 30 min at 35ºC and 500 rpm for oxidation. Immediately after oxidation, samples were diluted 50 times with 10% ACN, 0.1% BSA and 0.1% HCOOH and injected into the LC-MS/MS. This procedure resulted in the obtention of the several oxidized PTH forms, as displayed in Supplemental Figure 2.

7.1.3 Signal-to-Noise estimation

Signal-to-Noise (S/N) was defined according to the USP 621 and Ph.Eur. 2.2.46. The following equation was used:

$$S/N = 2H/h$$

where H was the height of the peak and h was the height of the noise calculated on a blank, on a distance corresponding to 5 times the width of the peak at half-height, centered on the expected retention time.

7.1.4 Contribution from blank matrix to signal of the analyte and internal Standard transitions

During each day of validation, a double blank sample (without any spiked PTH nor IS) was injected to confirm the absence of contribution of the matrix to the signal of any of the recorded transitions. A window of ± 30 s of the expected retention time was considered for the evaluation. There was no contribution of the matrix to either of the monitored transitions.

7.1.5 Contribution of the Internal Standard to native PTH response

According to CLSI C64 guidelines regarding the purity and contribution of the labelled internal Standard to the native response, the injection of a concentrated solution of internal Standard (at 5000.0 pg/mL) did not contribute to the response of the native PTH transitions (Delta mass is ~120Da between both species).

7.1.6 Assessment of interferences from hyperalbuminemia, icterus, hemolysis and lipidemia

The effect of hyperalbuminemia, icterus, hemolysis and lipidemia on the LC-MS/MS analysis was assessed according to CLSI EP07 by analyzing in a pool of human EDTA, in triplicate, increasing amounts of the potential interferent at five different interferant concentration levels, i.e., 0%, 25%, 50%, 75% and 100%.

The maximum acceptable difference was defined as one-half of the total acceptable error for PTH, i.e., 5%.

For hyperalbuminemia and icterus, stock solutions of albumin and bilirubin were prepared in water at 500.0 mg/mL and 5.0 mg/mL, respectively.

For hemolysis, interferent solutions were prepared from red blood cells obtained from the red fraction of an EDTA plasma tube. The red blood cells were lysed with demineralized water and centrifugation. Hemoglobin concentration was 10400.0 mg/dL, quantified on a Sysmex XS-800i.

For lipidemia, a 100.0 mg/mL fat emulsion prepared for intravenous administration was used as a stock solution.

The resulting concentrations of interferents are shown in supplemental Table 5.

The maximum acceptable difference was exceeded in hemolyzed samples with a hemoglobin concentration >5.2 mg/dL due to an isobaric interference with the internal standard. No interferences were found for the rest of the tested interferents. Interference plots are shown in Supplemental Figure 3.

7.1.7 Supplemental selectivity assessment

PTH fragments that could interfere with our MRM transitions were checked. In a prior study (1) the characteristics of the identified PTH fragments were described in Supplemental Table 3 from that article. From this study, we can hypothesize that, effectively, PTH 34-77 (z +7) shares a very similar *m/z* with the parent ion of PTH 1-84 (z +14) used in our method (as a qualitative transition, not for quantification). However,

even if we could not demonstrate chromatographic separation with this fragment or other fragments, it is highly improbable that they could interfere.

First, given the nature of the described fragments, already missing the N-term part of the peptide, at a given collision energy, the collision induced dissociation fragmentation patterns on the obtained product ions would likely differ and not interfere with the PTH 1-84 transition. Second, the ion ratios would differ between healthy and CKD patients (especially last stage), and we did not observe such an effect. Third, given the described chromatographic performance and observing the behavior of oxidized 1-84 PTH and 7-84 PTH forms, we would certainly expect, similar to Kritmetapak and colleagues (1), a resolution on the LC dimension of the identified possible interferant (34-77 PTH), which highly differs in size and presents a lower hydrophobicity.

7.1.8 Evaluation of Ion suppression through multiple matrices

A highly concentrated PTH sample was constantly infused with a Hamilton syringe at a ratio of 1900 pg/sec. With the help of a T-union, the LC elution line was attached to the syringe and connected together to the mass spectrometer. During the infusion, human and horse EDTA plasma samples, as well as solvent blanks, were injected to assess the ion suppression throughout the chromatographic run. A representative overlay of the T-infusion chromatogram monitoring the quantitative transition of PTH is shown in Supplemental Figure 6. We concluded that there were no ion suppression effects in the assessed matrices.

7.1.9 Data Acceptance Criteria

Before each validation run, the system was checked with our internal System Suitability Test sample, which served to assess an adequate LC and MS/MS performance before the run. Retention time, peak shape, intensity and ion ratios were monitored. The System Suitability Test was successful on all validation days.

During the validation run, we established the following acceptance criteria:

- Area of the lowest calibration point >2.5e4
- S/N of the lowest calibration point >5
- Ion Ratio shift <20%
- Calibrators Accuracy > 95%
- Acceptable RT shift <0.05 min
- Half-height peak width <0.07 min

All samples were checked individually during the validation regarding the mentioned acceptance criteria. All samples fell within the acceptable range.

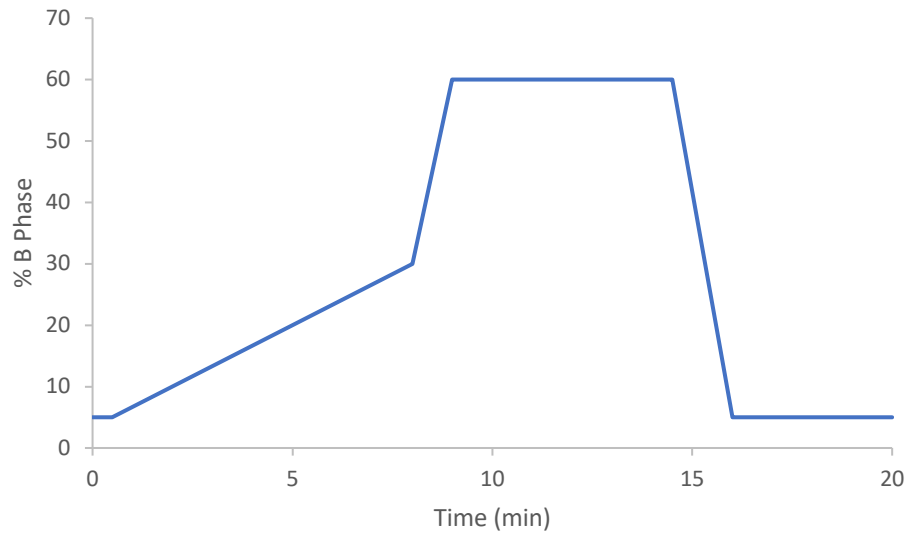
7.1.10 Concordance Correlation Coefficient

The concordance between the two compared immunoassays and the newly developed LC-MS/MS method was assessed by calculating the Concordance Correlation Coefficient (2) of the different groups of samples. Results are shown in supplemental Table 6. McBride (2005) suggested the different strengths of agreement according to the Concordance Correlation Coefficient value, indicating that values <0.90, 0.90-0.95, 0.95-0.99 or >0.99 denoted poor, moderate, substantial or almost perfect strength of agreement, respectively(3).

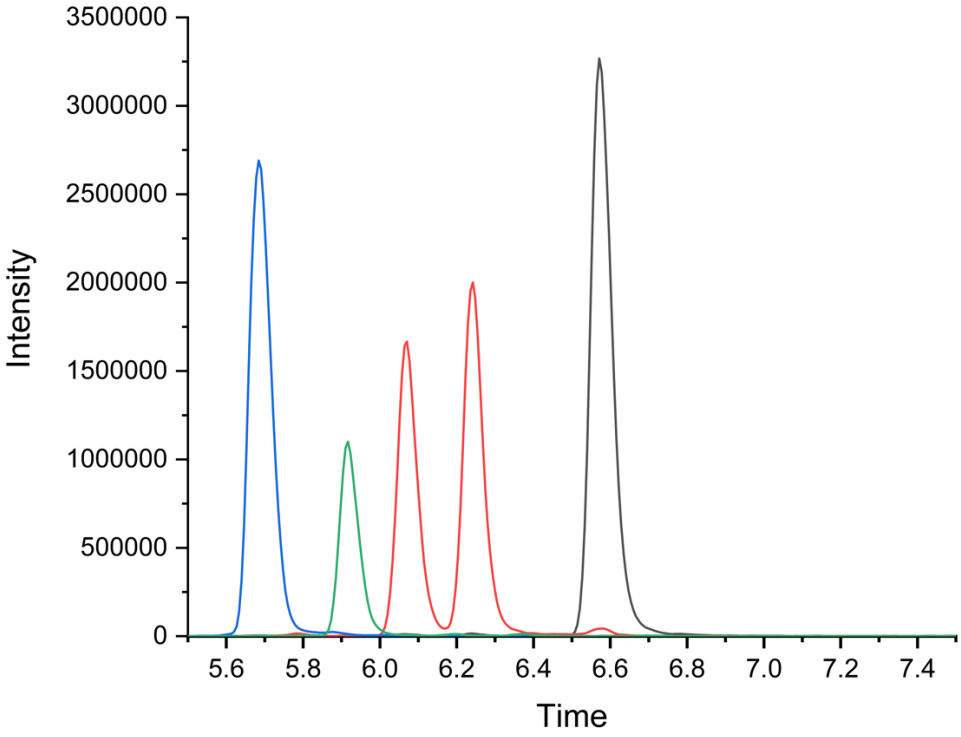
7.1.11 Estimation of WHO 95/646 Standard Uncertainty

