Perspective and priorities for improvement of parathyroid hormone (PTH) measurement – A view from the IFCC Working Group for PTH

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ABSTRACT

Parathyroid hormone (PTH) measurement in serum or plasma is a necessary tool for the exploration of calcium/phosphorus disorders, and is widely used as a surrogate marker to assess skeletal and mineral disorders associated with chronic kidney disease (CKD), referred to as CKD-bone mineral disorders (CKD-BMD). CKD currently affects more than 10% of the adult population in the United States and represents a major health issue worldwide. Disturbances in mineral metabolism and fractures in CKD patients are associated with increased morbidity and mortality. Appropriate identification and management of CKD-BMD is therefore critical to improving clinical outcome.

Recent increases in understanding of the complex pathophysiology of CKD, which involves calcium, phosphorus and magnesium balance, and is also influenced by vitamin D status and fibroblast growth factor (FGF) 23 production, should facilitate such improvement. Development of evidence-based recommendations about how best to use PTH is limited by considerable method-related variation in results, of up to 5-fold, as well as by lack of clarity about which PTH metabolites these methods recognise. This makes it difficult to compare PTH results from different studies and to develop common reference intervals and/or decision levels for treatment. The implications of these method-related differences for current clinical practice are reviewed here. Work being undertaken by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) to improve the comparability of PTH measurements worldwide is also described.

1. Introduction

Assessment of parathyroid hormone (PTH) concentration is of paramount importance in the exploration of disorders of calcium/phosphorus metabolism and in the

monitoring of patients with chronic kidney disease (CKD) in order to ensure that PTH concentrations are maintained within guideline limits [1]. With availability of convenient and highly precise and reliable automated immunoassay methods for its measurement, PTH is now routinely determined in most large clinical laboratories and can no longer be considered a specialist test.

Correct interpretation of a PTH concentration generally requires concomitant serum calcium concentration in order to evaluate whether PTH is physiologically appropriate (i.e. high calcium with low PTH or low calcium with high PTH) or not (high calcium/high PTH or low calcium/low PTH) for the calcium concentration. In clinical practice, however, patients may have normal calcium and high PTH or high or low calcium and normal PTH [1]. In such patients, measurement of phosphate, urinary calcium and 25-hydroxyvitamin D are mandatory.

PTH measurement is critical to the assessment of patients with primary hypoparathyroidism and primary or secondary hyperparathyroidism. However in many laboratories the majority of PTH measurements are now performed in patients with CKD. In a United Kingdom National External Quality Assessment Service (UK NEQAS) Survey of Practice carried out in 2005, 85% of respondents stated that <40% of the PTH assays carried out in their laboratories were for patients with primary hyperparathyroidism, while 73% reported that >60% of the assays carried out were for patients with renal disease [2]. The proportion of the latter is likely to have increased in the intervening period, reflecting the continuing worldwide increase in the number of patients with CKD.

A Kidney Disease Improving Global Outcomes (KDIGO) Working Group developed the term “CKD-mineral and bone disorders (CKD-MBD)” to encompass the systemic changes that occur in CKD patients [3]. These may include (a) metabolic

dysregulation of calcium, phosphorus, PTH, fibroblast growth factor (FGF23) and/or vitamin D and its metabolites, (b) bone disease or renal osteodystrophy as defined by abnormalities in bone turnover, mineralization, linear growth and/or strength, and (c) calcification of extra-skeletal tissues include both vascular and other soft tissues [3]. It was suggested nearly eighty years ago that development of parathyroid gland hyperplasia in patients with CKD-MBD represents a compensatory mechanism for the disturbed equilibrium occasioned by phosphate retention due to renal insufficiency. It was subsequently recognised that PTH plays a significant role in the aetiology and development of CKD-MBD [4] (Figure 1), as increases in PTH occur prior to abnormalities in both serum calcium and phosphate concentrations [5, 6]. A reduction in intestinal calcium absorption occurs when the glomerular filtration rate decreases. This reflects decreased production of 1,25 dihydroxy vitamin D [$(1,25(OH)_2D$] due to reduced renal 1α–hydroxylase activity which is mediated by increases in FGF23 and is associated with phosphate retention. PTH secretion increases in response to the changes in $1,25(OH)_2D$, calcium and phosphate [5]. However until the development of reliable methods for measuring PTH in the 1960s, the diagnosis of hyperparathyroidism in patients with CKD-MBD relied on assessment of the effects of increased PTH secretion on the skeleton.

