Problems with the PTH assays

Difficultés de dosage de la PTH

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Abstract

Even if the first assay for parathyroid hormone (PTH) was published in the early 1960s, its determination remains a challenge even today. Indeed, in the circulation, PTH is present in its active form (PTH 1–84), but many PTH fragments can also be present. These fragments accumulate when renal function declines and are recognized, at different extents, by the 2nd generation (“intact”) PTH assays that are widely used in the clinical laboratories. Some assays, called “3rd generation PTH” do not recognize these fragments, but are not available everywhere. Hence, different problems are also linked with PTH determination. Among them, one can cite the lack of a reference method, the lack of standardization of the assays and, sometimes, the lack of consistent reference range. We can also point out stability problems and a large intra-individual variation. A workgroup is working on these problems under the auspices of the IFCC and we hope that some of these problems will be resolved in the next years. In this article, we will discuss all the possible issues of PTH determination.

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Keywords: Parathyroid hormone measurement; PTH fragments; PTH assays

Résumé

Bien que le dosage de l’hormone parathyroïdienne (PTH) ait été l’un des premiers immunodosages à être publié dans les années 1960, il reste encore très problématique aujourd’hui. En effet, la PTH circule sous sa forme active (PTH 1–84) à côté de nombreux fragments qui s’accumulent dans l’insuffisance rénale et qui sont reconnus par les dosages de PTH de 2e génération (dite « intacte »), employés par la plupart des laboratoires de biologie clinique. Certains kits, dits de 3e génération, ne reconnaissent pas ces fragments, mais ils ne sont pas disponibles partout. D’autres problèmes sont également rencontrés lors du dosage de la PTH. Parmi ceux-ci, nous pouvons citer l’absence d’une méthode de référence et le manque de standardisation des trousse de dosages. Les valeurs de référence ont également souvent été établies de façon critiquable, des problèmes de stabilité sont à noter et la variation intra-individuelle de la PTH est particulièrement importante. Un groupe de travail a été constitué sous les auspices de l’IFCC pour essayer de répondre à certains de ces problèmes. Dans cet article, nous tenterons de faire la lumière sur ces difficultés évoquées.

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Mots clés : Dosage de l’hormone parathyroïdienne ; Fragments de PTH ; Trousses de PTH

1. The different generations of PTH assays: what they measure and what they don’t

The human parathormone (\textit{PTH}) gene is located on the short arm of chromosome 11 [1]. 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 principal form of the hormone stored in and secreted from the parathyroid glands. Its metabolism is performed in the liver, releasing 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 [2]. These fragments are cleared from the circulation through the kidneys, as PTH itself [3], thus accumulating in the blood of patients suffering from chronic kidney diseases (CKD) [4]. These fragments also have a higher half-life than the (1–84) PTH itself.

The description of the first immunoassay for PTH was published by Berson and Yalow in 1963 [5]. This assay, and those who used the same design in the following years, used a single antibody directed towards the C-terminal part of the peptide. If they have been important for a better comprehension of the phosphocalcic metabolism and helped for the diagnosis of primary and secondary hyperparathyroidism, they totally lacked specificity. In 1987, Nichols Diagnostics launched an Immunoradiometric (IRMA) kit called “Allegro” [6]. This IRMA used a pair of different antibodies: a capture antibody directed against the (39–84) portion of the PTH molecule, and a 125I labelled pair of different antibodies: a capture antibody directed against the (39–84) portion of the PTH molecule, and a 125I labelled antibody directed towards the (13–24) portion of the peptide. This “sandwich” assay was thus not influenced any more by the C-terminal fragments, which were measured with the first-generation assays. This “second generation” assay kit and the ones that followed were globally called “intact” PTH assays as they were thought to measure only the full-length (1–84) PTH. During the following years, several similar assays, either IRMA or fully automated chemiluminescent assays have become available on the market. Even if these second generation assays gave results much more consistent with the clinic, they still overestimated the degree of secondary hyperparathyroidism in CKD patients [7,8]. Indeed, some of them presented elevated “intact” PTH concentrations whereas there was no histological feature of low turnover bone disease. In 1998, it was demonstrated that several “intact” PTH assays recognized, with various cross-reactivities (from approximately 50% to 100%), a PTH molecule different from the (1–84) PTH, which co-eluted in HPLC with a synthetic (7–84) PTH fragment [9]. This fraction was then called the “non-(1–84) PTH” or, more generally, “(7–84) PTH”. In 1999, the first “third generation” PTH assay was developed by Scantibodies Laboratories [10]. This IRMA, called “Whole PTH assay” or “Biointact PTH”, used an anti-C-terminal antibody similar to those of the “intact” PTH assays, but an anti-N-terminal antibody directed against the very first amino acids (1 to 4) of the peptide. Thus, this IRMA did not measure the “non-1–84” PTH fragments anymore. In the last years, different automated third generation assays have become available on the market. If these assays do not recognize the C-terminal fragments, they however cross-react with a N-terminal molecular form of parathyroid hormone (amino-PTH), a PTH phosphorylated on the serine in position 17, overproduced in parathyroid carcinoma [11,12] and in rare cases of severe primary hyperparathyroidism [13]. When samples coming from these patients are run with 2nd and 3rd generation assays calibrated against the same material in parallel, the values obtained with the 3rd generation assay are greater than those obtained with the 2nd one, which never happens with other sample types. In this case, the 2nd generation PTH ratio is inverted and can be used as a screening or monitoring tool for parathyroid cancer [12,14–17].