The WHO 95/646 PTH Standard's uncertainty was estimated following the WHO/BS/09.2115 technical report, which established the 95/646 standard ampoule content of PTH 1-84 in a study conducted by 13 laboratories, 5 of which used AAA. In our work, we solely accounted for the AAA outcomes. The WHO excluded one of the five participating laboratories. The mean of the laboratory means was 98.52 µg, which was used as the true PTH 1-84 content per ampoule. The uncertainty associated with this value was determined using the mean of the four laboratories' claimed precision (% CV). The uncertainties related to trueness were not taken into account, because the deviation from the target value (100 µg) was only an indication and could be used as a reference value.

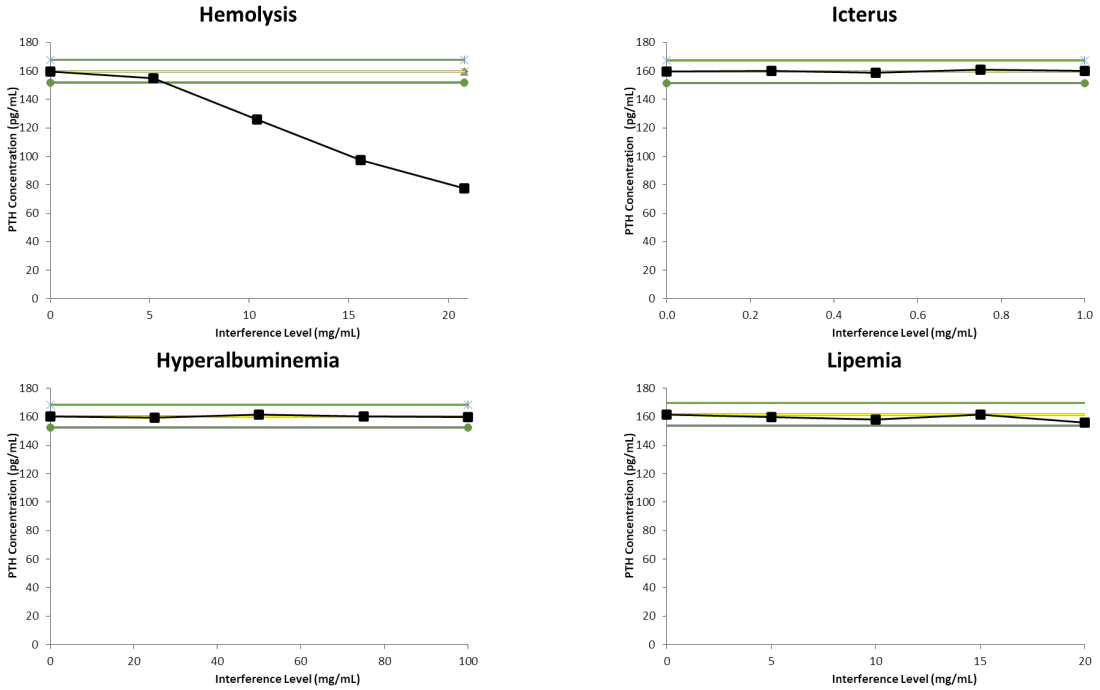
7.1.12 Supplemental Figures



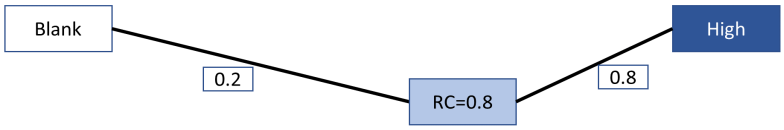
Supplemental Figure 1: Binary Flow LC gradient. Total flow: 0.30 mL/min. Phase A: H_2O + 0.4% HCOOH , 5% DMSO. Phase B: ACN+ 0.4% HCOOH , 5% DMSO.



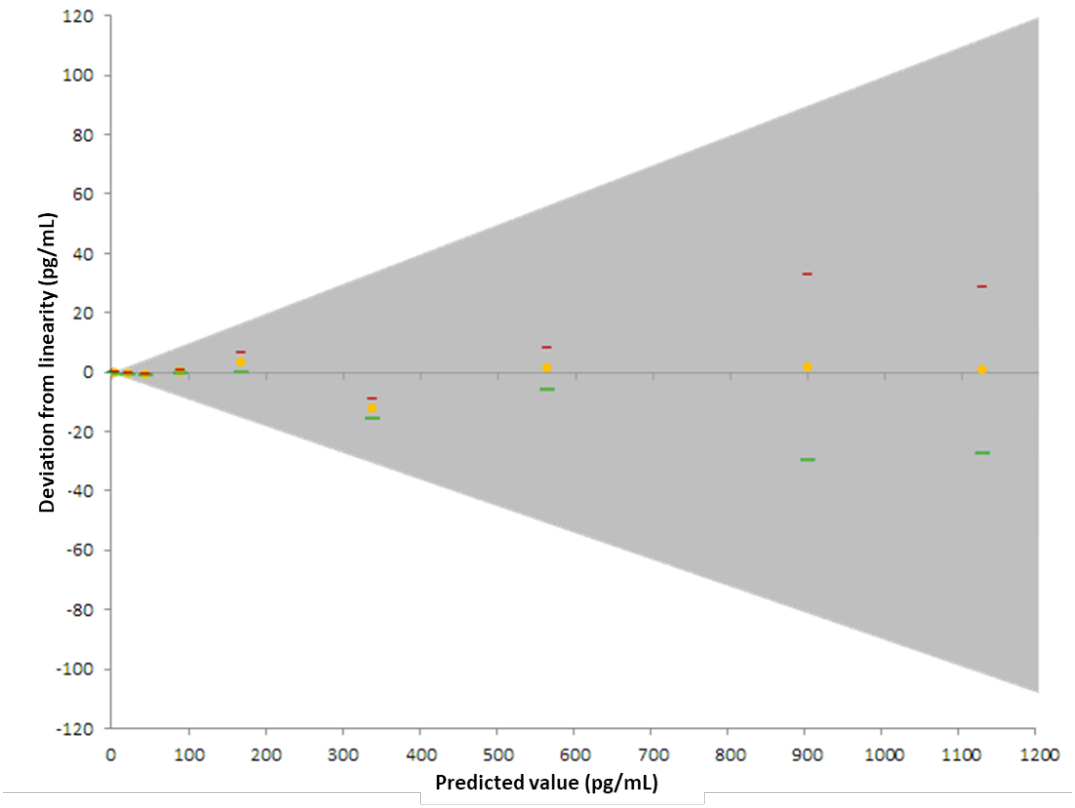
Supplemental Figure 2. LC separation of 1-84 PTH, oxPTH (oxidized in Methionine 8, 18 and both) and 7-84 PTH forms. Transitions are represented from left to right in: Blue: $[PTH(7\text{-}84)+12H]^{+12}$; Green: $2ox[PTH+13H]^{+13}$; Red: $ox[PTH+12H]^{+12}$ and Black: $[PTH+12H]^{+12}$.