PTH circulates in different molecular forms, including the "intact" (whole) molecule (PTH 1-84) and various truncated forms (e.g. PTH 7-84 and smaller fragments). These truncated forms may be recognised to different extents in different immunoassays (Table 1) as previously reviewed [7, 8]. Early radioimmunoassays (RIAs) developed in the 1960s and 1970s frequently detected inactive fragments and had relatively poor clinical sensitivity and specificity. Second generation immunometric assays (IMAs) developed in the late 1980s were initially thought to be

specific for the whole PTH molecule (PTH 1-84) but were subsequently found to recognise other circulating fragments as well [9]. Third generation assays which are analytically specific for PTH (1-84) are also available. However whether these assays are of improved clinical value as compared to second generation assays has not yet been established [10] and they are not yet widely adopted in clinical laboratories. Third generation assays provide results in CKD patients that are approximately 50-60% lower than those obtained with second generation assays and about 15% lower than those in subjects without CKD [11], exacerbating the already significant between-method differences in results observed for second generation methods [12] and (previously) for first generation methods.

These differences, together with a perceived view that there is inadequate evidence to link PTH measurements with adverse skeletal and/or cardiovascular events, have recently prompted questioning as to whether PTH measurement in patients with CKD is even appropriate or whether it represents a dangerous substitute for identification and use of more precise and reliable biomarkers [11]. Additional factors contributing to the concerns raised about routine use of PTH measurements in the management of CKD patients include issues associated with sample stability, biologic variability and sampling site (e.g. central venous catheter sampling vs peripheral blood sampling) [11].

In a strong rebuttal, it has been pointed out that while PTH assays have shortcomings and international standardisation is urgently required, PTH remains the best available biomarker with which to guide treatment of CKD-MBD patients, particularly those with PTH concentrations toward the extremes of the KDIGO recommendations [13]. In such patients, monitoring PTH on a regular basis and instituting treatment to decrease elevated PTH concentrations is essential [14], with prospective trials

required to determine whether trends in biomarker concentrations could guide therapeutic decisions [15]. Bone alkaline phosphatase (bone ALP) measurements have not consistently been shown to be superior or additive to PTH and primarily provide information on skeletal function. As PTH is a uraemic toxin, with systemic effects in CKD patients reaching far beyond the bone (e.g. proximal myopathy, growth retardation in children, anaemia, neurotoxicity, pruritus and cardiomyopathy) due to the ubiquitous location of the PTH receptor in multiple tissues, its measurement provides additional clinically relevant information [10].

While PTH measurement probably remains the best clinically available tool to discriminate the extremes of bone turnover [15], it seems likely that both bone ALP and PTH can be used to help guide decisions as a "blended approach" [11, 14] when the PTH is two to nine times the upper limit of normal, in accord with KDIGO recommendations [13]. Serial measurements of PTH are also recommended by KDIGO, beginning in CKD Stage 3 since marked changes in PTH even within the target PTH range suggest a need for early initiation or change of therapy [16].

Most nephrologists consider there is already sufficient evidence linking high or very low PTH with adverse outcomes in patients with CKD-MBD. However better understanding of the complex disease processes and biological interactions involved would be expected to help improve clinical outcome for CKD-MBD patients and further research is highly desirable. Whether for research or clinical use, measurement of PTH should in future be underpinned by well-standardised and well-characterised PTH assays, with evidence-based international guidance outlining pre-analytical and other requirements to be followed when designing study protocols. Such rigorous attention to detail will be essential to enable reliable comparison of results from different studies and centres. It is salutary to note that the poor agreement in PTH

results obtained in different methods was a contributory factor when KDIGO recommended widening recommended target PTH ranges from three to five times the upper limit of normal to two to nine times.

