Abstract: The analysis of intact parathyroid hormone (PTH) (PTH1-84) is useful in the diagnosis of hyper- and hypocalcaemia, hyperparathyroidism, and in the prevention of bone mineral disorders in renal patients. The analysis is complicated by the presence of PTH fragments, which may accumulate in renal failure and cross-react in immunoassays, including the most recent third-generation immunoassays. Large variability exists between different commercially available assays. This article reviews the current literature on PTH testing, with emphasis on the use of mass spectrometry-based methods, and considers the important sources of variation which still need to be addressed prior to the development of much needed candidate reference methods for PTH analysis. Recently, mass spectrometric methods have been developed for the quantitation of PTH1-84 using surrogate tryptic peptides, but even these methods are subject to significant interferences due to the presence of newly observed modified PTH species, such as oxidised and phosphorylated PTH variants, which can accumulate in patient samples. Further work, including: 1) the use of high-resolution mass spectrometry; and 2) the analysis of PTH without prior protease digestion, is required before these approaches can be considered as reference methods against which other methods should be harmonised.

Keywords: immunoaffinity; liquid chromatography-mass spectrometry (LC-MS); mass spectrometry; parathyroid hormone (PTH).

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Introduction

Parathyroid hormone (PTH) is a relatively small (84 amino acid) single-chain peptide synthesised by the parathyroid glands and is the main hormone responsible for the regulation of plasma-ionised calcium. The intact active form of the hormone (PTH1-84) is produced by the sequential cleavage of the initially translated peptide, pre-pro-PTH (115 amino acids), via pro-PTH (90 amino acids). Neither pre-pro-PTH nor pro-PTH are detectable by immunoassay in plasma samples [1]. PTH1-84 is released into the circulation in response to low extracellular-ionised calcium (Ca\(^{2+}\)), and is regulated by a classical negative feedback mechanism (Figure 1) mediated through calcium-sensing receptors found on parathyroid cells. Circulating PTH1-84 is rapidly cleared (plasma half-life approximately 2–4 min), being metabolised by both the liver and kidneys [2].

Circulating PTH1-84 acts to increase plasma calcium concentrations through several classically described mechanisms [3]. These include: 1) stimulation of bone resorption and calcium reabsorption in the kidney, mediated through PTH interaction with G-protein coupled PTH/PTH-related peptide (PTHrP) receptors expressed by osteoblasts and distal renal tubular cells, respectively; and 2) stimulation of the enzymatic 1-α-hydroxylation of 25-hydroxyvitamin D in the kidneys, which in turn increases calcium absorption in the small intestine. By
increasing renal phosphate excretion in the proximal tubules, PTH also acts to decrease serum phosphate concentrations.

PTH also has significant anabolic effects on bone formation, and for this reason PTH-based biotherapeutic anabolic agents including recombinant forms of full-length PTH1-84, such as Preotact® (Nycomed), and truncated forms, e.g., recombinant PTH1-34 (Teriparatide®, Eli Lilly) are widely used clinically, sometimes in preference to traditional anti-resorptive treatments, such as bisphosphonates, for the treatment of osteoporosis in post-menopausal women at risk of fracture [4, 5].

Clinically, PTH1-84 measurement is useful in the diagnosis of hyper- and hypocalcaemia, in the differentiation of primary and secondary hyperparathyroidism, e.g., related to parathyroid cancers, and in the prevention of bone mineral disorders in renal patients.

**Assay variability – causes, concerns and the need for harmonisation**

The analysis of ‘true’ PTH1-84 is not straightforward – there exists a surprisingly heterogeneous range of PTH-related peptides [6]. As will be discussed, both the exact composition and possible biological functions of PTH fragments remain to be fully elucidated as does the variable influence of these fragments on currently available analytical methods for PTH testing [2, 7]. PTH1-84 can be proteolytically cleaved within the parathyroid gland prior to secretion, or cleavage can occur through the actions of endoproteases in hepatic Kuppfer cells [8, 9]. Certain synthetic PTH fragments have been demonstrated to antagonise the biological activity of PTH1-84 both in vitro and in vivo [10, 11]. The discovery of circulating PTH fragments in the late 1960s and 1970s was the main driver for the development of second- and third-generation immunoassay methods for PTH analysis.