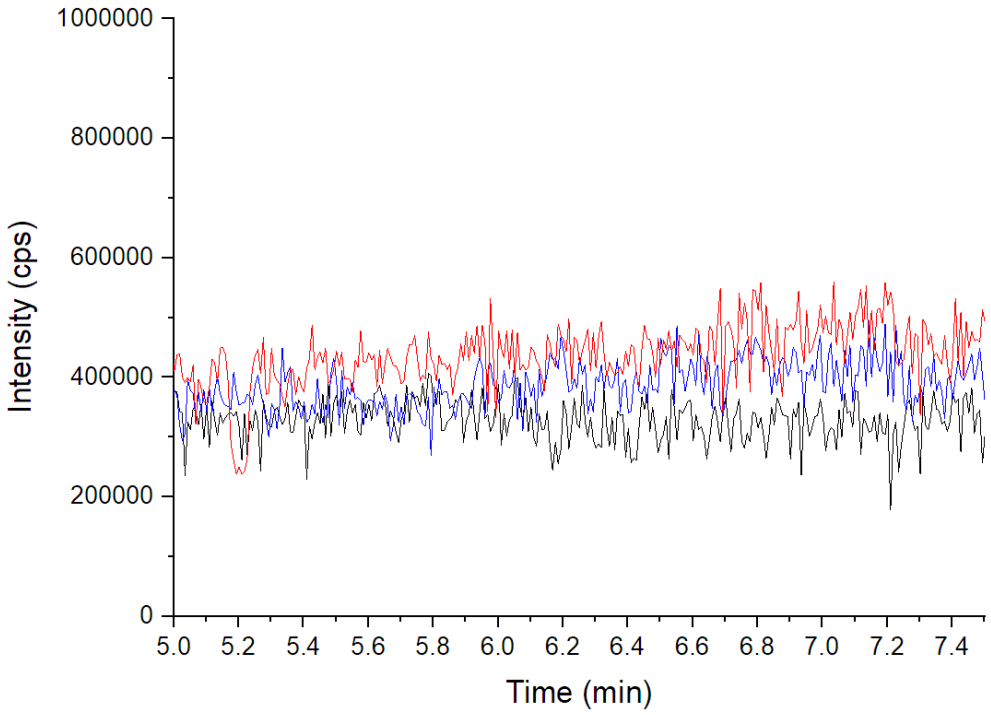
Supplemental Figure 3: Interference profile for Hemolysis, Icterus, Hyperalbuminemia and Lipemia, according to CLSI EP07. Green interval represents the maximum allowed difference (5%) and yellow line the “no interference” PTH concentration.



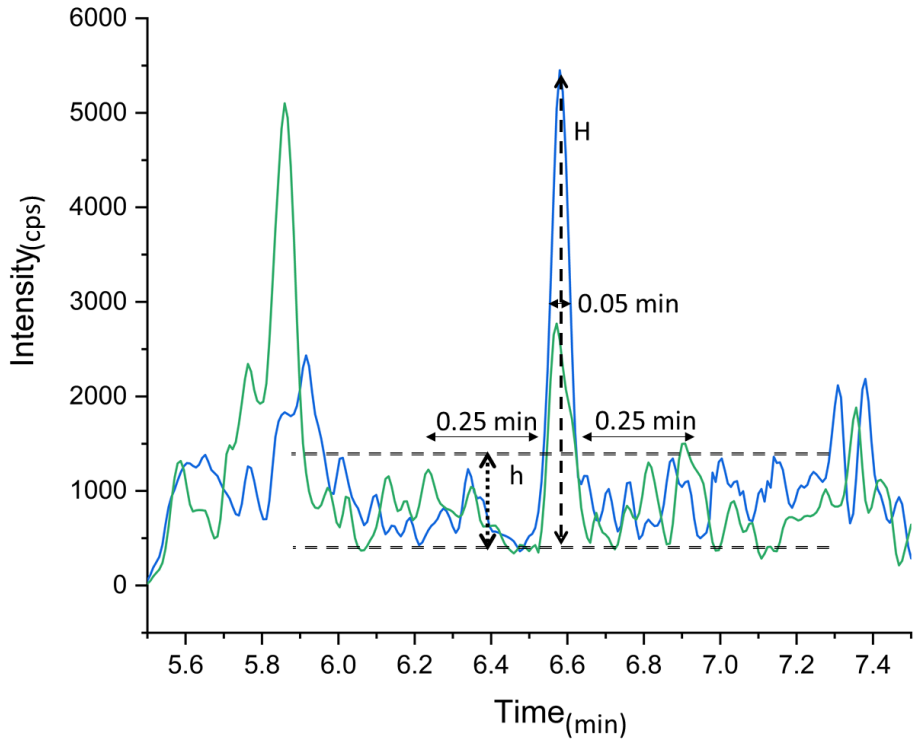
Supplemental Figure 4: Proportional mixing of high concentration EDTA human plasma pool and blank EDTA horse plasma. Adapted from CLSI EP06.



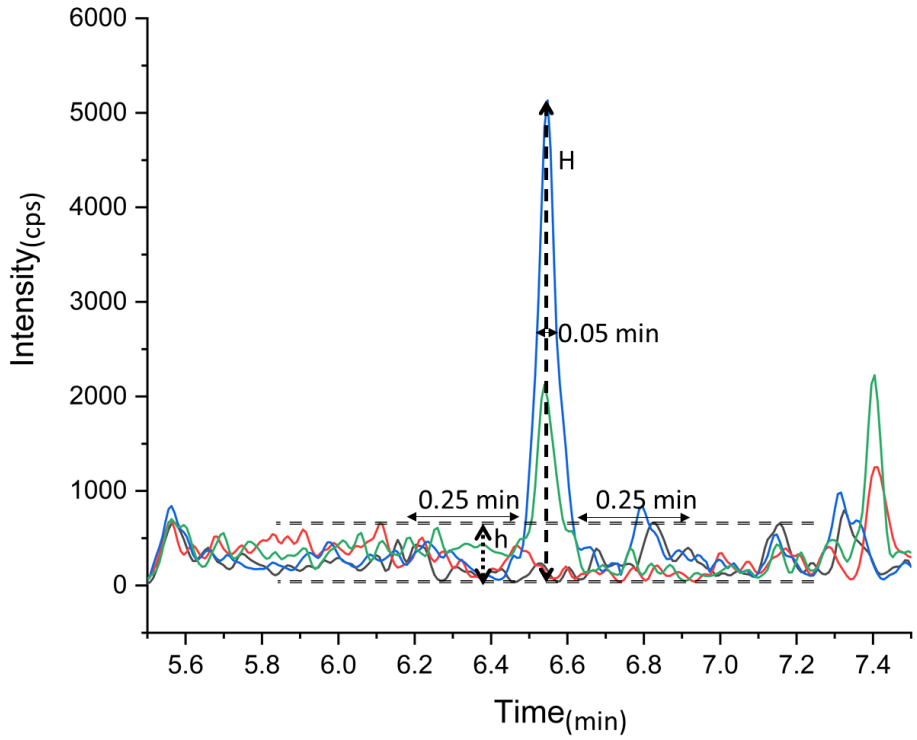
Supplemental Figure 5: Linearity profile according to CLSI EP06. Plain yellow dots correspond to the observed values. Red and green lines represent the upper and lower limit of the CI. Shaded area shows ±5% ADL. Abbreviations: ADL, allowable deviation from linearity; CI, confidence interval.



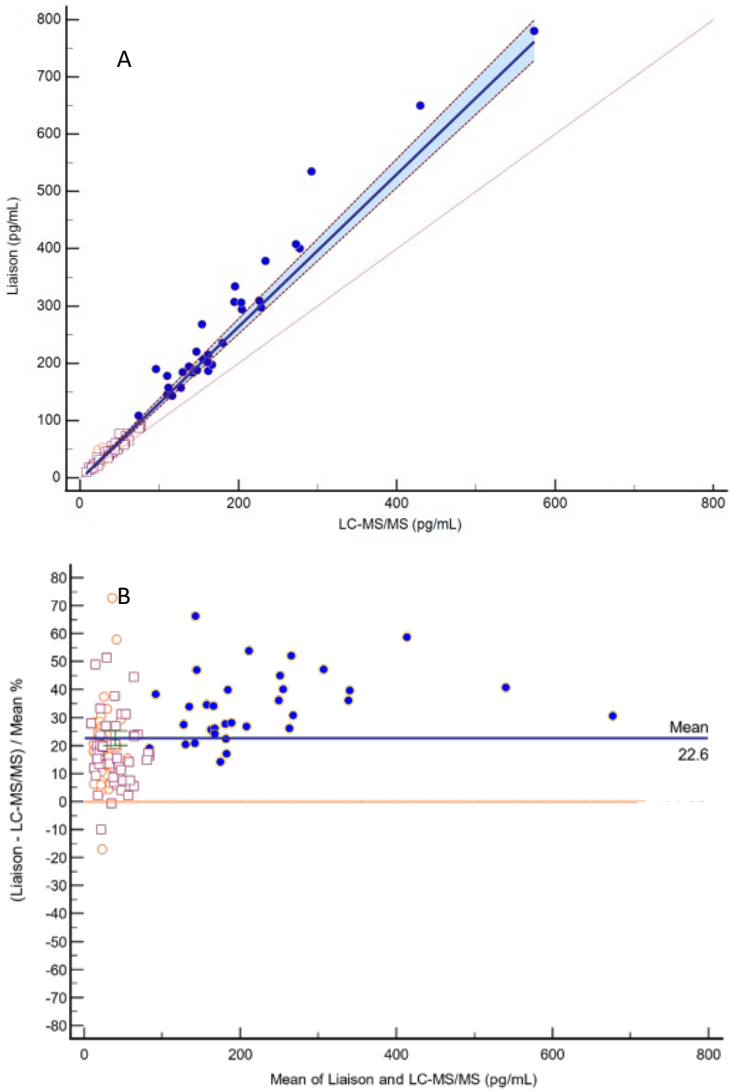
Supplemental Figure 6. Representative overlayed extracted ion chromatogram for PTH 1-84 (786.30 > 840.70) for ion suppresion assessment on different matrices. In red, horse EDTA; In blue, human EDTA; In black, blank sample.



