Opinion Paper

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Considerations in parathyroid hormone testing

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Abstract: Parathyroid hormone (PTH) is a major player in phosphocalcic metabolism and its measurement is very important for the correct diagnosis and treatment of several diseases. PTH determination represents the paradigm of quality in laboratory medicine as many variables in the pre-, intra-, and post-analytical phases strongly affect the value of the clinical information. Analytical determination of PTH has been rendered difficult by the presence, in the circulation, of truncated fragments that can cross-react with the antibodies used for its determination. In addition, pre-analytical phase is complicated by the lack of stability of the peptide and the best sample to use for its determination remains controversial, as well as sample handling and storage. PTH secretion is also affected by circadian and seasonal rhythms and by physical exercise. Finally, from the post-analytical perspective, establishment of reliable reference ranges requires further efforts as the selection criteria for reference subjects should take into consideration new variables such as gender, race and vitamin D levels. Finally, clinical guidelines have recently revised and improved the criteria for a correct interpretation of PTH values.

Keywords: calcium metabolism; clinical guidelines; diurnal and seasonal rhythms; parathyroid hormone; reference ranges; sample of choice; stability.

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Introduction

The measurement of the human parathyroid hormone (PTH) represents the paradigm of quality in laboratory medicine as many variables in the pre-, intra-, and post-analytical phases strongly affect the value of the clinical information (Table 1). In fact, in addition to analytical problems due to the presence in circulation of different molecular species, PTH results are influenced in the pre-analytical phase by diurnal and seasonal rhythms and poor standardization of sample collection (time, sample of choice, preservation and storage conditions). In the post-analytical phase, the interpretation varied over time according to different guidelines. However, as recently highlighted by Souberbielle et al. "*the quality of the PTH assay is of paramount importance because it contributes to the therapeutic decision*" [1] and, therefore, major efforts should be done to improve the accuracy and reliability of laboratory practice.

PTH secretion and action

The *PTH* gene is located on the short arm of chromosome 11 [2]. PTH is synthesized as a large polypeptide (pre-pro PTH) containing 115 amino acids that undergoes two successive proteolytic cleavages to yield the 84 amino-acids PTH, the main form of the hormone stored in and secreted from the parathyroid glands. PTH is a major player in calcium metabolism. Decreases in plasma ionized calcium (Ca^{2+}) are detected by the calcium-sensing receptor, a G-protein coupled receptor of 1078 amino acids which consists of a large N-terminal extracellular domain, seven transmembrane helical domains and an intracellular C-tail segment [3]. This receptor is present in the organs that secrete calcium-regulating hormones (the parathyroid glands and the C-cells of the thyroid) and in the target tissues of these hormones (renal tubules of the kidney, bone and intestine). It plays a key role in the control, by the ionized calcium levels, of PTH secretion and in the renal reabsorption of calcium. PTH acts primarily on kidney and bone where it binds to cells expressing the type 1 PTH/PTHrP

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Table 1: Variables in the pre-, intra-, and post-analytical phases that strongly affect the value of the clinical information of a PTH result.

Pre-analytical phase	Intra-analytical phase	Post-analytical phase
Short-term PTH stability	Cross-reactivity with fragments accumulating in kidney diseases for 2nd generation PTH assays	Establishment of reference ranges in vitamin D replete subjects
Long-term PTH stability	Cross reactivity of amino-PTH (hyperproduced in parathyroid carcinoma) with 3rd generation PTH assays	Large intra-individual variation
Choice of serum or EDTA plasma	Oxidized and non-oxidized PTH recognized by the assays whereas non-oxidized PTH is the only active form	Impact of ethnicity on reference range
Time of sampling	Simultaneous need to have PTH and calcium to correctly interpret PTH results	Impact of mean calcium intakes on the reference range
Short half-life of the peptide in the circulation		Impact of age on the reference range
Fasting status		

receptor (PTH1R). This G-protein coupled receptor is also characterized by seven transmembrane domains. When it binds PTH, the PTH/PTHrP receptor transduces its signal through activation of protein kinases A and C pathways [4]. We now know that the very first N-terminal amino-acids of the PTH molecule are necessary for this interaction [5]. In the kidney, PTH stimulates the reabsorption of calcium in the distal tubule and the activity of the 1-alpha hydroxylase in the renal proximal tubule, thus enhancing the synthesis of 1,25 dihydroxy-vitamin D. This active metabolite of vitamin D increases the intestinal absorption of calcium (and phosphorus) and exerts an endocrine feedback on the secretion of the peptide. PTH also decreases the renal reabsorption of phosphate in the proximal tubule (which decreases serum phosphate) through the endocytosis of the apical sodium dependant cotransporter Npt2a [6]. In bone, PTH can induce a rapid release of calcium from the matrix. At longer term, PTH induces changes in Ca metabolism by its action on osteoblasts and indirectly on osteoclasts. Briefly, a continuous production of PTH will stimulate bone resorption by enhancing the transformation of pre-osteoclasts into active osteoclasts, through the osteoblasts. Indeed, PTH stimulates the osteoblastic production of the receptor activator for nuclear factor κ B ligand (RANK-L) together with Interleukin-1 and 1,25 $(OH)_2$-vitamin D and inhibits the expression of osteoprotegerin, an inhibitor of the RANK receptor. Precisely, this receptor is present on the surface of the pre-osteoclasts and its activation by the RANK-L will induce the transformation of pre-osteoclasts into active osteoclasts.