It is perhaps ironic that it is in patients with renal failure, in whom the accurate analysis of PTH1-84 is clinically important for the prevention of mineral bone disorders and for guiding expensive drug treatments, that the accumulation of PTH fragments is the most pronounced. A review of circulating PTH isoforms based on immunoassay by D’Amour [11] concluded that PTH1-84 accounts for just 20% of the ‘total’ circulating PTH in normocalcaemic individuals, and can be as little as 5% in patients with end-stage renal failure, depending upon which assay is used for measurement.

Numerous publications have highlighted discrepancies between ‘PTH1-84’ results from the same sample analysed using different analytical platforms [2, 7, 12]. Indeed, it has been proposed that the difference between two different immunoassay results could provide a useful index for the diagnosis and prognosis of parathyroid carcinomas [13-15].

There is an obvious clinical need for the harmonisation of PTH testing. The variability which exists in PTH testing should be considered a critical governance issue in patients with chronic kidney disease [16]. Given this variability in results produced using different methodologies, some clinicians question the relevance of
measuring PTH at all in renal patients [2, 17]. Often, assay preference is governed more by reliance upon a method with which they have the most familiarity and clinical experience than by absolute analytical accuracy [8]. It is hoped by many that the establishment of an International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group for PTH [18] will aid the development of reference methodologies, as well as harmonise the testing of PTH towards a traceable reference material (e.g., the World Health Organization Recombinant PTH1-84 International Standard 95/646, available through the National Institute for Biological Standards and Control, NIBSC – www.nibsc.org). Until such time as a reference method is developed and validated, which can be used to establish target values based on reference materials, the IFCC group are working towards the development of assay-specific target values (more information on the IFCC working group is available through http://www.ifcc.org/ifcc-scientific-division/sd-working-groups/parathyroid-hormone-wg-PTH/).

The following sections aim to review the current literature on the development of PTH assay methods, with particular emphasis on the influence of circulating PTH fragments on the accuracy of analytical methods designed to measure intact PTH1-84. The use of mass spectrometry (MS)-based approaches for both the identification of PTH fragments and for the development of quantitative PTH assays will be reviewed, and important considerations for the development of analytical methods that are harmonised towards a standardised reference method will also be discussed.

### Historical context

Last year marked the 50th anniversary of the first competitive radioimmunoassay (RIA) developed for the analysis of PTH [19]. During the intervening 50 years there can be no doubt that a significant number of developments and improvements have been made to assay specificity. This has been achieved both through an increased understanding of the biology of PTH (and its fragments) in different patient groups, as well as through improvements in the instrumentation and processes used for analysis. A timeline summarising the most important developments in PTH testing is outlined in Figure 2.

The development of the first radioimmunoassay for PTH by Berson et al. [19] pre-dated the identification of truncated PTH forms. Needless to say, the lack of specificity of the single-site antibody used in these early RIAs (typically directed towards the mid-region of the PTH1-84 sequence – the region of greatest antigenicity) meant significant cross-reactivity from circulating PTH fragments, and thus overestimation of the true PTH1-84 concentrations. In some samples from patients with advanced renal disease, this overestimation could be several orders of magnitude when compared to individuals with normal renal and parathyroid function [20]. In the 1970s and early 1980s, a number of amino-terminal (N-terminal) RIAs were developed. As these were targeted more towards the biologically functioning regions of PTH1-84, they proved more useful than earlier more carboxy-terminal (C-terminal) RIAs for the diagnosis of renal bone disease. However, owing to important developments in the identification of PTH fragments

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**Figure 2** The historical development of PTH assays.
and early work towards characterisation of their bioactivity (see “PTH fragments and variants – influences on assay development”), both these assays were quickly superseded by second-generation two-site immunometric assays and, later still, third-generation immunoassays (Figure 2).

