Parathyroid Hormone (PTH) Assays and Applications to Bone Disease: Overview on Methodology

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Abstract

Biologically active parathyroid hormone (PTH) is an 84-amino acid-long hormone that mediates calcium homeostasis and is responsible for normal functions of bone and kidney. Accurate assessment of circulating PTH is essential for the diagnosis of hyperparathyroidism, bone diseases, and chronic kidney disease (CKD). Immunoassays have been extensively used for the measurement of PTH in biological fluids for more than 60 years. Besides several pre-analytical

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factors, differences in types of immunoassays influence estimation of PTH in clinical samples. First-generation PTH radioimmunoassays use a single antibody which detects fragmented PTH and does not entirely reflect levels of biologically active PTH. Second-generation assays use two antibodies directed against distinct N-terminal (12–20/26–32) and C-terminal (39–84) epitopes, respectively, to detect intact PTH (iPTH), but these antibodies also cross-react with N-terminally truncated PTH fragments. To avoid such cross-reactivity, the third-generation assays came which use an N-terminal antibody directed against the first 4 amino acids of PTH with identical C-terminal antibody, as used in second-generation assays. Both second- and third-generation assays are equally good in diagnosis of primary hyperparathyroidism (PHPT) and CKD patients. Third-generation assays are superior in performing intraoperative PTH measurement for predicting successful parathyroidectomy in PHPT patients. The ratio of PTH levels determined by the third-generation over the second-generation assay is another useful tool in detecting parathyroid carcinoma and severe PHPT. Relative measurements of PTH (1–84) and PTH (7–84) in clinical samples may provide insights in their biological roles in CKD. Recently, developed liquid chromatography-assisted mass spectrometry-based PTH assays are more accurate in quantitation of PTH, but require sophisticated instrumentation and expertise. The utility of such advanced assays to differentiate various modified forms of PTH (phosphorylation, oxidation, etc.) needs to be further explored in bone-related pathologies.

Keywords

Parathyroid hormone • Intact PTH • Amino-PTH • Immunoassay • Primary hyperparathyroidism • Bone • Renal osteodystrophy • Chronic kidney disease

List of Abbreviations

CAP	Cyclase-activating PTH
CIP	Cyclase-inactivating PTH
CKD	Chronic kidney disease
CLIA	Chemiluminescence immunoassay
C-PTHR	C-terminal PTH receptor
ECLIA	Electrochemiluminescence immunoassay
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IO-PTH	Intraoperative PTH
IRMA	Immunoradiometric assay
LC-MS/MS	Liquid chromatography-assisted mass spectrometry
n-oxPTH	Non-oxidized PTH
N-PTH	Amino-PTH
oxPTH	Oxidized PTH

PHP	Pseudohypoparathyroidism
PHPT	Primary hyperparathyroidism
PTH	Parathyroid hormone
PTH1R	PTH/PTHrP 1 receptor
RIA	Radioimmunoassay
SHPT	Secondary hyperparathyroidism

Key Facts

Parathyroid Hormone

- Secretory product of chief cells of parathyroid glands.
- JB Collip (1925) prepared the parathyroid gland extract and was the first to demonstrate physiological role of parathyroid glands in calcium regulation.
- In 1963, Berson et al. developed the first RIA-based immunoassay for estimation of human and bovine PTH in biological fluids.
- Complete amino acid sequence of human PTH was identified in the year 1978 by Keutmann et al.
- PTH (1–34) is biologically equivalent to the intact PTH (1–84) molecule (Potts et al. 1971).
- PTH is very fragile and gets degraded rapidly in the liver and kidney.
- In circulation, PTH is present as intact as well as different fragments like N-terminal PTH fragments (4–84, 7–84, 10–84, and 15–84), mid-region fragments, and C-terminal fragments (34–84, 35–84, 37–84 and 45–84).

Monoclonal Antibodies

- Polyclonal antibodies are those antibodies that originate from different B-cell clones that recognize diverse epitopes of a specific antigen.
- The major disadvantages of polyclonal antibodies are batch-to-batch variation in production and multiple specificities.
- Monoclonal antibodies are antibodies that are produced from a single clone of cells and have specificity for a single epitope of an antigen.
- The technique for synthesis of monoclonal antibodies is first devised by Köhler and Milstein in the year 1975.
- Monoclonal antibodies are widely used in diagnostic and research assays as well as in immunotherapy for diseases.

Biological Modifications of Parathyroid Hormone

- Posttranslational modifications are mainly limited to N-terminal region of PTH; only two amino acids methionine and serine are known to be posttranscriptionally modified.
- Methionine presents at the 8th and 18th position in PTH and can be single or double oxidized, but methionine 8 oxidizes more slowly than methionine 18.
- Oxidized PTH variations are most common modifications and but are biologically less active than non-oxidized PTH variants.

- Serine at the 17th position can get phosphorylated and form another N-terminal molecular form, amino-PTH.