PTH metabolization, performed in the liver, will release different amino-truncated PTH fragments in the circulation. These fragments are generally called C-terminal fragments because they have kept the carboxyl-terminal part of the peptide [7]. They are cleared from the circulation through the kidneys, as PTH itself [8], thus accumulating in the blood of patients suffering from chronic kidney diseases (CKD) [9]. These fragments have a higher half-life than the (1-84) PTH itself.

PTH determination

The presence, in the circulation, of the fully, untruncated, active peptide next to these truncated fragments have rendered PTH determination very complicated [10]. Indeed, what we call now “intact” PTH assays or second-generation assays, recognize both 1-84 PTH and large N-truncated fragments, that used to be called “7-84”-PTH (even if the presence in plasma of the 7-84 fragment has recently been challenged [11]). Intact PTH assays remain however the most used ones worldwide.

Third generation PTH assays are specific for the 1-84 PTH and do not cross-react with the C-terminal fragments, but they cross-react with a N-terminal molecular form of PTH (amino-PTH), a PTH phosphorylated on the serine in position 17, overproduced in parathyroid carcinoma [12, 13] and in rare cases of severe primary hyperparathyroidism [14]. These assays are not as used as the “intact” PTH ones, since two manufacturers only (namely DiaSorin and Roche) present an automated version of these assays, even not available in every country.

Finally, since 1984, it is known that the PTH peptide contains two methionines in position 8 and 18 and that these methionines can be oxidized. Oxidation of these methionines leads to a loss of biological activity of the peptide [15]. The different immunoassays available on the market do not make any distinction between the oxidized and non-oxidized PTH and thus cross react with

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this inactive form. This may be of particular importance in hemodialyzed patients, who suffer from intense oxidative stress and, recently, some results have shown that non-oxidized PTH correlated better with clinical events [16, 17], leading to the question on the potential interest to develop a fourth-generation PTH assay, which would only recognize the non-oxidized (1-84) PTH [18]. However, to our knowledge, no studies have ever proved that this oxidation occurred in vivo and not in vitro, which could be expected if the samples were not treated properly in due time. Indeed, the pre-analytical phase is of major importance to obtain reliable PTH results.

PTH determination: the pre-analytical phase

Pre-analytical handling of samples

PTH is known as being quite unstable and many papers have been published on PTH stability. The debate is also quite intense regarding the vehicle in which blood should be drawn for PTH determination, either EDTA-containing or gel-containing blood collection devices. Prior to discuss these important points, we should remember that the WHO has already published guidelines in 2002 regarding the use of anticoagulants in laboratory investigations [19]. These guidelines state the advantage of using serum or plasma and among the points that are mentioned, we can summarize the ones that may impact PTH determination as follows:

Advantages of using plasma

1) Time saving: Plasma samples can be centrifuged directly after sample collection, unlike serum, in which coagulation is completed after 30 min.
2) Higher yield: 15%–20% more in volume of plasma than of serum can be isolated from the same volume of blood.
3) Prevention of coagulation-induced interferences: coagulation in primary and secondary tubes that were already centrifuged, may block suction needles of the analyzers when serum tubes are used.

Disadvantages of plasma over serum

1) The addition of anticoagulants may interfere with certain analytical methods or change the concentration of the constituents to be measured (contamination with cations: NH^{4+}, Li^{+}, Na^{+}, K^{+}).
2) Assay interference caused by metals complexing with EDTA (e.g., inhibition of alkaline phosphatase activity by zinc binding, inhibition of metallo-proteinases, inhibition of metal-dependent cell activation in function tests, binding of calcium (ionized) to heparin – and we may also add to EDTA).
3) Interference by fibrinogen in heterogeneous immunoassays.