Supplemental Figure 7. Extracted ion chromatogram of a Human EDTA plasma sample at 5.8 pg/mL. The calculated S/N was 8:1. In blue, $[PTH+12H]^{+12}$ and in green $[PTH+13H]^{+13}$.

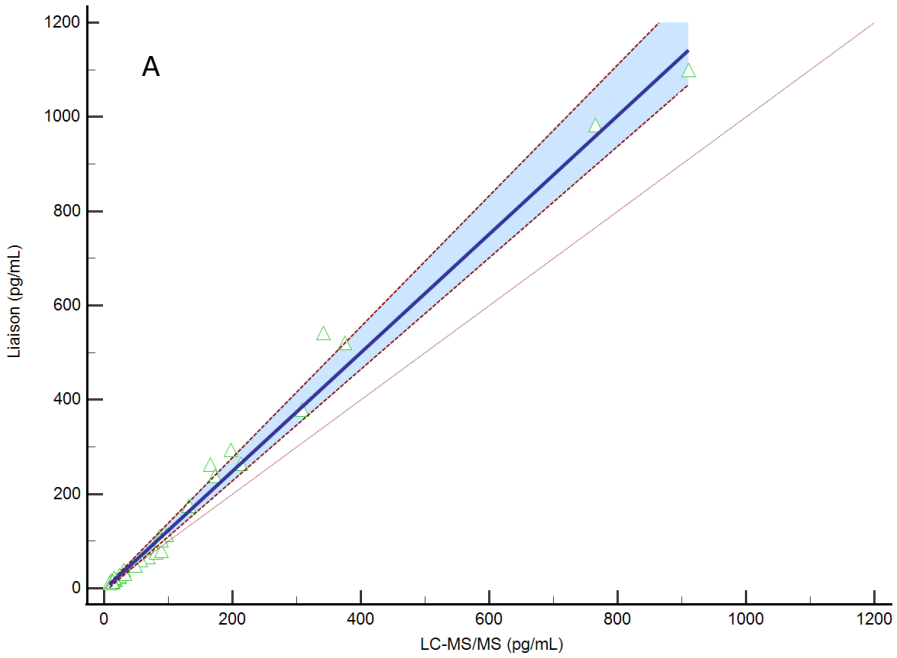


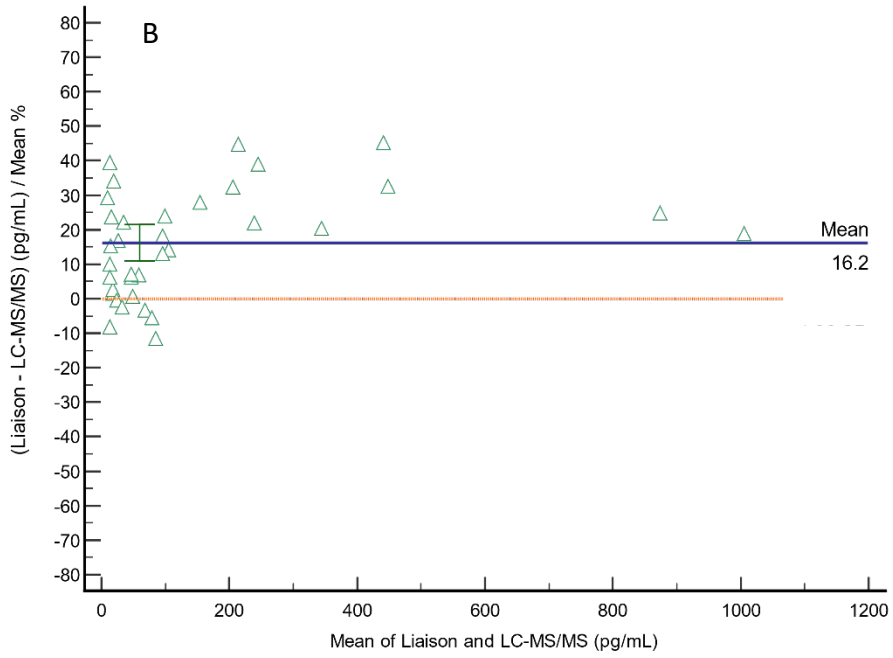
Supplemental Figure 8. Extracted ion chromatogram of a blank horse plasma sample (gray) and spiked with PTH at 5.7 pg/mL (black). The calculated S/N was 10:1. Blue and green XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$ in a LOQ sample. Black and red XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$ in a blank sample. Abbreviations: XIC, extracted ion chromatogram; LOQ, limit of quantification.



Supplemental Figure 9. A) Liaison and LC-MS/MS Passing-Bablok regression and Bland-Altman plots for non-CKD, CKD and DIA (n=124). Intercept pg/mL (95% CI): -2.90 (-4.49 to -1.58). Slope (95% CI): 1.33 (1.28 to 1.40). R^2=0.979. B) Bland-Altman plot for non-CKD, CKD and DIA. Mean of the difference from the two measurements is represented by the solid line at 22.6%. Dashed lines indicate values at ±1.96 SD of the mean differences. Empty circles and squares represent non-CKD and CKD samples respectively. Plain circles represent DIA samples.

Abbreviations: CKD, Chronic kidney disease. DIA, hemodialyzed. CI, Confidence interval. SD, Standard deviation.

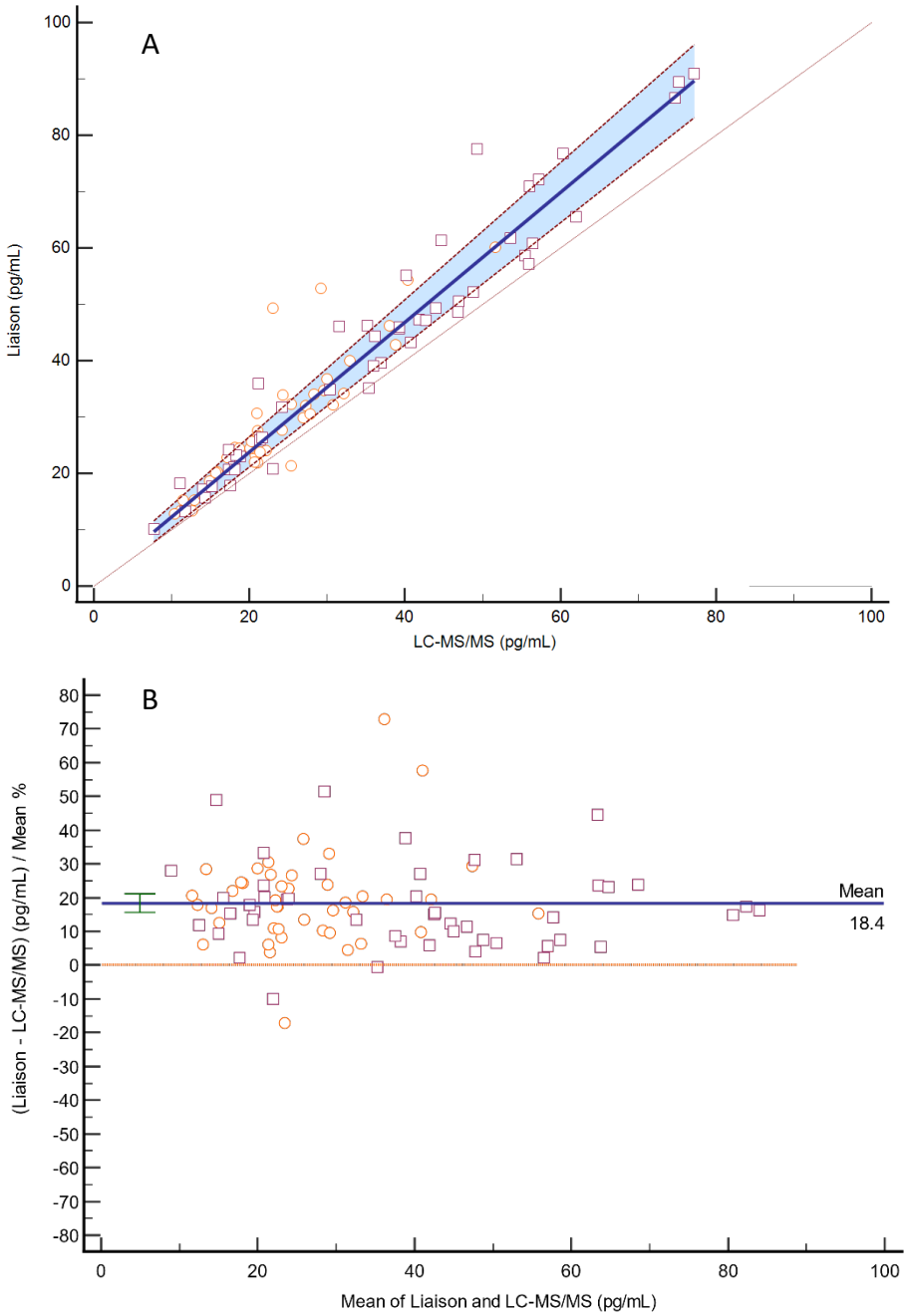

A
Liaison (pg/mL)
LC-MS/MS (pg/mL)
0
200
400
600
800
1000
1200


B
(Liaison - LC-MS/MS) (pg/mL) / Mean %
Mean of Liaison and LC-MS/MS (pg/mL)
Mean
16.2
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
200
400
600
800
1000
1200