Here we review how work undertaken by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)Working Group for PTH will contribute to achieving those objectives. With the ultimate aim of facilitating development of a complete reference measurement system for PTH determinations, current Working Group objectives include

(1) Achieving standardisation of commercially available PTH measurement methods in terms of the same International Standard and implementing this worldwide,

(2) Defining inclusion and exclusion requirements for an appropriate panel of plasma or serum samples with which to establish reference intervals and then to establish such a panel, and

(3) Facilitating development of a candidate reference measurement procedure (RMP) for PTH(1-84) to a standard that would enable its adoption by IFCC member national societies and its subsequent inclusion of the RMP in the methods supported the IFCC Reference Laboratory Network.

This standardisation initiative is ambitious but advances in mass spectrometric (MS) techniques enable more precise definition of what PTH methods measure, which is an essential pre-requisite for development of a reference measurement system [17].

Where feasible, standardisation is preferred to harmonization and is likely to be more readily sustained in the long-term as harmonization requires maintaining continuity and consistency between different reference pools of sera or plasma.

Measurement of PTH by immunoassay – current state of the art

Within-method performance of current automated PTH methods is excellent, with within-laboratory within-method coefficients of variation (CVs) <10% demonstrated over concentration ranges tested by external quality assessment (proficiency testing) schemes [18]. In contrast, between-laboratory between-method CVs are generally >20% [19].

Between-method variation in results - clinical consequences

Such method-related differences in PTH results have been convincingly demonstrated for some years [7, 18, 20] and have recently been confirmed in a study in which variations of up to 4.2-fold in PTH concentrations were observed when PTH was measured using five different methods in EDTA plasma from twenty-one haemodialysis patients [12]. Figure 2 shows the 3.4-fold difference in results observed for one of these patients. Applying Renal Association guidelines current in the UK at the time, 7/19 (37%) of the study patients would have been considered to have a different category of bone turnover by the highest reading PTH immunoassay than by the lowest reading immunoassay. Decisions as to whether medical treatment (cinacalcet) or parathyroidectomy should be recommended could also have varied in up to 15/19 (79%) of the patients studied [12].

Observed variation in PTH results – contributory factors

In general the major factors that contribute to between-method variation in immunoassay results for any analyte include lack of knowledge about what is the most clinically relevant PTH analyte to measure, poor calibration or lack of calibration against an internationally recognised reference material or reference measurement procedure, differences in antibody specificities and/or method design such that different isoforms are measured in different assays, and method vulnerability to clinically relevant interferences [19]. In order to establish what is

most clinically relevant to measure, i.e. the measurand, it is of course first necessary to know what PTH isoforms current methods are measuring. However some helpful indication of accuracy of calibration, differences in antibody specificities and vulnerability to interferences can be acquired from carefully designed external quality assessment distributions [21].

Relative recovery of purified PTH (1-84). Participants in the UK National External Quality Assessment Service (UK NEQAS) scheme for PTH receive 24 lyophilised PTH specimens annually, at two monthly intervals. While most of these specimens contain pooled EDTA plasma from patients with CKD-MBD, some contain known amounts of synthetic human PTH(1-84), enabling assessment of relative recoveries, i.e. an indication of accuracy. Figure 3 shows the correlation between cumulative bias in the UK NEQAS PTH scheme and mean % recovery for three recent recovery experiments. Cumulative bias is a statistically valid estimate of deviation from the consensus mean target over a period of time, usually 4 to 6 months in the PTH scheme.

The data, which are consistent with those from previous years, suggest that if PTH methods were accurately calibrated in terms of the same commutable International Standard, between-method agreement would improve.

Assessment of recognition / cross-reaction of purified PTH (7-84). A similarly designed experiment using highly purified PTH(7-84) confirmed significant differences in recognition of highly purified PTH(7-84) (Figure 4). As expected the 3rd Generation DiaSorin method did not recognise this fragment. In patient specimens that may contain PTH(7-84), the variable recognition observed is likely to contribute to the between-method differences in results observed for the other methods.