Finally, since 1984, it is known that the PTH peptide contains 2 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 [18]. The different immunoassays available on the market do not make any distinction between the oxidized and non-oxidized PTH and thus cross-react with 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 [19,20], leading to the question on the potential interest to develop a fourth generation PTH, which would only recognize the non-oxidized (1–84) PTH [21].

The 3 main PTH molecules (1–84 PTH, 7–84 PTH and N–PTH) are shown in Fig. 1 and the different PTH characteristics of the different PTH generation assays are summarized in Table 1.

Table 1

Main PTH circulating fragments and their recognition by the various PTH assay-generations.

<table>
<thead>
<tr>
<th></th>
<th>1st generation assays</th>
<th>2nd generation assays</th>
<th>3rd generation assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most common identifications</td>
<td>C-PTH assays, Mid-PTH assays</td>
<td>“Intact” PTH assays</td>
<td>Whole PTH assay, Ca-PTH assay, BioIntact PTH assay</td>
</tr>
<tr>
<td>Availability</td>
<td>1960s to 1980s RIA (competition assays)</td>
<td>1987-ongoing IRMA, Chemiluminescence, (Sandwich assays)</td>
<td>1999-ongoing IRMA, Chemiluminescence (Sandwich assays)</td>
</tr>
<tr>
<td>(1–84) PTH (mostly 7–84 PTH) recognition?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-(1–84) recognition?</td>
<td>Yes</td>
<td>Yes (with various cross-reactivity)</td>
<td>No</td>
</tr>
<tr>
<td>C-terminal fragments recognition?</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Amino-PTH recognition?</td>
<td>Yes</td>
<td>Depends on the epitope of the N-terminal antibody. No if the epitope is proximal (13–24), Yes if the epitope is distal (26–32) like Roche Elecsys PTH</td>
<td>Yes</td>
</tr>
<tr>
<td>Oxidized PTH recognition?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Fig. 1. Amino acid sequence of (A) 1–84 PTH, (B) 7–84 PTH, and (C) amino-PTH (as the 1–4 amino acids of the 1–84 PTH are absent). In most 2nd generation assays, the labeled antibody is directed towards the 15–20 portion of the 1–84 PTH molecule and thus may bind to both 1–84 PTH and 7–84 PTH but not to the N–terminally phosphorylated hPTH, stated of hPTH, stated

2. Standardisation of the PTH assays

In 1974, at its 26th meeting, the Expert Committee on Biological Standardization of the World Health Organization (ECBS, WHO), asked the National Institute for Biological Standards and Control (London), to obtain material of human origin, which could serve as an international reference preparation (IRP). In December 1978, a nominal amount of 150 μg of hPTH, stated
to be more than 95% pure, was donated to WHO as a proposed IRP. Ampoules, labelled 79/500 were prepared in 1981, evaluated in a multicentric study and became the International Reference Preparation (IRP) of Parathyroid Hormone, Human, for Immunoassay [22]. The results of this study emphasized the heterogeneity of the results obtained in the different homemade or commercially available immunoassay systems used at that time. Due to, mainly, lack of stability and reconstitution problems, the WHO standard has really never been used. Since then and up to now, each manufacturer still continues to use his own “homemade” PTH standard to calibrate its assays, leading to a lack a comparability between the results obtained with the different assays available on the market, particularly for CKD patients [23–26]. However, recently, the International Federation of Clinical Chemistry (IFCC) has established a working group who first met in September 2011 to encourage manufacturers to calibrate their PTH assays with a newly established International Standard (IS 95/646). In our opinion, however, standardization of PTH assays could totally be achieved with 3rd generation PTH assays only. Indeed if it is possible to standardize 2nd generation PTH assays in healthy individuals, as soon as glomerular filtration rate decreases, accumulation of non-1–84 fragments occurs. These fragments cross-react at different extents with the antibodies used in the 2nd generation assays, leading thus to the loss of the standardization. As many patients for whom a PTH determination is requested can potentially suffer from CKD, hoping a full standardization of the results with 2nd generation assays appears thus as a dream, whereas it seems possible with 3rd generation assays, for whom there is no interference with the non-1–84 fragments, as we have recently shown [27].