Supplemental Figure 10A: Liaison and LC-MS/MS Passing-Bablok regression for the EQCs (n=35). Intercept pg/mL (95% CI): -2.53 (-8.17 to -0.04). Slope (95% CI): 1.26 (1.05 to 1.18). R^2=0.989.

Supplemental Figure 10B. Bland-Altman plot for the EQCs. Mean of the difference from the two measurements is represented by the solid line at 16.2%. Dashed lines indicate values at ±1.96 SD of the mean differences.

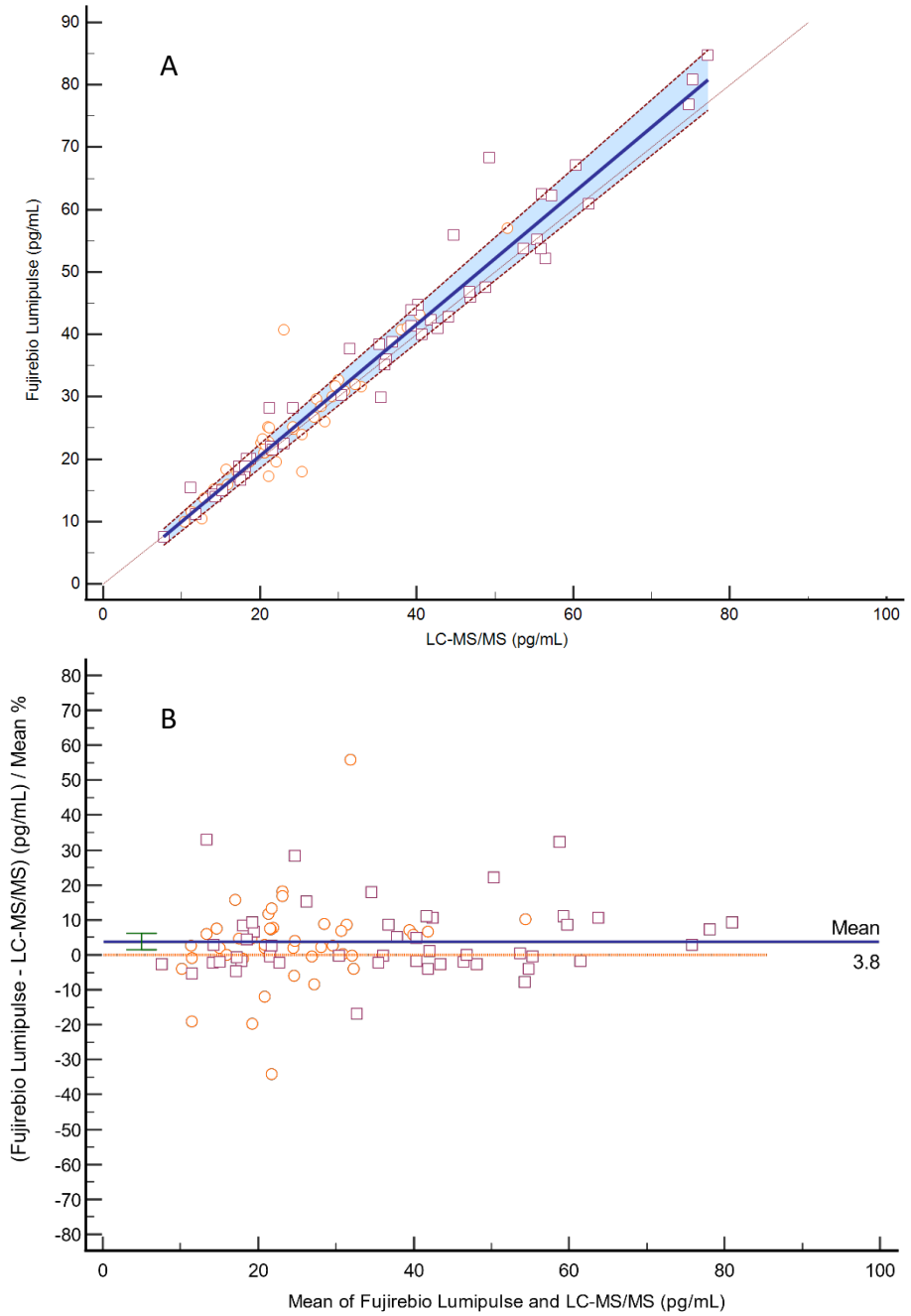
Abbreviations: EQC, External quality control. CI, Confidence interval. SD, Standard deviation.



Supplemental Figure 11. A) Liaison and LC-MS/MS Passing-Bablok regression for Human EDTA samples <100.0 pg/mL (LC-MS/MS), excluding DIA (n=91). Intercept pg/mL (95% CI): 0.78 (-0.51 to 2.21). Slope (95% CI): 1.15 (1.08 to 1.22). R^2=0.925. B) Bland-Altman plot for Human EDTA samples <100.0 pg/mL (LC-MS/MS), excluding DIA (n=91). Mean of the difference from the two measurements is represented by the solid line at 18.4%.

Dashed lines indicate values at ±1.96 SD of the mean differences. Empty circles and squares represent non-CKD and CKD samples respectively.

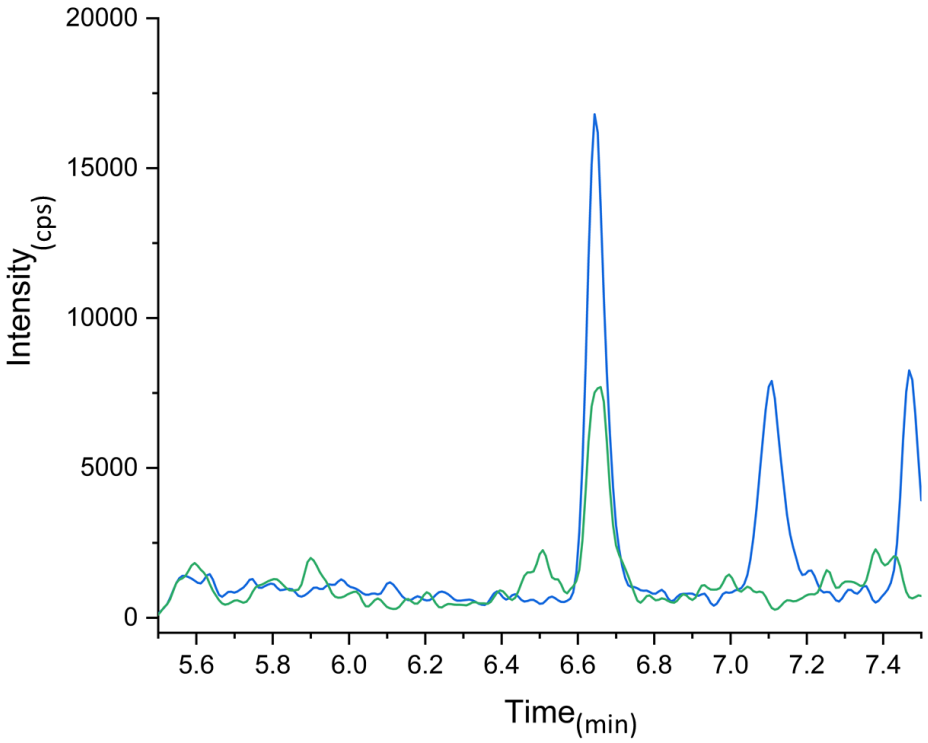
Abbreviations: CKD, Chronic kidney disease. DIA, hemodialyzed. CI, Confidence interval. SD, Standard deviation.