**PTH fragments and variants – influences on assay development**

Our understanding of the structure of circulating PTH fragments in normal individuals, and especially in patients with parathyroid pathology or renal impairment, has been aided by a number of developments in analytical technologies. These include: 1) faster, simpler antibody production; 2) more precise epitope mapping techniques, allowing for a more informed comparison of results between different immunoassays; 3) the ability to synthesise custom recombinant peptides as controls or ‘model’ PTH fragments in vitro; 4) peptide sequencing tools, such as Edman degradation radio-sequencing; 5) the improvement of chromatographic techniques and; more recently 6) MS-based detection.

**Second-generation immunoassays**

It was the discovery of N-terminally truncated PTH fragments (e.g., PTH34-84) at high concentrations in patients with terminal renal failure, plus the knowledge that the biological activity of PTH1-84 resided within the N-terminal region, that led to the development and introduction of second-generation (or ‘intact’), two-site immunoassays [21, 22]. These intact PTH assays typically have a solid-phase capture antibody directed towards the C-terminal region of PTH (amino acids 39-84), and a detection antibody directed towards the N-terminus (Figure 3), usually towards amino acids 12-24, though a few second-generation assays were developed with a detection antibody epitope directed towards the 26-32 region [23–25]. These assays were thought to be far more specific, since they avoided cross-reactivity with C-terminal PTH fragments. They were certainly an improvement upon the early single-antibody, mid-region, C-terminal RIAs as judged by correlation with clinical findings [26, 27]. In this form, these assays were widely adopted by many clinical laboratories, reference ranges for different patient groups were developed, and indeed, they are still used by many to this day for routine PTH testing.

**Third-generation immunoassays**

Based on findings from a parathyroid function study in 1993 [28], Brossaud et al. [29] developed a chromatographic method to separate C-terminal PTH fragments from PTH1-84 prior to immunoassay analysis (second-generation). They reported that, in addition to the known C-terminal PTH fragments missing the entire N-terminus (e.g., PTH34-84), there existed a second group of larger C-terminal PTH fragments with a partially preserved N-terminus, which accumulated in renal failure. They later established that the fragmentation occurred somewhere within the first 19 amino acids of the PTH N-terminus based on comparison of second-generation immunoassays with different detection antibody epitopes [24]. These fragments were collectively described as ‘non-1-84’ PTH fragments, and were demonstrated to cross-react in second-generation immunoassays using recombinant PTH7-84, a commercially available peptide, as a surrogate for this group of peptides. In renal failure, the cross-reactivity of these non-1-84 fragments was shown to account for up to 60% of total immunoreactive PTH, depending upon which immunoassay was used [11, 23–25, 29, 30]. These observations supported clinical findings that second-generation
immunoassay results did not always correlate with other clinical and biochemical results [31, 32].

This led to the development of third-generation (‘whole PTH’) assays [33, 34], in which the detection antibody epitope was targeted further towards the N-terminus of PTH1-84, to amino acids 1-4, thus avoiding cross-reactivity with the newly observed large C-terminal PTH fragments (Figure 3). Despite the improved performance of these assays in some patient groups [35], some anomalous results were still reported [36–38].

‘Amino-PTH’

D’Amour and co-workers explored the exact structure of non-1-84 PTH fragments, using the array of second- and third-generation immunoassays that were then available, plus Edman degradation radio-sequencing with PTH with $^{35}$S-labelled methionine residues. Samples from healthy controls were compared with individual clinical samples and sample pools from patients with confirmed parathyroid pathology and/or renal failure. Ex vivo tissue samples from patients with confirmed primary and secondary hyperparathyroidism were also used to generate cell lines in vitro. Findings included first, that PTH7-84 was likely to be the most abundant form of the non-1-84 PTH cross-reacting in second-generation immunoassays; second, fragments corresponding to PTH4-84, PTH8-84, PTH10-84 and PTH15-84 were also present; and third, that another non-1-84 PTH fragment, over-expressed in severe hyperparathyroidism and parathyroid cancers, cross-reacted with third-generation but not second-generation immunoassays with a 12-20 epitope [11, 24, 25, 30]. It was suggested that this PTH variant (termed ‘amino-PTH’ in some references), which appears to have an intact N-terminus, may represent a modified variant form of PTH, with a modification in the 15-20 region. This would explain its lack of cross-reactivity with second-generation immunoassays [23, 39].

