Problems with the PTH assays

Difficultés de dosage de la PTH

Etienne Cavalier, Pierre Delanaye, Laurent Nyssen, Jean-Claude Souberbielle

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

The human parathormone (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
La synthèse de l'hormone stockée et sécrétée par les glandes parathyroïdiennes. Son métabolisme se produit dans le rein, libérant différents fragments tronqués d'aminoacides PTH dans le circulation. Ces fragments sont généralement appelés fragments C-terminaux parce qu'ils maintiennent la partie carboxyle-terminale de la protéïne [2]. Ces fragments sont clairement du circuit vers les reins, et PTH lui-même [3], et accumulent dans le sang des patients souffrant d'affections chroniques des reins (CKD) [4]. Ces fragments aussi ont une durée de vie plus longue que le (1–84) PTH lui-même.

La description de la première immunoassay pour PTH a été publiée par Berson et Yalow en 1963 [5]. Ce test, et ceux qui ont utilisé la même technique dans les années suivantes, utilisant une seule anticorps dirigé vers la C-terminale de la protéïne. Si elle a été importante pour une meilleure compréhension du métabolisme phosphocalcique et a aidé pour le diagnostic de la dysfonctionnement phénylhydrazine et secondaire hyperparathyroïdie, ils étaient totalement spécifiques. En 1987, Nichols Diagnostics a lancé un Immunoradiométrique (IRMA) kit appelé "Allegro" [6]. Il a IRMA utilisé un paire de différences anticorps: un anticorps capture dirigé contre le (39–84) portion of the PTH molecule, and a 125I labelled antibody reconnaissant le (13–24) portion of the peptide. Ce "sandwich" test a donc pas influencé plus longtemps par la C-terminale fragments, qui étaient mesurés avec le test de première génération. Ce "second generation" kit de test et les uns qui ont suivi étaient globalement appelés "intact" PTH tests as they were thought to measure only the full-length (1–84) PTH. Pendant les années suivantes, plusieurs similar tests, either IRMA or fully automated chemiluminescent tests have become available on the market. Even if these second generation tests 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 tests 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 test was developed by Scantibodies Laboratories [10]. This IRMA, called "Whole PTH test" or "Biointact PTH", used an anti-C-terminal antibody similar to those of the "intact" PTH tests, 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 automatisé third generation tests have become available on the market. If these tests 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 tests calibrated against the same material in parallel, the values obtained with the 3nd generation test are greater than those obtained with the 2nd one, which never happen with other sample types. In this case, the 3rd 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 tests are summarized in Table 1.

<table>
<thead>
<tr>
<th>Main PTH circulating fragments and their recognition by the various PTH assay-generations.</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>&quot;Intact&quot; PTH assays</td>
<td>Whole PTH assay, Ca-PTH assay, BioIntact PTH assay</td>
</tr>
<tr>
<td>Availability Methodology</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>
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 calcemia 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, labor 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].