Improving PTH method comparability – IFCC activity

Achieving calibration of all PTH assays in terms of a single internationally recognised standard such as WHO PTH IS 95/646 [22] is a major goal of the Working Group. At least 2-3 years may be required to achieve this as re-standardisation of commercial methods is complex and time-consuming. While diagnostic companies supporting the IFCC project are in principle supportive of this move, demonstration of the commutability of the standard is a pre-requisite [23]. For this it is necessary to show experimentally that the standard material and fresh patient specimens exhibit the same analytical response (regression line slope about 1.0) when measured by two different methods, repeating this activity for all relevant method pairs. The IFCC Scientific Division has recently established a Working Group which is developing a protocol for formal assessment of commutability that will be used by the PTH Working Group.

2. Pre-analytical considerations relevant to measurement of PTH

Defining inclusion and exclusion requirements for a panel of patient specimens appropriate for investigating commutability or establishing reference intervals for PTH is complex and requires consideration of many potentially confounding factors. The most important of these include specimen type and stability, biological variability and vitamin D status. As for many analytes, there are few published reports on these important issues. Nevertheless, using a rigorous population, intervention, comparator, outcome (PICO) approach, a comprehensive electronic search of relevant sources up to 6th December 2012 has been undertaken on behalf of the PTH Working Group [24]. Only 83 of 5511 papers screened both met the strict criteria defined in the paper for inclusion in the systematic review and were relevant to one or more of the three PICO questions developed. These all related to specimen type or stability and led to some of the recommendations summarised in Table 2. A number of studies described other potential pre-analytical influences on PTH concentrations (e.g. potential effects of

serum separator tubes [25]) but often reports were few and/or inconsistent. Potential influences included food ingestion, vegetarian diet, strenuous exercise, gender, race and menopausal status. There were also many limitations to the studies included, most of which used as comparator a sample that had been frozen at baseline, a possible confounding factor [24]. No direct published comparisons of PTH stability with second versus third generation assays were found but such a study would be desirable as it is possible that the peptide fragments detected by the second generation assays are less stable than the intact molecule detected by third generation assays. In a study published after the systematic review, bovine thrombin in rapid serum tubes (RST) was found to decrease PTH results relative to results obtained in serum separator tubes (SST) by an average of 14.1% after 4h at room temperature [26]. Similar results were reported in a second study in which a -15.3% bias was observed for RST tubes in one automated method [25]. Authors of the first study suggest that thrombin cleavage of PTH may lead to conformational changes that variably affect the antigenicity of epitope regions on the molecule and emphasise the importance of validating and verifying blood collection tubes[26].

Developing specifications for reference panels of plasma for PTH – IFCC activity

Sourcing appropriate clinical specimens is critically important for commutability and other studies to establish or validate metrological traceability, as has been highlighted in a recent article which describes difficulties encountered in a similar thyroid hormone standardisation project [27]. The IFCC Working Group for PTH is therefore carefully considering how best to avoid such pitfalls when developing specifications for the planned reference panel, taking heed also of recommendations from the systematic review [24]. The same considerations are relevant when acquiring specimens for assessment of commutability.

Whether and how vitamin D status needs to be taken into account remains controversial. As noted above, the definition of vitamin D sufficiency, which may be regarded as the 25 hydroxyvitamin D (25OHD) concentration above which PTH cannot be suppressed further, varies widely [28] (Table 2). Recently, it has been reported that the optimal concentration of 25OHD above which suppression of PTH occurs progressively diminishes in CKD patients and is more than twice that currently recommended for the general population [29]. It has also been suggested that the optimal 25OHD concentration may be higher in CKD patients compared with the general population [30]. Two recent guidelines on the diagnosis and management of asymptomatic primary hyperparathyroidism strongly recommend that subjects with vitamin D insufficiency should be excluded when establishing reference intervals for PTH [28, 31]. Reaching consensus about how vitamin D insufficiency should be defined is difficult, for reasons that have been recently reviewed [1]. These include the between-method variability of current 25OHD assays, which is currently being addressed by the Vitamin D Standardization Program [32]. Additional factors requiring consideration include diurnal and circadian variation of PTH, renal function, and other variables including age, gender, body mass index and race.