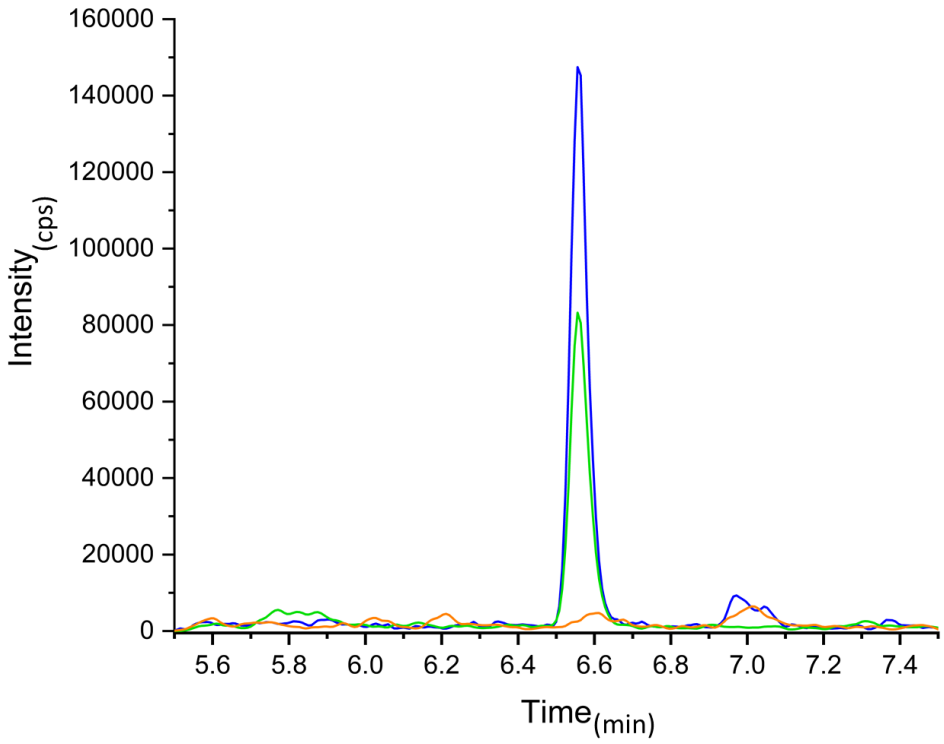
Supplemental Figure 12. A) Fujirebio Lumipulse and LC-MS/MS Passing-Bablok regression for Human EDTA samples <100.0 pg/mL (LC-MS/MS), excluding DIA (n=91). Intercept pg/mL (95% CI): 0.56 (-1.53 to 0.32). Slope (95% CI): 1.05 (1.00 to 1.10). R^2=0.954.

Supplemental Figure 12. B) Bland-Altman plot for Human EDTA samples <100.0 pg/mL (LC-MS/MS), excluding DIA (n=91). Mean of the difference from the two measurements is represented by the solid line at 3.8%. Dashed lines indicate values at ±1.96 SD of the mean differences. Empty circles and squares represent non-CKD and CKD samples respectively.

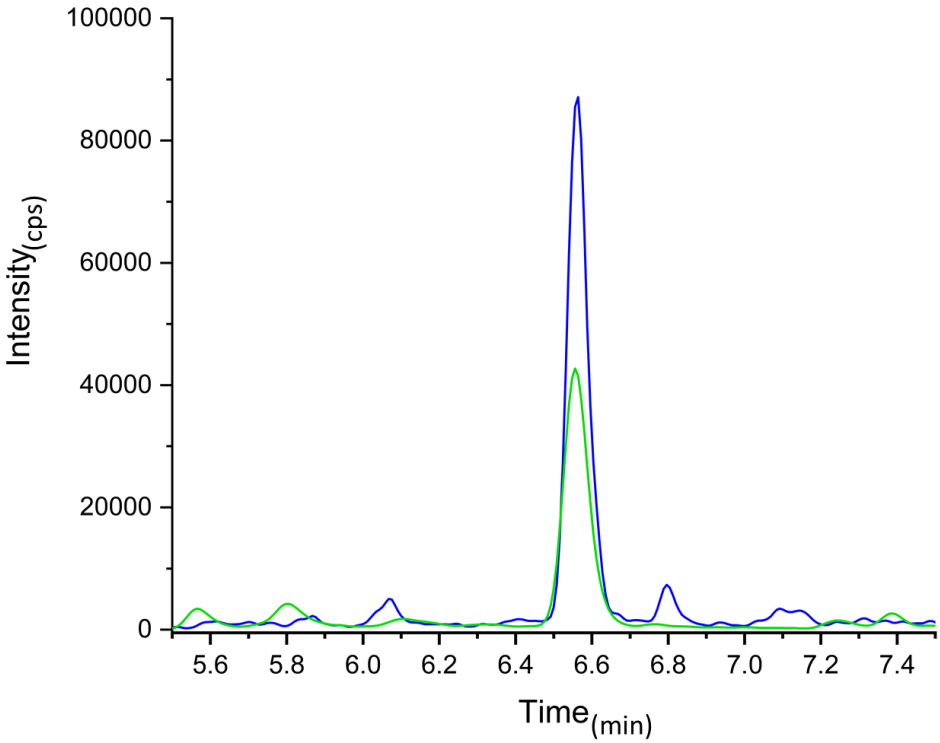
Abbreviations: CKD, Chronic kidney disease. DIA, hemodialyzed. CI, Confidence interval. SD, Standard deviation.



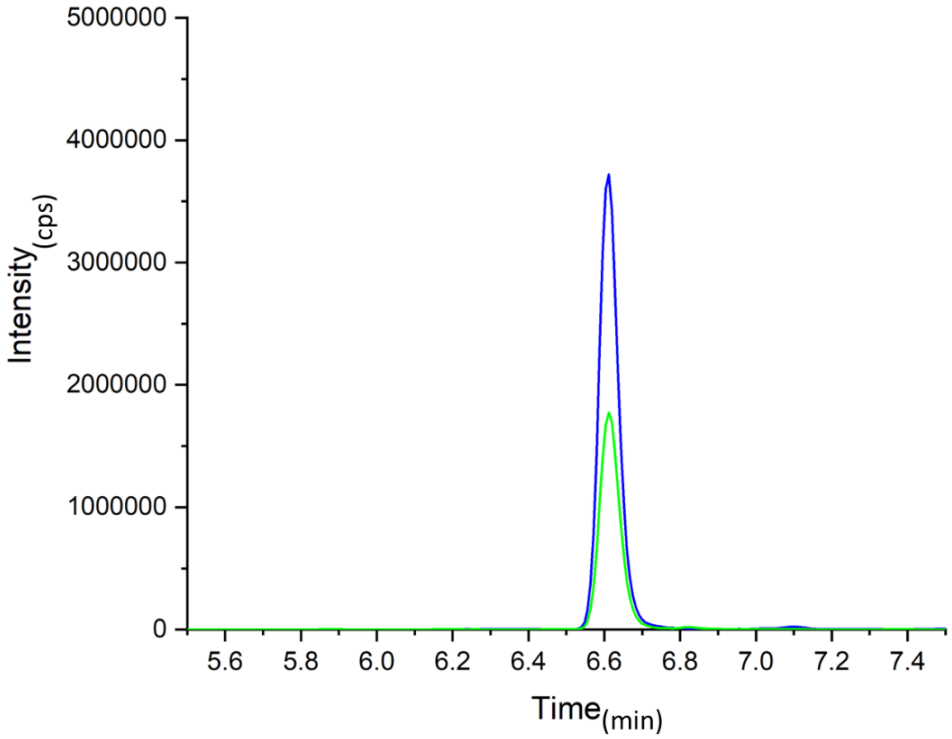
Supplemental Figure 13. Extracted ion chromatogram of a human EDTA plasma at 32.0 pg/mL. Blue and green XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$ respectively. Abbreviation: XIC, extracted ion chromatogram.



Supplemental Figure 14: Extracted Ion Chromatogram of a CKD EDTA sample at 234.1 pg/mL. Blue and green XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$ respectively. Yellow XIC corresponds to $ox[PTH+12H]^{+12}$. Abbreviation: XIC, extracted ion chromatogram.