Mass spectrometry

In the last 10–15 years, liquid chromatography coupled with mass spectrometric detection (LC-MS or tandem MS, LC-MS/MS) has become a widely applied technique in clinical chemistry laboratories. It has largely been used for the quantitative analysis of small-molecules (i.e., <1000 Da), such as steroids and related hormones (e.g., testosterone and vitamin D metabolites) and drugs and their metabolites. Modern LC-MS instruments are highly sensitive, and coupled with appropriate sample preparation techniques, can often achieve limits of detection comparable with immunoassays (e.g., pmol/L for some analytes). However, it is the combination of chromatographic separation with a detection system based on mass-to-charge (m/z) ratio, which provides a specificity of LC-MS that is far superior to that of immunoassay-based detection [10, 40, 41].

The analysis of more complex proteins and peptides using MS instrumentation was for a long time restricted to research laboratories and biomarker discovery/validation workflows. This work has typically been low-throughput, using micro-flow or nano-flow LC separations over hours rather than minutes. However, recent work has focused on the development of higher throughput, more robust, quantitative protein/peptide analyses suitable for the rigours of clinical chemistry work. The MS analysis of proteins and peptides can be done either directly using the intact analyte(s) in a so-called ‘top-down’ approach, or in a ‘bottom-up’ approach following digestion with a protease such as trypsin, and by monitoring specific (tryptic) peptides as surrogate markers. The approach used depends on the target analyte; large proteins with m/z values exceeding the operating range of the MS instrument require proteolytic digestion. Further, some tryptic peptides offer better ionisation efficiency or better chromatographic peak shape than the intact species, and so the digestion step can be used to increase assay sensitivity. In the same manner as with quantitative small molecule MS analysis, isotopically labelled internal reference standards can be synthesised, either for whole peptides/proteins or proteolytic peptides, and matrix-based calibration standards can be produced, ideally from reference standards, such as the PTH1-84 reference standard mentioned earlier.

For low abundance proteins/peptides such as PTH1-84 (and PTH fragments) in serum, the complexity of the sample matrix necessitates thorough sample preparation prior to LC-MS analysis. For some small peptides, techniques traditionally used for small molecule analysis such as solid-phase extraction (SPE) may be useful, and have been successfully applied in a number of clinical assays, e.g., hepcidin [42] and insulin [43]. For larger proteins, or for those requiring more extensive analyte enrichment to allow detection at physiological concentrations, e.g., PTH [44], immunoaffinity extraction (sometimes referred to as immunocapture or immunoextraction) is a novel approach which is increasingly being used [45–48].

Matrix-assisted laser desorption ionisation coupled with time-of-flight MS (MALDI-TOF MS) has been widely used for peptide identification in proteomics studies. This technique has a wide m/z range, offers high-resolution
MS, and a number of sophisticated de-convolution software packages are available to aid spectral interpretation. Furthermore, although MALDI-TOF cannot be directly connected to chromatographic separation techniques, these instruments can be used with immunoaffinity sample preparation and HPLC fraction collection methods to allow identification of low abundance proteins and peptides. Two studies using immunoaffinity coupled with MALDI-TOF MS to identify PTH fragments have been published. In 2006, Zhang et al. [10] immunoextracted PTH fragments from healthy controls, patients with chronic renal disease, and from healthy post-menopausal women given recombinant human PTH1-84. Extracted PTH fragments were analysed by: 1) MALDI-TOF MS following fractionation on a capillary LC system; and 2) directly by nano-LC-TOF-MS. Four PTH fragments (PTH34-84, PTH37-84, PTH38-84 and PTH45-84) were identified as the major circulating fragments in samples from renal patients and from healthy volunteers given PTH1-84 (at estimated concentrations from 10 to 100 pmol/L). All of these fragments, with the exception of PTH45-84, were also found in control samples. In 2010, Lopez et al. [49] carried out similar experiments, again using immunoaffinity to enrich the PTH fragments. However, this time the immunoaffinity process was automated by having the antibody (polyclonal goat anti-human PTH39-84) immobilised on a micro-columns embedded into pipette tips [mass spectrometric immunoassay (MSIA)]. In samples (n=12) from patients with renal failure, PTH fragments observed were consistent with those observed by Zhang et al. previously [10]. In addition, novel fragments with truncated N- and C-termini were also observed; these corresponded to PTH28-84, PTH48-84, PTH34-77, PTH37-77 and PTH38-77 (Figure 4). Lopez et al. also went on to develop a quantitative assay for these PTH fragments using a triple quadrupole mass spectrometer (see ‘Quantitative LC-MS/MS PTH assays’). It is especially interesting to note that in neither study were larger C-terminal PTH fragments observed, including the widely cited PTH7-84 variant to which the development of third-generation immunoassays can be largely credited.