Primary Hyperparathyroidism

- A major pathological condition of parathyroid glands and the third most common endocrine disease.
- PHPT is defined as hypercalcemia in association with inappropriately elevated concentrations of PTH.
- PHPT is most commonly observed in postmenopausal women and men over the age of 50 years, with women predominating by approximately 3:1.
- Classical clinical features of PHPT were first described by Fuller Albright (1948) as a pentad of bone pains, kidney stones, abdominal groans, psychic moans, and fatigue overtones.
- With recent advances in screening programs and assays, it is now possible to diagnose PHPT much earlier before it develops into symptomatic disease without obvious signs of hypercalcemia or PTH excess.
- In many developing countries such as India, Brazil, and China, most of the PHPT cases are still presented with classical clinical symptoms.

Teriparatide

- Teriparatide is a recombinant PTH (1–34) molecule.
- It is an anabolic agent for the treatment of osteoporosis in postmenopausal women and men with primary or hypogonadal osteoporosis who are at high risk of fracture.
- Once daily administration of teriparatide preferentially stimulates bone formation in trabecular and cortical bone.
- Teriparatide activates the osteoblasts (bone-forming cells) more than osteoclasts (bone-resorbing cells).

Definition of Words and Terms

Amino-PTH	A newly described molecular form of PTH with intact first 5-amino acids at N-terminal and phosphorylation of serine residue at 17th position.
Chronic kidney disease	Chronic kidney disease is a pathological condition of kidney characterized by progressive loss of kidney function and can lead to renal failure, which can be fatal without dialysis or kidney transplant.
Electrochemiluminescence immunoassay	It is in vitro chemiluminescent assay that is initiated electrically and generates highly reactive species from stable precursors on the surface of the electrode. The highly reactive species reacts with a chemiluminescent substrate to produce light.

Epitope	The epitope is a part of a molecule or an antigen that is being recognized by a specific antibody or T-cell receptor, also known as antigenic determinant.
Intraoperative PTH	Intraoperative PTH refers to the PTH levels measured after minimum invasive parathyroidectomy to predict the success of surgery which is indicated by at least 50% decrease in PTH concentration within 10 min. from pre-incision PTH value.
Mass spectrometry	Mass spectrometry is an analytical technique to quantify the amount or type of molecules in a sample on the basis of the mass-to-charge ratio. The function of the mass-to-charge ratio for ion signal is plotted and known as mass spectrum.
Parathyroid hormone/parathyroid hormone-related peptide 1 receptor	PTH1R is a G-protein-coupled transmembrane receptor. Its activity is mediated by Gsα and activates adenylate cyclase and phosphatidylinositol–calcium second messenger system.
PTH (7–84)	It is the biologically active fragment of PTH (1–84) which starts from the 7th amino acid (lysine); binds to a different receptor, the C-terminal PTH receptor; and performs antagonistic function to PTH.
PTH IS 95/646	It is the recombinant human PTH (1–84) considered as the first international standard of human PTH (1–84) recognized by the World Health Organization.
Radioimmunoassay	It is an analytical technique to quantify the amount of a molecule using radiolabeled or radiotracer antibody directed against a specific region (epitope) of that molecule.
Renal osteodystrophy	Renal osteodystrophy is a metabolic bone disorder of kidney which is mainly due to failure of kidney to maintain normal levels of calcium and phosphorus in circulation. Renal osteodystrophy includes osteitis fibrosa cystica (OFC), adynamic bone disease, and osteomalacia.

Introduction

Parathyroid hormone (PTH), a major regulator of mineral homeostasis, is produced by chief epithelial cells of the parathyroid glands. It is synthesized as a 115 amino acid pre-pro-PTH precursor molecule which undergoes two successive proteolytic

cleavages leading to the formation of pro-PTH and its biologically active form, intact PTH (iPTH) (84 amino acids), respectively. PTH molecules are stored in secretory granules. In response to external stimuli, especially, decreased extracellular ionized calcium concentration, these secretory granules fuse with cell membrane to release PTH in the bloodstream (Brown 2001). Circulatory PTH increases plasma calcium concentration by stimulating bone resorption and calcium absorption from the kidney. These actions are mediated through the PTH/PTHrP receptor or PTH1 receptor (PTH1R), a G-protein- coupled receptor that is ubiquitously expressed and abundantly found in the bone and kidney. PTH also increases the activation of 1-alpha hydroxylase enzyme in the kidney that converts inactive vitamin D to active vitamin D (1,25-dihydroxyvitamin D) to increase calcium absorption in the small intestine. PTH also decreases the phosphorus concentration in circulation by decreasing phosphorus absorption in the proximal tubule of the kidney. Overall, PTH has a positive role in bone formation, and recombinant form of PTH is used as a therapeutic anabolic agent in the treatment of bone diseases.