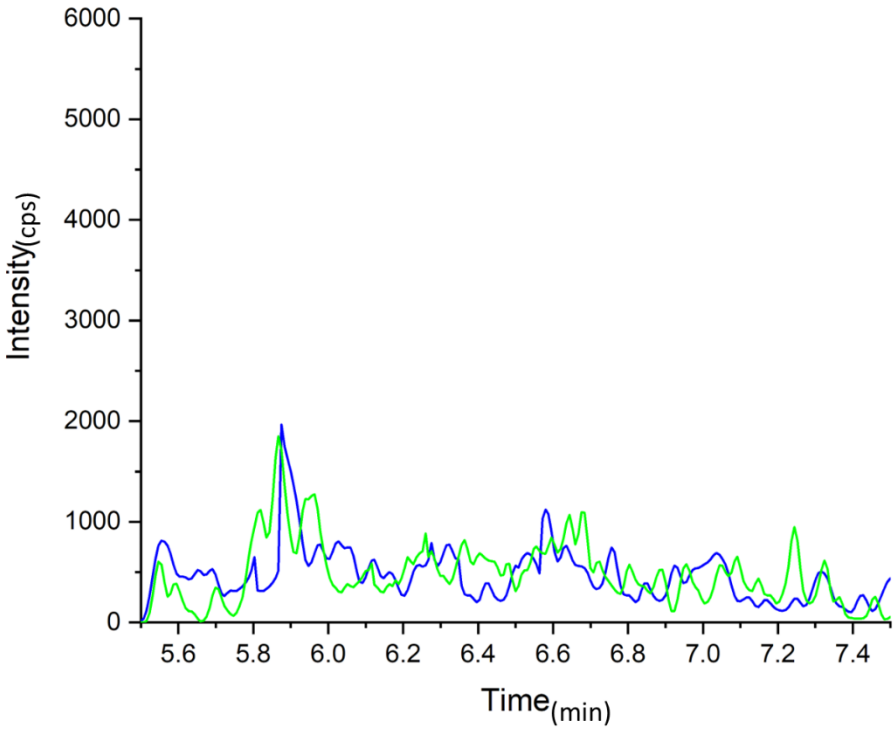


Supplemental Figure 15: Extracted Ion Chromatogram of a EQC sample at 87.4 pg/mL. Blue and green XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$ respectively. Abbreviation: XIC, extracted ion chromatogram.

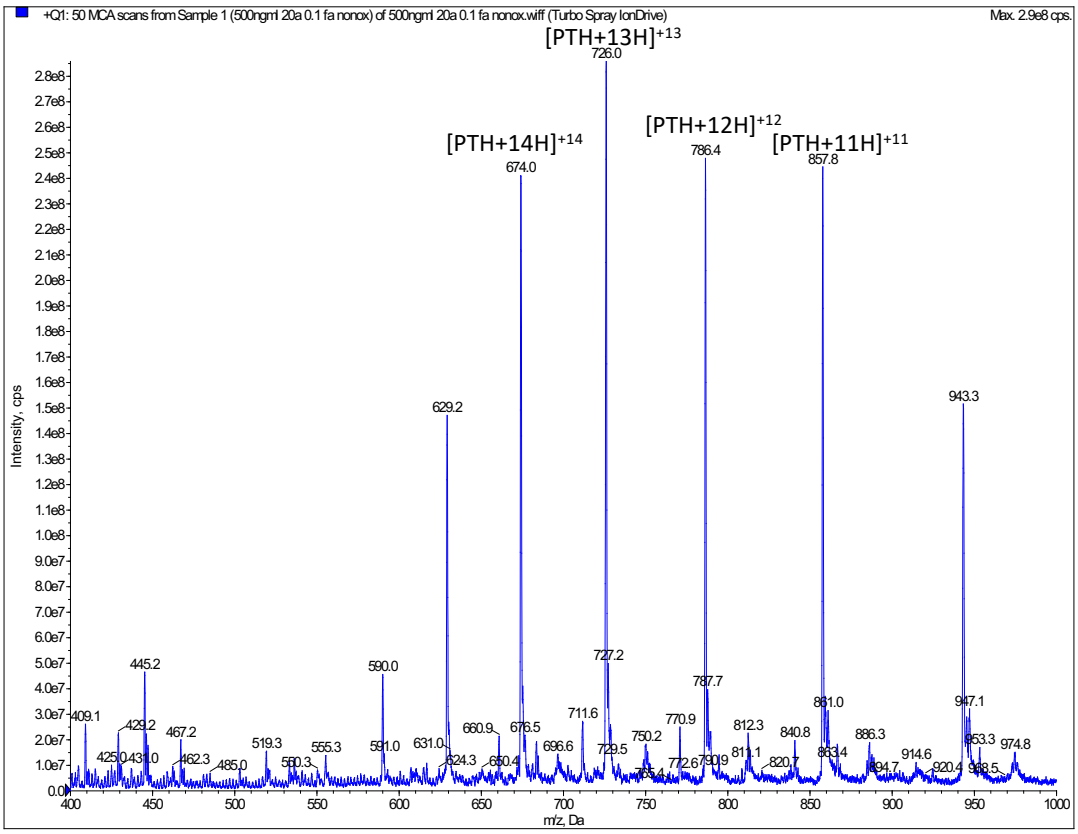


Supplemental Figure 16. Carryover assay. Extracted ion chromatogram of a 5000.0 pg/mL of 1-84 PTH. Blue and green XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$.

Abbreviations: XIC, extracted ion chromatogram



Supplemental Figure 17. Carryover assay. Extracted ion chromatogram of a blank sample, following a 5000.0 pg/mL injection of 1-84 PTH. Blue and green XIC correspond to $[PTH+12H]^{+12}$ and $[PTH+13H]^{+13}$. Abbreviations: XIC, extracted ion chromatogram.



Supplemental Figure 18. Q1 Scan of a WHO 96/646 1-84 PTH standard, with the 4 most intense species explored for this work.

7.1.13 Supplemental Tables

Supplemental Table 1. Binary Flow gradient at a flow rate of 0.300 mL/min.

Time (min)	% B
0.0	5
0.5	5
8.0	30
9.0	60
14.5	60
16.0	5
20.0	5

Supplemental Table 2. Optimized compound MRM parameters.

			MRM transitions					
Precursor ion	RT (min)	Product ion	Natural compound	Isotopically labeled	CE	CXP	EP	DT
$[PTH+12H]^{+12}$	6.60	$[y82+11H]^{+11}$	786.30 > 840.70*	796.60 > 851.80	24	29	10	70
$[PTH+13H]^{+13}$	6.60	$[y82+12H]^{+12}$	726.00 > 770.90	735.40 > 780.90	27.5	13	12	70
$[PTH+14H]^{+14}$	6.60	$[y82+13H]^{+13}$	674.10 > 711.70	682.90 > 720.85	24	29	10	70
$ox[PTH+12H]^{+12}$	6.00 6.19	$ox[y82+11H]^{+11}$	787.75 > 842.23	N/A	31.5	32	10	20
$ox[PTH+13H]^{+13}$	6.00 6.19	$ox[y82+12H]^{+12}$	727.23 > 772.13	N/A	27.5	13	12	20
$2ox[PTH+13H]^{+13}$	5.92	$2ox[y82+12H]^{+12}$	728.46 > 773.46	N/A	27.5	13	12	20
$[PTH_{(7-84)}+12H]^{+12}$	5.68	$[y76_{(7-84)}+11H]^{+11}$	732.80 > 776.90	N/A	27	30	10	20

* Transition selected for quantification.

RT: Retention Time, MRM: Multiple Reaction Monitoring. Entrance Potential (EP), Collision Energy (CE) and Cell Exit Potential (CXP), in volts (V). Dwell Time (DT) in msec.

Declustering potential = 75V.

Supplemental Table 3. Optimized MS parameters.

Parameter	Setting
Scan Type	MRM
Ionization Mode	ESI +
Curtain Gas	20*
Collision Gas	12*
Ion Source Gas 1	30*
Ion Source Gas 2	40*
Source Voltage	4.5 kV
Temperature	550 °C

*Sciex arbitrary units

Supplemental Table 4. Ion ratios (native and internal standard) in calibrators and human plasma samples.

Sample Name	Sample Type	Ion Ratio IS	Ion Ratio native
Cal 0.49	Standard	2.39	2.20
Cal 0.98	Standard	2.14	2.09
Cal 2.54	Standard	2.41	2.33
Cal 5.08	Standard	2.05	2.23
Cal 10.09	Standard	2.14	2.30
Cal 27.12	Standard	2.51	2.18
Cal 50.37	Standard	2.39	2.19
Cal 78.49	Standard	2.43	2.11
Sample 1	Unknown	2.08	2.01
Sample 2	Unknown	2.33	2.08
Sample 3	Unknown	2.32	2.11
Sample 4	Unknown	2.39	2.46
Sample 5	Unknown	2.43	2.05
Sample 6	Unknown	2.01	2.14
Sample 7	Unknown	2.34	2.12
Sample 8	Unknown	2.39	2.26
Sample 9	Unknown	2.23	2.40
Sample 10	Unknown	2.21	2.26
Sample 11	Unknown	2.28	2.17
Sample 12	Unknown	2.21	2.05

Supplemental Table 5. Sample composition for the study of interferences.