Quantitative LC-MS/MS PTH assays

Recently, two groups have published quantitative LC-MS/MS methods for the analysis of PTH1-84 in clinical samples [44, 49]. With these methods now available, it has been recognised that the development of a candidate reference method for PTH is now a possibility [16]. Both methods utilise immunoaffinity extraction with a C-terminal anti-PTH capture antibody, either immobilised onto polystyrene beads [44] or onto customised MSIA pipette tips [49]. In both methods, following washing steps to remove non-specific binding, the captured PTH1-84 and related fragments are digested using trypsin. Selected tryptic peptides are then quantified using LC-MS/MS. Absolute quantification is carried out by preparation of calibration standards.

Figure 4  Reconstructed MSIA MALDI-TOF MS spectra representative of samples from patients with renal failure (blue) and from healthy controls (red). Reproduced from Lopez et al. [49], with permission from Mary Lopez.
using recombinant human PTH1-84. Kumar et al. [44] used isotopically labelled (15N) PTH1-84 as the internal reference standard, whereas Lopez et al. [49] used individual isotopically labelled tryptic peptides (one for each tryptic peptide monitored). Both groups used the quantification of the 1-13 amino acid tryptic peptide (SVSEIQLMHLNLGK) as a surrogate for the measurement of PTH1-84. Both methods demonstrated good analytical performance, linearity and appropriate limits of quantitation (39.1 [44] and 16 pg/mL [49], respectively, from 1 mL sample). Kumar et al. also tested their assay for the 1-13 tryptic peptide for interference from other PTH fragments (PTH1-44, PTH7-84, PTH43-68, PTH52-84 and PTH64-84), PTHrP, and in haemolysed, lipaemic, and icteric samples. No significant interferences were identified.

In addition to the 1-13 peptide, Lopez et al. also included SRM transitions to monitor the presence of other tryptic peptides, specific to other PTH fragments. For example, the 7-13 tryptic peptide was used as a surrogate for PTH7-84. Echoing the findings using MALDI-TOF MS, the 7-13 tryptic peptide could not be detected (LLoQ 16 pg/mL) in the samples tested [49, 50]. We have since replicated this method in our laboratory using over 300 samples from patients with differing degrees of renal impairment [50–52].

**Modified PTH variants**

As well as the many truncated forms of PTH that appear to exist, investigation of the amino acid sequence of the PTH N-terminus reveals that possible post-translational modifications can occur at a number of sites (Figure 5). For example, in vitro work using bovine and human PTH dating back to 1984 demonstrated that phosphorylation of serine residues in the PTH N-terminal region was possible (e.g., post-translational phosphorylation at serine 17), and indeed accounted for up to 20% of the total PTH species present [53]. It was concluded by D’Amour et al. [23] that the amino-PTH variant(s) which were cross-reacting with third-generation immunoassays could include phosphorylated PTH, but that further work was required to demonstrate this.