Along with iPTH, different carboxyl (C)-terminal fragments are also released into circulation. These fragments are formed within parathyroid glands as well as in the liver and are eliminated by glomerular filtration and subsequent degradation in the kidney. In circulation, PTH undergoes proteolytic cleavage into C-terminal fragments, mid-region fragments, and N-terminal fragments. Being a small polypeptide, PTH is highly fragile in nature. The circulating half-life of PTH (1–84) in blood is only 2–4 min; however, C-terminal fragments, especially PTH (7–84) known as "N-terminal truncated PTH," have five to ten times longer half-life and make approximately 80% of circulating immunoreactive PTH in normal individuals. Recent discoveries such as identification of PTH (7–84) as another biologically active form of PTH, identification of C-terminal PTH receptor (C-PTHR), and an amino molecular form of PTH, amino-PTH (N-PTH), which is distinct from PTH (1–84), have made the understanding of PTH physiology more complex. These findings suggest multifaceted roles of PTH and its forms and their possible involvement in pathophysiological conditions.

Altered synthesis and secretion of PTH is involved in various diseases of the parathyroid glands, bone, and kidney, like hyperparathyroidism, hypoparathyroidism, and metabolic bone diseases. Therefore, accurate and reproducible measurement of PTH in clinical samples is an important prerequisite for diagnosis and treatment of these diseases.

Evolution of PTH Assays

Immunoassays are being used for the measurement of PTH in biological fluids for more than 60 years. An immunoassay is a technique to detect or quantify any analyte in blood or body fluids employing the inherent property of an antibody to bind to a specific region of the molecule, termed as epitope. Estimation of PTH in biological fluids has witnessed a constant evolution and has become much easier following the development of antibodies directed against specific epitopes on the hormone

together with a better understanding of the physicochemical properties of PTH. The first PTH immunoassay was developed in the 1960s based on the radioimmunoassay (RIA), generally referred as first-generation PTH immunoassays (Berson et al. 1963). Initial RIA for PTH used single polyclonal antibody directed against the PTH extracted from bovine (porcine) glands and later from human parathyroid adenoma glands. Identification of amino acid sequences of PTH in year 1978 demonstrated that initial PTH RIA detected only the mid-region or C-terminal fragments of PTH. However, quantification of PTH by one RIA assay differed from another assay under similar experimental/clinical conditions. In the early 1980s, N-terminal PTH RIA was developed that could quantify PTH (1–34) which is biologically equivalent to PTH (1–84). The N-terminal assays had better predictive values in disease setup compared to mid-region and C-terminal assays since it could detect biologically active part of PTH. C-terminal assays had limited clinical value as C-terminal fragments have little biologically activity and low clearance rate from the kidney. Major limitations of these assays were low affinity and sensitivity of antibodies, availability of the antisera, and limited applicability to bone disease in CKD patients. These assays were in use till the 1980s.

In the late 1980s, immunometric assays were introduced for most of the hormonal estimations. These assays use two different antibodies directed against two distinct epitopes, one against the N-terminal and another against C-terminal region of the PTH (Nussbaum et al. 1987). One antibody is known as capture antibody bound to solid support and second antibody bound to isotope labeled and used as signal antibody. Thus, these assays measure only "intact (1–84) PTH." By definition, iPTH assay means measurement of a PTH molecule that binds and activates PTH1R. Later, radioisotopes were replaced by enzyme-based chemiluminescent substrates (luminol/peroxidase system) or electrochemiluminescent compounds (ruthenium/tripropylamine). These second-generation assays proved to be superior to RIA and also had better reproducibility in CKD patients and demonstrated good correlation with bone histomorphometric findings from renal osteodystrophy patients. However, these assays overestimate PTH levels in secondary hyperparathyroidism (SHPT) and CKD patients as C-terminal fragments, especially PTH (7–84) are cleared from kidney and form major part of total circulating PTH (90–95%). To overcome this problem and to measure only iPTH, in the year 1999, an immunoassay was developed using signaling antibody directed against the first 4 amino acids of the PTH molecule. These assays are known as "whole" or "bioactive" or "bio-intact" PTH assays. These assays detect only those PTH molecules which have these N-terminal 4-amino acids and are thought to theoretically measure biologically active PTH. In the year 2005, D'Amour et al. demonstrated that an amino-terminal form of PTH (N-PTH) cross-reacted with the antibodies in the third-generation assays. By using HPLC purification, it was identified that N-PTH has a phosphorylated serine amino acid at 17th position. Third-generation PTH assays are expensive and not widely used in diagnostic laboratories. Further, to quantify only PTH (1–84), liquid chromatography-assisted mass spectrometry (LC-MS/MS)-based PTH estimation methods have been developed (Kumar et al. 2010; Lopez et al. 2010). MS-based PTH measurement utilizes immunoaffinity

extraction with a C-terminal anti-PTH capture antibody, followed by digestion with trypsin, and finally quantification by LC-MS/MS method. These mass spectrometry-based PTH assays have high sensitivity and provide accurate estimation of iPTH molecule.