Concentration of interferant	**Pool 1**	**Pool 2**	**Pool 3**	**Pool 4**	**Pool 5**
	0%	25%	50%	75%	100%
Interferant volume	0 µL	50 µL	100 µL	150 µL	200 µL
Solvent Volume (H_2O LCMS)	200 µL	150 µL	100 µL	50 µL	0 µL
Sample volume	800 µL	800 µL	800 µL	800 µL	800 µL
Hemoglobin (Stock= 104 mg/mL)	0 mg/mL	5.2 mg/mL	10.4 mg/mL	15.6 mg/mL	20.8 mg/mL
Bilirubin (Stock concentration = 5 mg/mL)	0 mg/mL	0.25 mg/mL	0.5 mg/mL	0.75 mg/mL	1 mg/mL
Lipemia (Stock= 100 mg/mL)	0 mg/mL	5 mg	10 mg/mL	15 mg/mL	20 mg/mL
Hyperalbuminemia (Stock 500 mg/mL)	0 mg/mL	25 mg/mL	50 mg/mL	75 mg/mL	100 mg/mL

Supplemental Table 6. Concordance Correlation Coefficient between the two immunoassays and LC-MS/MS . Abbreviations: EQC, External quality control; DIA, hemodialyzed.

	CONCORDANCE CORRELATION COEFFICIENT VS LC-MS/MS		
	GROUP		
	EQC	<100 pg/mL (excluding DIA)	Human EDTA (all)
FUJIREBIO LUMIPULSE 3RD GEN PTH	0.99(0.99-0.99)	0.97 (0.96-0.98)	0.95(0.93-0.96)
DIASORIN LIAISON 3RD GEN PTH	0.95(0.93-0.97)	0.89 (0.85-0.92)	0.89(0.87-0.91)

7.1.14 References

1. Kritmetapak K, Losbanos LA, Hines JM, O'Grady KL, Ulmer CZ, Vesper HW, et al. Chemical Characterization and Quantification of Circulating Intact PTH and PTH Fragments by High-Resolution Mass Spectrometry in Chronic Renal Failure. Clin Chem. 2021;67:843–53.

2. Lin LI. A Concordance Correlation Coefficient to Evaluate Reproducibility. Biometrics. 1989;45:255–68.

3. McBride G. A proposal for strength-of-agreement criteria for Lin's Concordance Correlation Coefficient. NIWA Client Rep. 2005; HAM2005-06.

Chapter 8. List of Publications.

First author

Le Goff C.*, Farré-Segura J.*, Stojkovic V., Dufour P., Peeters S., Courtois J., Nizet A., De Vos N., & Cavalier E. (2020). The pathway through LC-MS method development: in-house or ready-to-use kit-based methods? Clinical Chemistry and Laboratory Medicine.

doi:10.1515/cclm-2019

Farré-Segura J., Fabregat Cabello N., Calaprice C., Nyssen L., Peeters S., Le Goff C., & Cavalier E. (2021). Development and validation of a fast and reliable method for the quantification of glucagon by liquid chromatography and tandem mass spectrometry. Clinica Chimica Acta. doi:10.1016/j.cca.2020.11.004

Farré-Segura J., Le Goff C., Lukas P., Cobraiville G., Fillet M., Servais A.-C., Delanaye P., & Cavalier E. (2022). Validation of an LC-MS/MS Method Using Solid-Phase Extraction for the Quantification of 1-84 Parathyroid Hormone: Toward a Candidate Reference Measurement Procedure. Clinical Chemistry 68 (11) 1399-1409.

doi:10.1093/clinchem/hvac135

Co-author

Fabregat Cabello N., **Farré-Segura J.**, Huyghebaert L., Peeters S., Le Goff C., Souberbielle J.-C., & Cavalier E. (2017). A fast and simple method for simultaneous measurements of 25(OH)D, 24,25(OH)2D, and the Vitamin D Metabolite Ratio (VMR) in serum samples by LC-MS/MS. Clinica Chimica Acta.

doi:10.1016/j.cca.2017.08.024

Le Goff C., **Farré-Segura J.**, Dufour P., Kaux J.-F., & Cavalier E. (2020). Intense sport practices and cardiac biomarkers. Clinical Biochemistry 79 1-8.

doi:10.1016/j.clinbiochem.2020.02.008

Le Goff C., Kaux J.-F., **Farré-Segura J.**, Stojkovic V., Ancion A., Seidel L., Lancellotti P., & Cavalier E. (2020). Evolution of the slopes of ST2 and galectin-3 during marathon and ultratrail running compared to a control group. Clinical Chemistry and Laboratory Medicine 58 (2) 314-321.

doi:10.1515/cclm-2019-0555

Demeuse J., Huyghebaert L., Determe W., Schoumacher M., Grifnée E., Massonnet P., Dubrowski T., Rechchad M., **Farré-Segura J.**, Peeters S., Cavalier E., & Le Goff C.*(2023). Development and validation of an LC-MS/MS method for the simultaneous quantitation of angiotensin (1-7) (1-8) (1-9) and (1-10) in human plasma. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences 123943.

doi:10.1016/j.jchromb.2023.123943

Cavalier E., & **Farré-Segura J.** (2023). In Reply to A Comment about Analytical Performance Specifications for the Combined Measurement Uncertainty Budget in the Implementation of Metrological Traceability of Parathyroid Hormone. Clinical Chemistry.

doi:10.1093/clinchem/hvad193

Cavalier E., **Farré-Segura J.**, Lukas P., Gendebien A.-S., Peeters S., Massonnet P., Le Goff C., Bouquegneau A., Souberbielle J.-C., Delatour V., & Delanaye P. (2023). Unveiling a new era with liquid chromatography coupled with mass spectrometry to enhance parathyroid hormone measurement in patients with chronic kidney disease. Kidney International.

doi:10.1016/j.kint.2023.09.033

Massonnet P., Grifnée E., **Farré-Segura J.**, Demeuse J., Huyghebaert L., Dubrowski T., Dufour P., Schoumacher M., Peeters S., Le Goff C., & Cavalier E. (2023). Concise review on the combined use of immunocapture mass spectrometry and liquid chromatography for clinical applications. Clinical Chemistry and Laboratory Medicine.

doi:10.1515/cclm-2023-0253

Oral communications

Fabregat Cabello N., **Farré-Segura J.**, Huyghebaert L., Peeters S., Le Goff C., & Cavalier E. (2017). A fast and simple method for simultaneous measurements of 25(OH)D, 24,25(OH)2D, and the Vitamin D Metabolite Ratio (VMR) in serum samples by LC-MS/MS (powerpoint). MSACL, Salzburg, Austria.

Farré-Segura J., Fabregat Cabello N., Nyssen L., Peeters S., Le Goff C., & Cavalier E. (2018). A newly developed LC-MS/MS method for the quantitation of Glucagon in plasma shows the lack of specificity of a former immunoassay method. 5th Annual Conference MSACL (Mass Spectrometry: Applications to the Clinical Lab) 2018 EU, Salzburg, Austria.

Farré-Segura J., Calaprice C., Peeters S., Demeuse J., Le Goff C., & Cavalier E. (2019). Development of an Immunoprecipitation Method for the Analysis of Intact Parathyroid Hormone (PTH) by LC-MS/MS. MSACL 2019 EU, Salzburg, Austria.

Poster Presentations

Le Goff C., Zelzer S., Huyghebaert L., **Farré-Segura J.**, Fabregat Cabello N., Hermann M., & Cavalier E. (2019). Method comparison of the 24,25(OH)2D3 measurement with two different LC-MS/MS assays. Euromedlab 2019, Barcelona, Spain.

Huyghebaert L., Demeuse J., **Farré-Segura J.**, Peeters S., Grifnée E., Vanden Broeck N., Collard G., Dufour P., Massonnet P., Dubrowski T., Cavalier E., & Le Goff C. (2022). Validation of an LC-MS/MS method for the quantitation of plasma renine activity (PRA) in human. RBSLM Congress.

Massonnet P., Grifnée E., Demeuse J., Huyghebaert L., Peeters S., **Farré-Segura J.**, Le Goff C., & Cavalier E. (03 May 2022). Workshop on principles in proteomics: MALDI Imaging. Belgian Proteomics Association Conference (BePAc) 2022: A proteomics perspective on protein function in space and time.

Huyghebaert L., Demeuse J., Grifnée E., Massonnet P., **Farré-Segura J.**, Peeters S., Le Goff C., & Cavalier E. (August 2022). Simultaneous detection and quantification of angiotensin I, II, 1-7, and 1-9 by LC-MS/MS in human plasma. International Mass Spectrometry Conference (IMSC), 2022, Maastricht, Netherlands.

Massonnet P., Peeters S., Grifnée E., Demeuse J., Huyghebaert L., **Farré-Segura J.**, Delanaye P., Le Goff C., & Cavalier E. (August 2022). Iohexol quantitation and possible degradation kinetics in human urine using mass spectrometry coupled to liquid chromatography (LC). International Mass Spectrometry Conference (IMSC), 2022, Maastricht, Netherlands.