**Oxidised PTH**

The discovery of the anabolic effects of PTH, and the subsequent use of recombinant PTH analogues as biotherapeutic agents was an important development, and one which can be used to further our analytical ability to measure PTH variants in clinical samples. For these agents to be produced and licensed for use by regulatory authorities such as the FDA, extensive purity and drug stability testing is required. Much research is carried out to investigate the use of solvents and other excipient formulations to stabilise protein-based drugs. A number of studies using MS on the stability of PTH1-34 formulations have therefore been carried out. In addition, analytical methods to identify and characterise degradation products, plus in vitro studies to assess their potential biological activity have also been developed. The N-terminus of PTH1-84 contains a number of residues prone to

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**Figure 5** Modified PTH forms and tryptic cleavage sites. Highlighted are the amino acid residues within the N-terminus which may be, or have been shown to be, oxidised (dark grey) or phosphorylated (light grey).
chemical degradation. Amongst other possible degradation products such as deamidation of asparagine (ASN, N) residues, methionine (MET, M) oxidation is likely to be the most prevalent. In vitro studies using high-resolution MS to identify oxidation products, following exposure of these compounds to peroxides, have demonstrated that the methionine residues at positions 8 and 18 of PTH1-34 and PTH1-84 can become oxidised [38, 54]. Methionine 8 oxidises more slowly than methionine 18 [55], and both methionine residues can be singly or doubly oxidised (to sulphoxides and sulphones, respectively). It is also well established that all oxidised PTH variants are less biologically active than non-oxidised PTH [38, 56–60]. With these findings considered in the context of clinical PTH measurement, Hocher et al. [38, 61] raised a monoclonal antibody against oxidised PTH, and immobilised this antibody in microcolumns. High-resolution MS was used to demonstrate the efficiency of the antibody prior to use. These affinity columns were then used to remove oxidised PTH in clinical samples from haemodialysis patients prior to analysis of non-oxidised PTH species using immunoassay (second-generation, Roche PTH assay). It was demonstrated that a large, yet highly variable, proportion (>90% in some cases) of the total PTH in these samples was present in an oxidised form [38, 61].

Phosphorylated PTH

In our laboratory, we have employed a selected reaction monitoring (SRM) MSIA method similar to that of Lopez et al. [49]. The presence of phosphorylated PTH was looked for in clinical samples from patients with differing degrees of renal impairment. The immunoaffinity capture methodology and trypsin digestion steps were unchanged from the method described by Lopez et al., but additional SRM transitions were added to the MS method to allow the detection of the phosphorylated 14-20 tryptic peptide (HLNS(phosphor)MER) and we used a custom-synthesised heavy isotope-labelled phosphorylated PTH14-20 internal reference standard. We were able to identify phosphorylated PTH in a number of clinical samples from patients with renal impairment and parathyroid cancer. In those samples where phosphorylated PTH was detected (n=58), a large range of phosphorylated PTH14-20 concentrations was observed [median (range) 52 (32–767 pg/mL)] [51, 52]. Further work is still required to determine the biological activity, or the significance of circulating phosphorylated PTH variants. An immunoassay for the detection of phosphorylated PTH has also been developed [62].

PTH analysis – considerations and future perspective

Despite its clinical importance, it is clear that the analysis of PTH for clinical diagnostics and patient management is highly complex. Whilst the development of three generations of immunoassays have been key to our improved understanding of the biology of PTH and PTH fragments, there remains an obvious need for the harmonisation of PTH testing between laboratories. Further work is also required to better understand the exact structures of the PTH species that can be observed in different clinical settings. MS methods will be critical for the future development of this work – permitting unambiguous identification of PTH forms based on their m/z ratio and fragmentation characteristics, as opposed to inferring the structure of PTH fragments by differential antibody cross-reactivity.

However, MS is not foolproof. Whilst the assay performance data (e.g., precision and accuracy, calibration linearity) look encouraging for the two existing quantitative MS methods for PTH1-84, both may be subject to interference in real clinical samples. As discussed, modified PTH1-84 and potentially modified PTH fragments have been observed in clinical samples, with phosphorylation at serine-17 and oxidation at methionine-18. When using LC-MS/MS, and monitoring the tryptic peptide 1-13 as the surrogate for PTH1-84 using SRM, the concentration of PTH1-13 is actually a total of at least three species: PTH1-84, oxidised PTH1-84 (at methionine-18) and phosphorylated PTH1-84 (as serine-17), since the site of modification is after the tryptic cleavage site. There is thus no way in which to distinguish between these variants (Figure 5). In the same way, our failure to detect PTH7-84 in patient samples may be explained by modification by oxidation at methionine-8, so that it was ‘missed’ when using SRMs towards the 7-13 tryptic peptide. Further work is necessary to evaluate this.