3. Development of a Candidate Reference Measurement Procedure for PTH

Advances in MS have enabled this technique to be applied to much larger and more complex clinically relevant analytes than small molecules such as the steroids for which MS reference measurement procedures are now well-established. Rigorous physicochemical techniques (e.g. mass spectrometric analysis) are required when developing reference measurement procedures, so it is advantageous that there are now several published methods for PTH measurement using MS [33-35]. These methods can provide accurate and precise PTH results as compared with

immunoassay [34] and can identify and quantify new and previously identified PTH fragments [35], which will in the future enable better understanding of the role of the PTH isoforms, thereby enabling definition of the clinically relevant compounds to measure (i.e. the measurands).

However further work is required before MS can provide a reference method against which other methods should be standardised [33].

Most problematically, while potentially more analytically specific, the analytical sensitivity of currently available MS methods does not match that of more sensitive immunoassay methods, although transfer of methods to higher resolution MS may overcome this difficulty. Proteolytic digestion of PTH prior to MS analysis is required, but it would be desirable to eliminate this step if possible. MS methods can also be vulnerable to significant interferences due to the presence of oxidised and phosphorylated PTH variants which may accumulate in patient samples [33]. Finally, some MS methods rely on a preliminary immunoadsorption step, which means that what is ultimately measured is influenced by the particular specificity of the antibody or antibodies selected and also (especially for a potential candidate reference method procedure) on their long-term stability and availability.

Developing a candidate reference measurement procedure for PTH – IFCC activity

In order to assess the feasibility of implementing a MS reference measurement procedure for PTH, three sets of 48 freeze-dried specimens that had previously been distributed through the UK NEQAS for PTH were analysed by MS at the Mayo Clinic [Rochester, Minnesota] using a previously published procedure [34]. Specimens were stored at -70°C on arrival and each set was reconstituted immediately prior to analysis. Results (Figure 5) confirm the feasibility of using the MS method as a candidate reference measurement procedure. Results were in excellent agreement with

the trimmed all laboratory consensus means used as targets in the UK NEQAS for PTH. However until a full reference measurement system is established it is not possible to determine whether the UK NEQAS targets for PTH represent “the truth”. Also problematically, the MS method used in this study is ten times less sensitive than typical immunoassay methods and the between-laboratory reproducibility of the MS method has yet to be demonstrated.

CONCLUSIONS

Clinical interpretation of currently available PTH assay results is clearly fraught with significant governance issues that may adversely affect confidence in the appropriate clinical management of CKD-MBD. The activities described above should facilitate more meaningful comparison and interpretation of national and international audit data and other studies as well as enabling better understanding of how PTH measurements should be used in the management of CKD, to benefit patient care optimally. Improving the standardisation of PTH methods is clearly feasible although ambitious, and the plans presented here will require support from many stakeholders. However there is no doubt that with sufficient participation and co-operation from the clinical and scientific communities, they are achievable.

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Table 1. Classification and characteristics of PTH assays

PTH method classification	**Usage in clinical laboratories**	**Method type**	**Antibody characteristics**	**Molecular forms recognised**
1st generation	1960s and 1970s	Radioimmunoassay	Polyclonal	Broad specificity – PTH and related fragments
2nd generation	From 1980s onwards	Immunometric assay (frequently referred to as "intact" PTH assays)	One antibody directed to the C-terminal and one to the N-terminal region (amino acids 1-34).	PTH (1-84) and some circulating fragments, especially PTH (7-84), but to lesser extent than 1st generation methods
3rd generation	From 2000s onwards	Immunometric assay (frequently referred to as "whole" or "bioactive" PTH assays)	One antibody directed to the C-terminal and one to the N-terminal region (amino acids 1-4).	PTH (1-84). Detection of a "big" molecular fragment has also been reported.