Earlier studies have shown that PTH can be oxidized at methionine residues present at position 8 and 18. The oxidized forms of PTH are biologically inactive but cross-react in third-generation assays. The oxidized PTH can be separated from non-oxidized PTH (active form) by using a specific antibody directed against oxidized PTH and further quantification by LC-MS/MS methods (Hocher et al. 2013). A timeline showing the development of different PTH assays is shown in Table 1.

With increasing knowledge of the heterogeneous forms of PTH, an optimized assay which is highly specific, sensitive, accurate, and inexpensive for the measurement of iPTH and its different molecular forms in routine clinical samples is thus yet to be achieved, and the field is still open for more research.

Pre-Analytical Factors Affecting Estimation of PTH

The prime requirement for any successful biochemical estimation is the provision of optimal pre-analytical conditions as there are several factors that may influence the analysis of samples as well as the final interpretation of results in context of the disease. Intact PTH is a relatively unstable molecule with varying secretory amounts and very low levels in circulation. PTH levels in circulation also vary during different pathophysiological conditions. In order to evolve consistency in PTH estimation, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has established a PTH-working group (Hanon et al. 2013) with following aims:

(a) To prepare good practice recommendations for the optimal pre-analytical handling of patients and samples
(b) To encourage worldwide implementation of recombinant international standard for PTH (IS 95/646) by manufacturers of diagnostic kits
(c) To establish inter-assay limits for harmonization among the different platforms
(d) To develop a standard reference measurement procedure for PTH
(e) To prepare a panel of reference plasma samples to establish standard reference intervals

Sample Type, Storage, and Stability

Ever since the inception of the PTH assays, measurements have been performed in whole blood, plasma, or serum separated in different blood collection tubes like plain tubes, tubes containing anticoagulants (potassium-ethylenediaminetetraacetic acid (EDTA), citrate, or lithium heparin), and/or gel-separating tubes. Studies have demonstrated that PTH is stable at room temperature in EDTA, whole blood for up to

Table 1 History of development of PTH assays

	Year	Group	Description
First-generation immunoassays Radioimmunoassay (RIA)			
1	1963	Berson et al.	Developed polyclonal antibody directed against PTH extracted from bovine (porcine) glands
2	1970	Fischer et al.	Developed antibodies against synthetic PTH peptides used for iodine labeling
3	1971	Patts et al.	Discovered that synthetic PTH (1–34) (amino-terminal fragment) is biologically active and functionally equivalent to PTH (1–84)
4	1977	Desplan et al.	Developed amino-terminal specific RIA PTH assay
5	1978	Keutmann et al.	Described complete amino acid sequence of human PTH
6	1980	Manning et al.	Developed first antisera against peptide extracted from human parathyroid adenoma glands
Second-generation immunoassays "Two-site" or "sandwich" intact PTH assays Two antibodies directed against distinct amino- and carboxyl-terminal regions			
7	1987	Nussbaum et al.	Developed first two-site immunoradiometric assay (IRMA) for PTH measurement
8	1996	Brossard et al.	Described a non-PTH (1–84) circulatory form, PTH (7–84) that interferes with intact PTH assays
9	2001	Divieti et al.	Identified carboxyl-terminal specific receptor (C-PTHR) that binds to PTH (7–84)
Third-generation immunoassays "Whole" or "bioactive" or "bio-intact" PTH immunoassay Signal antibody specifically directed against epitope of 1–4 amino acids of PTH			
10	1999	Scantibodies Clinical Laboratories	Developed the first third-generation assay, cyclase-activating PTH (CAP) assay
11	2005	D'Amour et al.	Identified amino-PTH (N-PTH) by HPLC that showed reactivity to third-generation assays
12	2006	Zhang et al.	Immunoextracted PTH fragments and analyzed by LC fractionation and then by MALDI-TOF MS in normal and diseased individuals sera
13	2010	Kumar et al.	Developed PTH LC-MS/MS assay using isotopically labeled recombinant PTH (1–84) as internal reference standard
14	2010	Lopez et al.	Developed quantitative PTH LC-MS/MS assay using individual isotopically labeled tryptic peptides as standard reference
15	2012	Hocher et al.	Generated antibody directed against oxidized PTH (inactive PTH) and measured oxidized PTH by nano-LC-MS/MS

24 h, and in plasma up to 48 h, but in case of clotted blood and serum, PTH suffers significant decline in immunoreactivity within 2–3 h after venipuncture (Stokes et al. 2011; Oddoze et al. 2012; Glendenning et al. 2002). We and others have also reported that PTH starts degrading at room temperature but remains within

acceptable range until 24 h (3.5–7.3% degradation), whereas after 48 h, samples show 9–14.8% degradation (Omar et al. 2001; Arya and Sachdeva 2014). Very few studies have directly compared the use of lithium heparin whole blood and EDTA whole blood and showed similar stability of PTH (Levin and Nisbet 1994; Teal et al. 2003; Stokes et al. 2011).