The analysis of PTH without prior digestion is one solution to the pitfalls of an SRM approach. Indeed, the acquisition of high-resolution, full-scan MS data may also be valuable in the future, since these data can be interrogated retrospectively for the presence of other fragments or modified PTH variants. Preliminary findings from our laboratory, plus reports from other groups using a similar approach [10], suggest that whilst PTH1-84 itself can be analysed ‘intact’, it accumulates multiple charges (from +16 through to +8) during the ionisation process in the MS source, with multiple isotopes at each charge state. This significantly limits the analytical sensitivity, as the MS signal is spread over multiple charge states and isotope
Table 1  Summary and future considerations for PTH testing.

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- A very large number of PTH variants exist, each of which may contribute to assay variability. The exact structures, and the biological significance, of some of these are yet to be determined.
- All three generations of immunoassay are subject to cross-reactivity from interfering PTH fragments/variants.
- As well as truncated PTH fragments, modified PTH forms (oxidised and phosphorylated) have been observed in clinical samples, sometimes at high concentrations. Oxidised PTH has less biological activity than non-oxidised PTH.
- Immunoaffinity extraction with a C-terminal capture antibody is an efficient sample preparation and pre-concentration step for PTH analysis using MS.
- LC-MS/MS methods which involve trypsin digestion result in the loss of important structural information – the 1-13 tryptic peptide is NOT a surrogate for PTH1-84, since phosphorylation at serine-17 and oxidation at methionine-18 cannot be distinguished from non-modified forms.
- PTH7-84 (or tryptic peptides representative of PTH7-84), has not yet been observed in clinical samples using MS-based techniques.
- Further analysis of PTH and related variants using high-resolution MS, without prior digestion, is necessary, but is especially challenging due to the very low concentrations likely to be found in clinical samples, and the fact that the intact PTH MS signal is spread across a number of charge states.
- MS methods have the capacity to not only identify, but also quantify, PTH fragments. Reference materials for PTH fragments as well as PTH1-84 are therefore required. Prospective studies correlating these concentrations to clinical observations are required.
- Pre-analytical conditions (e.g., sample collection and storage) still need to be considered. Stability of PTH and PTH fragments (especially after extraction from biological matrices) requires further investigation.
- Harmonisation of PTH testing remains critical, especially in certain patient groups such as those with renal failure.

Distributions. With the sensitivity of currently available MS instrumentation, the only way to overcome the sensitivity issues would be to use nano-flow LC separation and to concentrate analytes from much larger sample volumes (e.g., by immunoaffinity) prior to analysis – both approaches which are not suited to routine clinical chemistry analysis, but may be useful for research purposes or, indeed, a reference method for the standardisation of existing PTH immunoassays.

Conclusions

A traceable reference methodology for PTH1-84 is needed to harmonise PTH testing and reduce the variability which currently limits clinical application of this assay. Current PTH testing inadequacies are of grave concern for patients with renal failure, with potential influences on patient misclassification, inappropriate treatment outcomes and patient management [16, 17]. The establishment of the IFCC working group is an important step towards this goal, as are the analytical developments discussed in this review. Absolute quantitation of peptides and proteins by mass spectrometric techniques is still a relatively new field, and no consensus currently exists regarding the most reliable, robust approach for these types of analysis. The vast experience gained by using antibody-based capture and detection systems in the last 50 years will remain important as immunoaffinity techniques used to capture, enrich and even remove interfering PTH fragments, coupled to mass spectrometric detection could be the most useful tool in the future development of reference methods. It has been demonstrated that some PTH fragments show biological activity, and in fact that some C-terminal fragments show antagonistic calcaemic activity to PTH1-84 and PTH1-34 through a different receptor system [10, 11, 31, 63–65]. With this considered, future assay developments should not only aim to exclude these fragments as interferences in the measurement of true PTH1-84, but also include the independent quantitation of these fragments in different patient groups. A summary of the key points from this review is presented in Table 1.