Table 2. Evidence-based recommendations for good practice for PTH sample handling and acquisition

PICO questions	Recommendations	Strength of recommendation	Comments
In human blood samples, how stable is PTH in EDTA or lithium heparin whole blood or plasma compared to clotted whole blood at 4°C, -20°C and -80°C?	If blood samples for PTH measurement are taken into tubes containing EDTA, the plasma must be separated from the cells within 24h of venepuncture	Strong [24]	Consistent with guidance issued by the Clinical Laboratory Standards Institute and the World Health Organisation.
	If blood samples for PTH measurement are taken into "dry" tubes, the serum must be separated from the cells as soon as possible, and analyzed within 3-4h of venepuncture or stored at -20°C for later analysis.	Strong [24]	The main practical advantage of using serum is that calcium can then be measured in the same tube as PTH, since calcium (and bone-alkaline phosphatase) cannot be measured in EDTA plasma. If immediate transfer of the specimen to the laboratory can be guaranteed, this may be a preferred option.
	EDTA plasma samples for PTH measurement should be stored at 4°C and analysed within 72h of venepuncture	Strong [24]	An advantage of EDTA plasma over serum is that PTH in EDTA plasma has longer stability at RT than in serum and that delayed centrifugation to allow blood to clot is not needed Published evidence is inconsistent regarding the stability of PTH under frozen storage conditions. Should laboratories need to freeze plasma prior to PTH measurement, they should establish the stability of PTH in frozen plasma as measured with their own assay.
In human blood samples, does the sampling site affect PTH concentrations?	Blood samples for PTH measurement should always be collected from the same sample site (central or peripheral) for comparison both within and between individuals. Clinical guidelines should explicitly state whether targets refer to peripheral or central venous concentrations.	Strong [24]	This is particularly relevant in haemodialysis patients, for whom samples are often taken through a central line, as PTH concentrations are reported to be 30% higher in central blood compared to peripheral blood. Similarly, in patients undergoing parathyroidectomy with intra-operative PTH monitoring, central venous PTH concentrations were higher compared to peripheral venous PTH concentration. Knowledge of local practice is highly desirable.
In human blood samples, does the time of sampling affect PTH concentrations?	Season, latitude, renal function and vitamin D status (and perhaps age and race) should be considered and/or reported in all studies undertaking reference range determinations for PTH and when interpreting PTH results in individual patients. Reference intervals must be derived from same sample type (e.g. serum, EDTA plasma) than the one that is used routinely in patients samples.	Assessed as weak [24] to strong [28, 31]	This is a controversial area. It is difficult to assess whether the observed seasonal variation in 25OHD concentration is pathological and not normal physiology. The definition of vitamin D sufficiency also varies widely (e.g.25OHD concentration from 30 to 110 nmol/L) and the relationship between PTH and 25OHD is highly dependent on age. There are some concerns about the validity of the data identified and no studies addressed the relative diagnostic accuracy of PTH measurement at different times of the day. However, when recruiting a reference population to establish PTH reference values, exclusion of any subjects who are clinically likely to have either increased or decreased PTH concentration would seem appropriate.
	Except for dialysis patients in whom PTH is measured before the dialysis session, blood samples for PTH measurement should ideally be collected in the early morning in a fasting state and result interpreted against a reference interval derived for this sampling time and feeding status. Indeed, serum calcium, phosphorus and PTH display significant circadian variations and are influenced by food intake (especially calcium-containing foods).	Expert opinion	

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Figure 1. Schematic diagram showing the changes in calcitriol, FGF23 and PTH with increasing stage of CKD. [Figure adapted from Reference [6] and used with permission]