If these guidelines are very useful, they may not decipher if EDTA plasma should be preferred over serum: indeed, in favor of EDTA, we will speed up the turnaround time (TAT) by reducing the clotting time. This can be of great interest in clinical situations where a PTH result must be reported as soon as possible by the laboratory, like in parathyroid surgery, when the surgeon needs to objective the drop of PTH before ending the operation. One should admit that, besides this particular situation, the vast majority of PTH determinations can support to wait the clotting time of the sample. We think that the major advantage of using serum is that calcium concentrations can be evaluated on the same sample. Indeed, calcium concentrations are mandatory to interpret a PTH value. Thus, by using EDTA samples, two samples would be needed, one EDTA for PTH and one serum tube for calcium. KDIGO guidelines also recommend to measure bone alkaline phosphatase concentrations (bAP) next to PTH [20]. Most of bAP assays actually measure the activity of the enzyme (even if they report mass units) [21] and, as stated in the WHO guidelines, alkaline phosphatase is inhibited by EDTA. Thus, two samples would be needed once again. Finally, excess EDTA in underfilled tubes can inhibit signal production in methods that use alkaline phosphatase [22]. In conclusion, excepted for the particular case of parathyroid surgery, we believe that serum should be preferred over EDTA for routine testing.

PTH stability in EDTA or serum should be the paramount of our concerns. As previously stated, many papers have been published on the topic and a recent systematic review on the subject has been published in this journal [23]. As the authors of this review, we have to complain about the tremendous variability – due to both pre- and intra-analytical variables – between the studies, which does not help to decide whether EDTA should be preferred over serum or not. Our major concerns are mainly the definition of the "zero" point and the cut-off below which one can decide whether there is a degradation of the peptide or not. Regarding the "zero" point, most of the studies on PTH stability have used, as comparator, a sample that

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has been immediately frozen at –20 °C [24–31] or at –80 °C [32–34], but few of them used an unfrozen sample, immediately assayed after centrifugation [22, 35–37]. The authors that used a frozen sample as T0 did that on purpose to run all the samples in one batch in order to reduce the CV, but it is unclear if this could have some consequences on the final results.

Even more important is the cut-off below which it was decided if the peptide remained stable or not. Basically, two approaches have been followed by the authors: the purely statistical approach and the "biological" approach.

In the first approach, a statistical test was performed to see if there was a difference before/after storage or not, whatever the percentage of degradation. Very few authors used the "biological" approach, as recommended by the WHO guideline that defines the "maximum permissible instability", which should be smaller than half of the total error derived from the sum of biological and technical variability [19]. This guideline also defines the permissible storage time as the period of time of which the stability requirement of 95% of the samples is met. Oddoze et al. have also elegantly defined a total change limit (TCL) as the square root of $[(2.77*CVa)^2+(0.5*CVb)^2]$, with CVa and CVb being the analytical and biological coefficients of variation, respectively [34]. Accordingly, if we take the analytical and biological CV of PTH as determined by second and third generation by Gardham et al. [38] or by our group [39], the WHO limit should be <11.4 or 8.05% for the second generation and <14.02 and 9.7% for the third generation. The TLC should be <14.5 and 9.4% for the second and <13.7 and 16.7% for the third generation. As the TLC has been defined according to the acceptable change limit defined in the ISO 5725-6 [40] and takes into consideration a statistical factor Z ($=1.96*\sqrt{2}$) for a 95% confidence interval for a bidirectional change as well as half of the intra-individual variation, we think that the results of the TLC should be used to set up PTH stability.

Taking all this information into consideration, it remains difficult to set up one's mind on PTH stability. In plasma or serum, PTH does not seem to be degraded when conserved for long periods (>1 year) at –20 °C or –80 °C. However, for short-term conservation at room temperature, EDTA seems better than serum, even if this advantage tends to disappear when samples are kept at +4 °C.

Time of sampling

Hanon et al. have recently reviewed the circadian variation of PTH [23]. Briefly, PTH follows a bimodal rhythm, with a nocturnal acrophase a mid-morning nadir and a smaller afternoon peak, even if all the studies do not totally agree on peak times. Sensu stricto, it may thus be logical to collect samples for PTH determination during the nadir phase, i.e., between 10:00 and 16:00 [23, 41]. To correctly interpret PTH levels, we have however to consider calcium (and phosphate) concentrations. Surprisingly, very few papers have thoroughly studied the impact of a meal on calcium levels. Statland et al. have noted that a meal could increase calcium by a mean value of 0.04 mmol/L [42]. Even if this increase may seem of little importance, it is higher than the minimal allowable bias for total serum calcium and a patient suffering from mild hypocalcemia could become normocalcemic. In the same vein, a patient presenting calcium levels in the upper normal range could be considered as mildly hypercalcemic. Finally, the meal used in this study was not particularly rich in calcium as it consisted of 2–5 sandwiches (pork, herring, cheese) and a beverage (soda, beer or coffee). A breakfast could contain more calcium if milk or a dairy product is consumed and it is well known that large calcium doses (like the ones used in the calcium loading test) significantly decrease PTH levels.

Serum phosphate concentrations can also be influenced by a meal. In the same study, the participants increased their mean phosphate concentrations by 28%, which far exceeds the minimal bias of 5.1%. Furthermore, phosphatemia is subject to important circadian variations. Thus, a serum phosphate concentration is frequently measured together with calcium and PTH. These are other reasons to recommend a morning blood sample in a fasting state.