Stability of PTH has also been analyzed at lower temperatures such as, 4 °C, −20 °C, and −80 °C. Most studies compared the EDTA plasma and serum for PTH stability at 4 °C and showed that PTH is more stable in EDTA plasma (Parent et al. 2009; Oddoze et al. 2012; Evans et al. 2001). Combining all the available data, it can be concluded that at 4 °C PTH is stable in EDTA plasma for at least 72 h, serum for at least 24 h, and EDTA whole blood for at least 18 h. Few studies have assessed the stability of PTH in plasma or serum kept at −20 °C and −80 °C. In one contrasting report, Cavalier et al. (2007) have shown that when samples are kept at −20 °C, stability of PTH in serum and EDTA plasma is similar, but after 5 days, degradation of PTH was higher in EDTA plasma. Brinc et al. (2012) reported that PTH is unstable in serum and degraded up to 16% in 2 months when kept at −80 °C. Studies involving long-term storage have shown that PTH is stable in serum for 14 days and EDTA plasma for 12 months when kept at −20 °C and −80 °C, respectively, as analyzed by third-generation PTH assays (Inaba et al. 2004; Cavalier et al. 2012). Different commercial assays also influence data on PTH stability. At −80 °C, PTH was stable till 9 months in serum and only 2 months in EDTA plasma when analyzed by the DiaSorin Liaison method, but when assessed by the Roche Elecsys system, PTH was found to be stable until 2 years in EDTA plasma (Cavalier et al. 2007, 2009).

Therefore, based on the previous data and PTH working group of IFCC, blood samples for routine diagnostic PTH measurements should be collected in EDTA tubes; after venipuncture, plasma should be separated immediately and measured as soon as possible or should be stored at 4 °C and analyzed within 72 h (Hanon et al. 2013; Arya and Sachdeva 2014; Cavalier et al. 2007).

Sampling Site

The PTH working group strongly recommends that blood samples should be collected from the same site (central or peripheral) for comparison, both within and between individuals as central venous PTH concentrations were higher than peripheral venous concentrations.

Sampling Time

PTH has a circadian rhythm with higher secretion in the night (12–6 am) and nadir (8–10 am) in the morning with another slight higher secretion in the afternoon (Fig. 1). Studies have shown the variations in secretory nadir, but none have compared the effect of sampling time on the diagnostic accuracy of PTH measurement. Most of the studies suggest pre-noon time as the best suitable period for

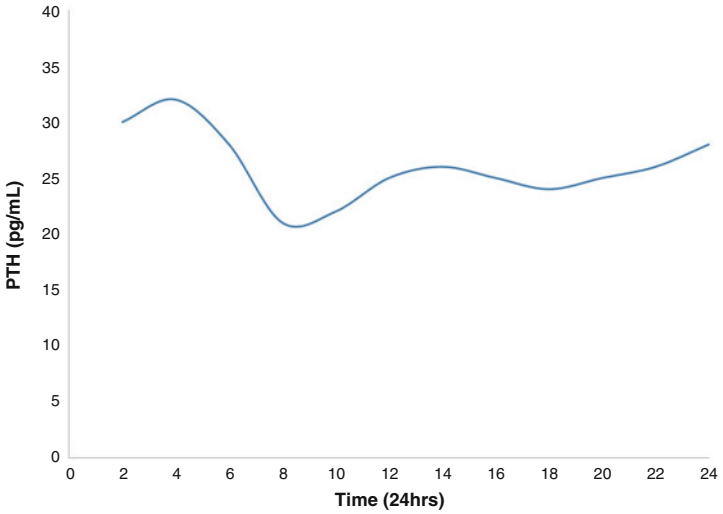

Fig. 1 Schematic representation of diurnal circadian rhythm of PTH secretion. Secretion of PTH is highest during sleep (12–6 AM), then decreases in morning (8–10 AM) and then again slightly increases in the afternoon (12–4 PM)

collecting blood samples for PTH measurements (Herfarth et al. 1992; Ahmad et al. 2003; Logue et al. 1989). Recently, Hanon et al. (2013) have suggested a broader time period between 10 am and 4 pm for collecting blood for PTH estimations. Calcium and phosphorus concentrations are influenced by meals (Statland et al. 1973) and that may finally alter the PTH concentration in the circulation; so, for practical purpose, blood should be collected in fasting condition and preferably before 10 am.

Other Factors

Few studies have tried to assess the effect of other factors like seasonal variation, fasting, diet and sleep, etc. PTH concentrations are influenced by season variability and are higher in the winter season than summer (Woitge et al. 2000). Continuous 2 days or more fasting also leads to loss of circadian rhythm (Schlemmer and Hassager 1999), whereas PTH levels are not affected by sleep (Chapotot et al. 1996). Intake of vegetarian diet also increases the PTH concentrations in blood (Moe et al. 2011).