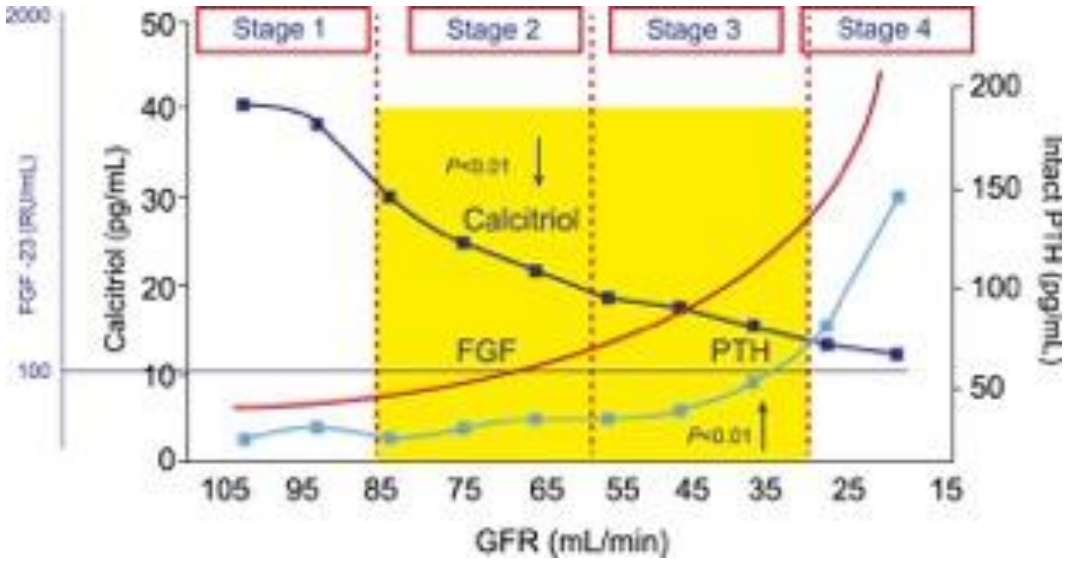

Figure 2. Between-method differences in the concentration of parathyroid hormone (PTH) observed in a typical single patient specimen. Reference intervals for the lowest and highest reading immunoassays were similar (1–6.5 and 1.2–7.6 pmol/L) respectively) [12]. [Figure from Reference [12] and used with permission.]