For all these considerations, in non-hemodialyzed patients, we still recommend to measure PTH levels in fasting status (generally earlier than 10 AM).This will also be mandatory if PTH levels are measured together with bone markers like CTX or PINP.

PTH determination: the post-analytical phase

An elevated serum PTH concentration (i.e., above the upper normal value) reflects either a secondary hyperparathyroidism (SHPT) when associated to hypocalcemia, or a primary hyperparathyroidism (PHPT) when associated with hypercalcemia. In normocalcemic patients, an elevated PTH necessitates a differential diagnosis between SHPT and normocalcemic PHPT which may be more challenging [43]. In hemodialyzed patients, KDIGO guidelines recommend to maintain the serum PTH levels within

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twice to 9 times the upper normal limit of the reference range [20]. Accordingly, the definition of the upper limit of the PTH normal range is of prime importance. This raises the question of the inclusion/exclusion criteria which should be applied when recruiting a reference population to establish PTH normal values. Exclusion criteria for this population can be defined as any situation possibly inducing an increase or a decrease in PTH concentration. Among these conditions, low serum 25-hydroxyvitamin D (25OHD) concentration is highly frequent in the general population [44] and should thus be prevalent in an apparently healthy group recruited to establish normal PTH values. Excluding subjects with vitamin D insufficiency from a reference population for serum PTH reference values seems thus logical and has been strongly recommended in the two most recent guidelines on the diagnosis and management of asymptomatic PHPT [45, 46]. We have also demonstrated in several studies that excluding subjects with a low serum 25OHD concentration from a reference population decreased the upper normal limit for serum PTH by 20%–35%, depending on the assay considered [44, 47–50]. The obvious consequence is that above-normal concentrations will be found more often in clinical practice. On the one hand, this will improve the diagnostic sensitivity of PTH measurements as serum PTH will be more frequently elevated in patients with either a true SHPT or PHPT. On the other hand, by decreasing PTH upper reference limit, one could argue that it could potentially reduce its diagnostic specificity (i.e., find a "high" PTH concentration in patients without any reason for an increased PTH secretion). However, we have already shown that establishing PTH reference ranges in vitamin D-replete subjects did not induce a decrease in diagnostic specificity, as there was no more than the expected 3% of above-normal PTH concentrations in 360 consecutive osteoporotic patients for whom no reasons for high PTH were found after examining their medical chart and extensive biological evaluation [51].

Besides 25(OH)D levels, renal function should also be taken into consideration when establishing PTH reference values. Indeed, PTH generally rises when estimated glomerular filtration rate (eGFR) is below 60 mL/min/1.73 m^2 [20]. As a decreased renal function may be present, but ignored, in some apparently healthy subjects (especially in those aged more than 60 years) a creatinine measurement to determine the eGFR is mandatory when establishing reference ranges. Other parameters like age, BMI, dietary calcium intakes and ethnicity may also influence PTH reference ranges and further studies are needed to determine whether reference PTH values should be stratified according to some of these parameters.

Analytical phase: standardization of PTH assays

Standardization of PTH assays is of importance to reduce inter-method variability [52], particularly in hemodialyzed patients [53]. An IFCC workgroup, in which representatives of relevant clinical and scientific professional organization and manufacturers of most PTH immunoassays are present is thus working on the current status of PTH measurement and on the identification of priorities for improvement. The group has proposed that the 95/646 WHO International Standard Parathyroid hormone 1-84, established by the Expert Committee on Biological Standardisation of the WHO in October 2009, become the reference standard for PTH assays calibration. This standard is made of recombinant, 1-84, human PTH and has recently been shown to be commutable [54]. The traceability of the assays to the WHO International Standard could be achieved with the recently published LC-MS/MS method allowing the quantification of serum 1-84 PTH after trypsic digestion [55]. In our opinion, however, a total standardization of PTH assays can only be achieved with third generation PTH assays and not with the second generation ones. Indeed, for these latter, even if standardization can theoretically be achieved in healthy populations, this will probably not be the case for CKD patients due to different cross-reactivity of the antibodies with the 7-84 PTH.

Conclusions

The debate on PTH is still active and many studies should still be undertaken to clarify some essential (but basic) points like stability, type of sample and time of sampling. Two of the authors (EC and JCS) participate to the IFCC Scientific Division Working Group on PTH, but totally disagree with the conclusions published by other experts that signed on behalf of this group [23]. For us, and in contrast with these colleagues, serum seems the most appropriate to measure PTH, mainly because it allows the simultaneous determination of calcium. PTH should also be measured in fasting status and good reference ranges should be established in a multicenter and multiethnic study in vitamin D-replete subjects with a normal renal function.

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