3. Reference method for PTH measurement

Liquid chromatographs coupled with mass spectrometers in tandem (LCMS/MS) have recently been used for PTH quantitative analysis [28,29]. While very sensitive, the main drawback of these techniques lies in sample preparation. Indeed, peptides as large as PTH have to undergo enzymatic digestion, followed by fragment quantification. PTH quantification is then possible, but the additional information (e.g. oxidation, new fragments, etc.) may be lost. Ideally, we need a technique selective and gentle enough to distinguish PTH and its derivatives in their native form. To date, this technique does not exist yet and there is currently no reference method for PTH determination.

4. Problems linked with the pre-analytical phase: sample stability, sample type and sampling time

4.1. Sample stability and sample type

A huge number of studies are dealing with PTH stability and a meta-analysis has recently been published on the subject [30]. The authors of this review, on behalf of the IFCC Workgroup for PTH standardization, have strongly recommended that EDTA blood samples should be taken for PTH measurement. Even if two of us (EC and JCS) are participating to this workgroup, they do not agree, for the moment, with these recommendations. Indeed, we think that the different studies on the subject present too much heterogeneity to set up such a recommendation. For instance, most studies have used as comparator a sample, which had been already frozen, and not a fresh one. Different approaches have also been used to define that a degradation had occurred or not and the percentage of decrease above which one can consider that a degradation had occurred is not the same across the studies. These studies either deal with samples that have been immediately spun, aliquoted and stored for different periods at different temperatures before determination or samples that have been kept as clotted blood or whole EDTA blood for different periods at different temperatures then spun and run. Finally, most of the studies have been published before (and sometimes far before) 2010, with reagents and instruments that are not available anymore in their original form – and the vast majority of these reagents are 2nd generation PTH assays. We think that a thorough study should be performed with an unbiased methodology to set up the best recommendations. Indeed if the results of this study show that PTH is as stable in serum as in EDTA plasma, this avoids taking an extra sample as calcium determination – mandatory for PTH interpretation – can only be achieved on serum.

4.2. Sampling time

A circadian rhythm for PTH exists, showing a nocturnal acrophase, a mid-morning nadir and a smaller afternoon peak, leading the authors of the IFCC working group on PTH recommendation to suggest that blood samples for PTH measurement should be collected between 10:00 and 16:00 – even if they consider this suggestion as a weak recommendation [30]. In our opinion, in non-hemodialyzed patients, a correct interpretation of a PTH result should mandatory be performed in conjunction with total serum (or ionized) calcium levels obtained on the same sample. As calcium is influenced by food intake, samples for PTH should always be taken when the patient is in fasting status.

5. Problems linked with the analytical phase: heterophilic antibodies interference

Just like any other immunoassays, PTH determination is prone to heterophilic antibodies interferences. This interference, due to the presence in the serum of the patients of immunoglobulins targeted against animal proteins, can lead to falsely elevated or decreased levels of the studied parameter. These interferences can lead to significant diagnostic errors and we have shown that patients presenting human anti-goat or anti-mouse antibodies could have large discrepancies in their PTH results [31–33]. In front of situations where the analytical results do not correspond to clinical expectancies, the dialog between laboratory specialists and clinicians must be initiated.

6. Problems linked with the post-analytical phase: reference values

Establishment of reference ranges is not an easy task [34]. First, a “reference population” needs to be defined and 120
samples for each sex and/or age category have to be taken in order to establish the ranges. This task is difficult, tedious, labour intensive, time consuming and difficult to achieve in all the laboratories, reason why the ISO 15189 guidelines that rule the quality in clinical laboratories ask them to “verify” the reference ranges provided by the manufacturers. For PTH, it is obvious that any patient suffering from primary or secondary hyperparathyroidism should not be included in the reference population. For that purpose, creatinine and calcium levels, as well as 25(OH)-vitamin D should have been performed to decipher whether a subject should be included or not. Unfortunately, most of the manufacturers have not taken these clinical aspects into consideration. As a consequence, when we have selected a reference population by selecting subjects presenting normal GFR and calcium levels, as well as a 25(OH)D level > 30 ng/mL, we have shown that the upper reference range for PTH provided by the manufacturers was generally overestimated by approximately 20% [24,35]. This may have significant clinical issues, in delaying the diagnostic of a primary or secondary hyperparathyroidism, or in leading to a bad classification of hemodialyzed patients according to the KDIGO guidelines. Of note, the last guidelines for the management of primary hyperparathyroidism clearly request that PTH reference range should be established in a population presenting normal 25(OH)D levels [36].

Even in 2015, PTH determination remains a difficult task. Pre-analytical, analytical and post-analytical problems are still observed, no standardization of the assays is available yet and a reference method should be developed. A workgroup is working on these problems under the auspices of the IFCC and we hope that some of these problems will be resolved in the next years.

Disclosure of interest

EC is consultant at DiaSorin and IDS.
EC has received lecture fees from Shire, Amgen, Pfizer, DiaSorin, IDS, Abbott, OCD and Roche.

References


[34] Cavalier E, Delanaye P. Establishment of a reference range: no easy task. J Bone Miner Res 2009;24(9), http://dx.doi.org/10.1359/jbmr.090322 [In press].