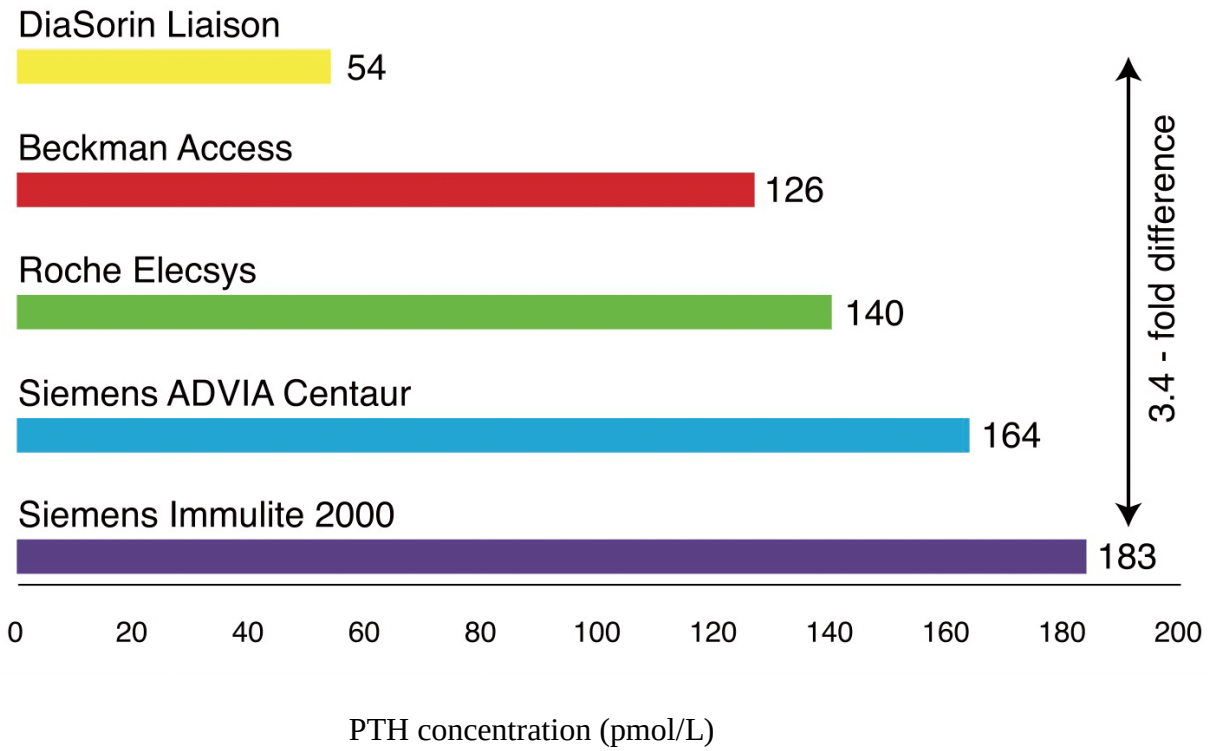

Figure 3. Relationship between mean method recovery of highly purified synthetic PTH(1-84) and cumulative bias from the consensus mean target. [The zero line on the y axis represents the consensus mean target.] [UK NEQAS (Edinburgh) data, 2015]

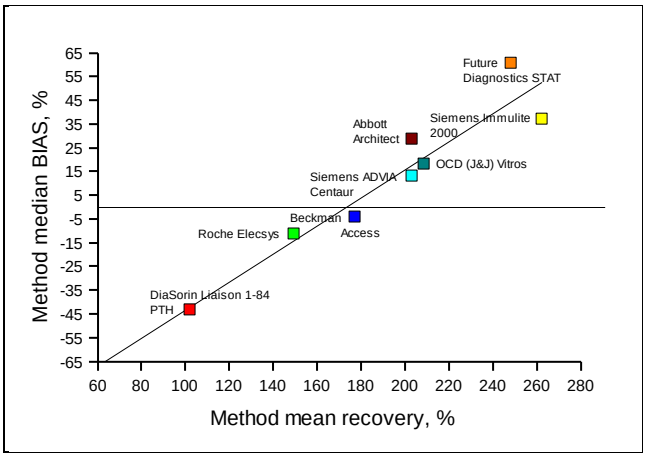

Figure 4. Relative recognition of PTH(7-84) in twelve commercially available PTH methods. [Highly purified PTH(7-84) was added to a pool of human EDTA plasma containing a measurable level of PTH(1-84). [UK NEQAS (Edinburgh) data, 2015] [Methods: A, IDS iSYS; B, Siemens Advia Centaur; C, DiaSorin Liaison N-tact II; D, Roche Elecsys; E, Tosoh AIA; F, Ortho Vitros; G, Abbott Architect; H, Future Diagnostics STAT; I, Beckman Access; J, Siemens Immulite; K, Siemens Immulite 2000; L, DiaSorin Liaison 1-84 PTH]

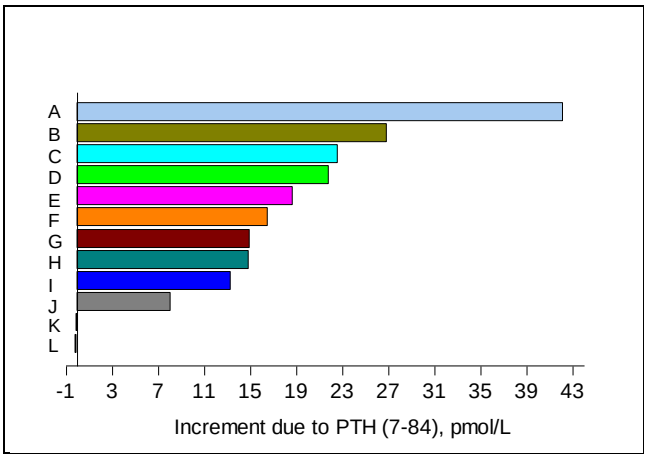

Figure 5. PTH results obtained by mass spectrometry plotted against the all laboratory trimmed consensus mean for 48 UK NEQAS specimens. Results are the average of three mass spectrometric analyses for each sample. Circles indicate specimens containing plasma from patients with CKD-MBD, squares indicate specimens containing PTH IS WHO PTH IS 95/646. [Passing-Bablok slope 0.9926; 1.0 pmol/L of PTH ~ 9.5 pg/mL]