Several areas associated to affect the PTH stability are still open for research like comparison of the third-generation with second-generation assays to check stability of PTH in EDTA samples, requirement of standard reference range data, and further comparison related to the effect of freezing temperature on the PTH stability by both second and third-generation assays.

Table 2 Optimum pre-analytical factors for estimation of PTH from blood specimens (Arya and Sachdeva 2014; Hanon et al. 2013; Cavalier et al. 2007; Woitge et al. 2000; Stokes et al. 2011; Oddoze et al. 2012)

Recommendation	Factor	Guidelines
# 1	**Type of sample tube**	Blood samples for PTH measurement should be taken in EDTA tubes, and plasma should be separated as soon as possible after venipuncture
# 2	**Sample storage**	PTH should be measured as soon as possible or should be stored at 4 °C and analyzed within 72 h
# 3	**Sampling site**	Blood for PTH measurements should be drawn from same (central or peripheral) site every time
# 4	**Time of collection**	Blood samples should be collected preferably before 10 AM, and results should be interpreted against a reference interval derived for this sampling time
# 5	**Season variations**	Consider season, vitamin D status, and latitude while reporting PTH levels, and interpret the results accordingly in individuals
# 6	**Transfer of samples**	Use pre-chilled EDTA tube after drawing blood transport on ice (without freezing), and use a cold centrifuge to separate plasma
# 7	**Protease inhibitors**	If available, addition of protease inhibitors (e.g., aprotinin) in sample tube before blood collection improves stability of PTH

Based on the data available till date, optimum pre-analytical conditions for PTH measurement have been summarized in Table 2.

PTH Immunoassays: Methods

First-Generation Immunoassays

The first-generation PTH immunoassays were based on competitive radioimmunoassay (RIA). In PTH RIA, radiolabeled hormone (^{125}I-PTH) and unlabeled PTH compete for binding to an immobilized antibody or antisera generated against PTH. Antiserum is prepared from immunized rabbits or guinea pigs with affinity-purified bovine PTH. Purified PTH is also used as standard as well as for the radioiodination of PTH.

Procedure: Antisera against PTH and a fixed amount of radiolabeled hormone (^{125}I-PTH) are incubated in a test tube followed by addition of a specific quantity of unlabeled PTH in samples. The unlabeled PTH competes with the ^{125}I-PTH for binding to the anti-PTH antibody and displaces a proportional amount of ^{125}I-PTH from the antibody. Following washing and precipitation of antigen antibody complexes, the amount of radioactivity in the tube is measured in a gamma counter. This process is continually repeated, using progressively higher concentrations of unlabeled PTH. Radioactivity remained or bound for each standard (**B**) is

compared to the zero standard having no unlabeled PTH ($\mathbf{B_0}$) using the following formula:

$$\%\mathbf{B}/\mathbf{B_0} = \frac{\textbf{Net cpm of standard or sample} \times \textbf{100}}{\textbf{Net cpm of zero standard}}$$

Then a standard-binding curve is plotted between concentration of the unlabeled PTH and the radioactivity (counts per minute, cpm) remaining in each tube (standard) ($\mathbf{\%B/B_0}$), with $\%B/B_0$ on y-axis and log of concentration of PTH on x-axis. By comparing the resulting radioactivity of unknown sample with the standard-binding curve, concentration of the PTH in the sample can be calculated.

Types: On the basis of epitopes used, PTH RIA can be categorized into three types – C-terminal (58–69, 76–84), mid-region (44–53), and N-terminal (1–34) PTH assay. No single assay estimates the complete PTH molecule. The N-terminal assay detects the biologically active form of PTH, as well as the intact PTH, and has a better correlation with the clinical conditions mainly diagnosis of metabolic bone disease in CKD patients than the other two types of assays (Solal et al. 1991; Coen et al. 1993).

Limitations: Generation of antibody or antisera, use of polyclonal antibody, availability of purified PTH, availability of the radiolabeled tracer molecule, radiolysis of radiolabeled PTH, and exposure to radioactive compounds are the major limitations of RIA for PTH determination.

Second-Generation Immunoassays

Second-generation immunoassays are two-site sandwich immunoassays that use two antibodies, the first N-terminal antibody as the signal antibody and second C-terminal antibody as a capture antibody. The capture antibody is immobilized on the solid surface to bind any PTH molecule that has a C-terminal sequence, and the signal antibody is labeled with a detection molecule to bind the N-terminal region of the PTH molecule. Different commercial assays are available that use the signal antibody directed against the proximal (12–20) or distal (26–32) N-terminal region of PTH. These assays are easy to perform and widely used for diagnosis of PHPT and renal osteodystrophy in CKD patients.

Limitations: Second-generation assays also detect PTH (7–84), which is a major C-terminal component of bioactive PTH. Initially, it was thought that PTH (7–84) is biologically inactive, but in vivo studies have shown that PTH (7–84) binds to uncharacterized C-PTHR and increases the bone resorption (Brossard et al. 1993; D'Amour and Brossard 2005).