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Lewis Couchman graduated with a BSc in Chemistry and Forensic Analysis from Loughborough University in 2005, and completed an MSc in analytical toxicology from the Queen Mary University of London in 2009. Currently, he works as a senior clinical scientist in the Departments of Clinical Biochemistry and Toxicology at King’s College Hospital, London, alongside studying for his PhD at the University of Leicester. His work focusses on the implementation of chromatographic and mass spectrometric techniques for the analysis of endogenous analytes, such as vitamin D metabolites, steroid hormones and small peptides in clinical diagnostics, but also the analysis of drugs and metabolites for toxicological investigations and therapeutic drug monitoring.

David Taylor received an honours degree and PhD in Biochemistry from the University of Leeds. After this he embarked on post-doctoral studies investigating protein-protein interactions in prion disease using in vitro techniques. In 2009 he switched focus and trained at King's College Hospital in a clinical laboratory, obtaining an MSc in Clinical Biochemistry, developing an interest in mass spectrometry. He now spends his time developing liquid chromatography-mass spectrometry methods for routine clinical applications including quantitative peptide hormone analysis.

Bryan Krastins investigates and develops automated solutions for the MS-compatible sample preparation of biofluids, tissues, and cells for protein biomarker discovery and verification. This includes developing and implementing robust automated sample analysis platforms, such as plasma workflows using magnetic bead technologies, and workflows for cell samples for differential analysis. He also supports ongoing biomarker discovery projects by collaborating with the scientific community and other parts of Thermo Fisher Scientific. In this regard, Bryan acts as an applications expert to assist Thermo Fisher Scientific’s product development organisation. He also interfaces with the scientific community and customers on technical and application topics through collaborations, publications and presentations at conferences and meetings. Bryan holds a MBA with concentration in international management and healthcare from Boston University’s School of Management, and a BSc in Chemistry and Mathematics from Franklin and Marshall College. Bryan joined the BRIMS Center in 2007. Prior to that, he spent 5 years with Harvard Medical School’s core proteomic facility. His roles included performing protein investigations for biomarker discovery research, and protein sequencing for the Boston medical and scientific communities using mass spectrometry.

Mary Lopez is currently Director of the Thermo Fisher BRIMS (Biomarker Research Initiatives in Mass Spectrometry) Center in Cambridge, MA where her responsibilities include the development of comprehensive, MS-based workflows from sample preparation to bioinformatic analysis for robust biomarker discovery and clinical validation. Previously, she held positions as Strategic Collaborations Leader and Analytical Proteomics Business Leader at Perkin-Elmer Life and Analytical Sciences and Executive Vice President of Proteomics Research and Development at Proteome Systems. Lopez was a Post Doctoral NIH Fellow at MIT and received her PhD in Botany from the University of Massachusetts, Amherst, MA in 1985. She currently serves on the board of the Scientific Advisory Board of the Clinical Proteomics Research Center, Massachusetts General Hospital. Lopez’s career has been focused on proteomics and, most recently, in the area of quantitative proteomics. She has worked primarily in industry and therefore has had the opportunity to access many cutting-edge technologies with a recent concentration on mass spectrometry. A focus has been the development of broad collaborations that unite industry and academia and result in novel products and workflows for the development of robust assays for disease-related biomarkers.

Caje Moniz is a consultant chemical pathologist, who has qualified in Biochemistry at the University of Sussex then trained in medicine at the Westminster Hospital Medical School before higher specialist training in metabolic medicine and chemical pathology at the Middlesex Hospital and King’s College Hospital, London. Currently he is Head of Department and Laboratory Director of the Blood Sciences and Reference Biochemistry departments at King’s College Hospital NHS Trust. He is a recognised teacher and Honorary Senior Lecturer at King’s College London and supervises academic research
in clinical and applied sciences. He is a prescribing physician and Clinical Lead for Osteoporosis and Metabolic Bone diseases. His laboratory research interests are in calcium and its regulatory hormones and he has contributed to several intervention trials for osteoporosis and Paget’s disease as well as developing laboratory tests into routine diagnostic clinical applications. In collaboration with Thermo Fisher Scientific, he is currently evaluating novel TurboFlow™ Mass Spectrometric technology in translational research and for developing total and free hormone assays, amongst them, vitamin D, vitamin D binding protein, PTH, insulin and hepcidin.