Third-Generation Immunoassays

Third-generation immunoassays, also known as "whole" or "bio-intact" or "bioactive intact" PTH assays, detect only full-length biologically active PTH and use

signal antibody directed against first 4-amino acids of N-terminal PTH and a capture antibody, identical to second-generation assays. Thus, the third-generation assays have an upper hand over second-generation assays in diagnosis of primary hyperparathyroidism and bone diseases.

Limitations: A non-1–84 PTH fragment, amino-PTH, is overexpressed in parathyroid carcinoma and severe primary hyperparathyroidism and shows cross-reactivity with third-generation assays. It was suggested that amino-PTH is phosphorylated at 17 serine residue that explains no cross-reactivity with second-generation immunoassays having signal antibody in distal N-terminal region (D'Amour et al. 2005; Rubin et al. 2007).

Types: Second-generation and third-generation PTH assays use similar detection systems. Different types of labels and label detection systems are available which are used in the second and third-generation PTH assays. The main aim of developing different detection systems was to increase the sensitivity and reduce background noise. Currently, almost all PTH estimations are performed using automated platforms. The major variants of second and third-generation immunoassays based on the detection systems are as follows:

1. Immunoradiometric assay (IRMA)
2. Chemiluminescence immunoassay (CLIA)
3. Electrochemiluminescence immunoassay (ECLIA)
4. Enzyme-linked immunosorbent assay (ELISA)

Immunoradiometric Assay (IRMA)

The immunoradiometric assay is a noncompetitive assay, which has higher sensitivity and specificity than classical RIA. In PTH IRMA, first, polyclonal/monoclonal N-terminal antibody is labeled with radioiodine (^{125}I) (signal antibody) rather than using radioiodine-labeled PTH as a tracer. Second, C-terminal antibody (capture antibody) is linked to a solid-phase support such as magnetic cellulose particles, glutaraldehyde-BSA, or polystyrene beads. First, plasma is incubated with the solid-phase antibody alone, effectively extracting the analyte from the sample. After washing, the secondary antibody labeled with ^{125}I is added. This antibody reacts with an alternative antibody binding sites on the solid-phase complex to form a solid-phase antibody-analyte-labeled antibody complex. A further washing step separates the complex from the unreacted/free labeled antibody. Then complex is analyzed on a gamma counter. Finally, a standard curve is constructed, and concentration of an unknown sample is calculated, which is directly proportional to the measured radioactivity, unlike classical RIA.

Chemiluminescence Immunoassay (CLIA)

In a chemiluminescence immunoassay (CLIA), the signal antibody is directed against the N-terminal region of the PTH and is conjugated to an isoluminol derivative (DiaSorin Liaison) or acridinium ester (Advia Centaur), while the capture antibody is directed against the C-terminal PTH and is immobilized on magnetic particles or biotinylated (immobilized on streptavidin-coated paramagnetic

particles). During incubation, PTH first binds to the solid-phase and then to N-terminal antibody. After incubation, the unbound/free material is washed, the starter reagent is added, and a flash chemiluminescence reaction is initiated. The light signal is measured by a photomultiplier as relative light units (RLUs) and is proportional to the concentration of PTH in sample.

Electrochemiluminescence Immunoassay

Electrochemiluminescence immunoassay (ECLIA) is based on a process in which highly reactive species are generated from stable precursors at the surface of an electrode. ECL immunoassays use biotinylated monoclonal C-terminal PTH-specific antibody and a monoclonal N-terminal PTH-specific antibody labeled with a ruthenium complex (tris (2, 2′-bipyridyl) ruthenium (II) complex ($Ru(bpy)_3^{2+}$). These antibodies after treatment with analyte samples attach to streptavidin-coated paramagnetic microparticles. The application of voltage to the immunological complexes leads to excitation of ruthenium complex that emits light which is detected by a photomultiplier. The amount of light produced is directly proportional to the amount of PTH in the sample. In the third-generation ECLIA assay, the signal antibody is directed against 1–5 amino acids of PTH, while in the second-generation assay, signal antibody is directed against a distal region (26–32) of N-terminal PTH. This makes amino-PTH (N-PTH) cross-reactive in both second- and third-generation PTH ECLIA.

Cyclase-Activating PTH (CAP) Immunoassay

Cyclase-activating PTH (CAP) refers to intact PTH molecules featuring the first 34-amino acids that bind to PTH1R and activate adenylate cyclase stimulating the synthesis of cyclic adenosine monophosphate (cAMP). On the contrary, cyclase-inactivating PTH (CIP) is another form of PTH that does not bind to PTH1R; therefore, cAMP doesn't get synthesized. Studies have shown that CIP is basically PTH (7–84) that binds to C-PTHR and lowers bone turnover through inhibition of osteoclast formation and differentiation resulting in an overall inhibition of bone resorption (D'Amour 2006; Nguyen-Yamamoto et al. 2001).

CAP immunoassay is the first third-generation IRMA assay developed by Scantibodies Clinical Laboratories, USA. CAP assay only quantifies PTH (1–84) without any cross-reactivity of PTH (7–84). Then CIP level can be calculated by subtracting the PTH levels determined by the CAP assay from the PTH levels determined by the iPTH assay. Finally, CAP/CIP ratio [PTH (1–84) /PTH (7–84) ratio)] can be calculated that has both theoretical and practical advantages over iPTH assay for CKD patients since they usually have large amounts of PTH (7–84) in circulation (Grzegorzewska and Mlot 2004).

Enzyme-Linked Immunosorbent Assay (ELISA)

Current available ELISA assays for determination of PTH are mainly limited to research applications and are available for both intact and total PTH measurement. Principally, in this assay, standards, controls, or samples are simultaneously incubated with an enzyme-labeled antibody and a biotinylated antibody in the well of a streptavidin-coated microtiter plate. Following the incubation period, each

Table 3 Comparison of first-, second-, and third-generation PTH immunoassays

	First-generation immunoassays	Second-generation immunoassays	Third-generation immunoassays
Methodology	RIA	IRMA, CLIA, ECLIA	IRMA, CLIA, ECLIA
Antibody	Single antibody directed against C-terminal, mid-region, or N-terminal PTH	Two antibodies for two distinct epitopes **Capture antibody:** against C-terminal PTH **Signal Antibody**: against N-terminal PTH	Two antibodies for two distinct epitopes **Capture antibody**: against C-terminal PTH **Signal Antibody**: against first 4 amino acids at N-terminal PTH
Detection	C-terminal, mid-region, or N-terminal at a time	Intact PTH and C-terminal fragment (7–84) PTH	Intact PTH and amino-PTH
Advantages	–	Excellent analytical quality and significant correlation with bone biopsy parameters	Measure only biologically active PTH
Limitations	Measures mainly fragments, low-analytical sensitivity, does not distinguish active and inactive PTH	Cross-reactivity with 7–84 and overestimation in CKD patients	No major superiority over second-generation assays

microwell is washed to remove any unbound components followed by the addition of a chromogenic substrate, such as tetramethylbenzidine (TMB). Finally, the reaction is terminated by the addition of stop solution, and the intensity of color formed is read at 450 nm on an ELISA reader. The intensity of the color is directly proportional to the concentration of PTH in the standard or the sample. In most of these assays, antibodies used are directed against distinct epitopes of PTH just as in second and third-generation immunoassays.

A comparison of first-, second-, and third-generation PTH immunoassays has been shown in Table 3.

Mass Spectrometry

In the last 10–15 years, liquid chromatography-assisted mass spectrometric (LC-MS/MS) detection has been widely applied for quantifying small molecules (<1 kDa), such as steroids, hormones (testosterone, vitamin D), drugs, and their metabolites from clinical samples (Rauh 2009). This technique is a combination of chromatographic separation with a detection system that determines the mass-to-charge (m/z) ratio of the analyte and provides far superior specificity over immunoassay-based detection. There have been published reports on quantitative LC-MS/MS methods

for the analysis of PTH (1–84) in clinical samples that have led to their application in diagnostics (Kumar et al. 2010; Lopez et al. 2010; Krastins et al. 2013).

Quantitative LC-MS/MS Assay: Albumin and IgG make 90% of the total proteins in plasma and mask the expression of proteins present in minimal concentrations. Therefore, quantification of low-concentration peptides like PTH in plasma by MS requires extensive sample enrichment. Immunoaffinity extraction (also referred as immunoextraction) is increasingly being used for sample enrichment. Immunoaffinity extraction of PTH is performed by using either C-terminal PTH capture antibody immobilized on polystyrene beads (Kumar et al. 2010) or by immobilizing the capture antibody (polyclonal goat antihuman PTH39-84) on micro-column embedded in pipette tips for automation (Lopez et al. 2010). After washing steps to remove nonspecific binding, the captured PTH (1–84) and related fragments are digested using trypsin. Selected tryptic peptides are then quantified on LC-MS/MS. Quantification of the first 1–13 amino acid tryptic peptide (SVSEIQLMHNLGK) is used as a surrogate for the measurement of PTH (1–84). The LC-MS/MS method has been calibrated using standard recombinant human PTH WHO IS 95/646. For internal reference control, isotopically labeled intact (^{15}N) PTH (1–84) or isotopically labeled tryptic peptides are used. LC-MS/MS PTH quantification methods have good analytical performance, linearity, and sensitivity. MS analysis has nonsignificant interference with any of the other PTH fragments [PTH (1–44), PTH (7–84), PTH (43–68), PTH (52–84), and PTH (64–84)] or other PTH-related forms like PTHrP, amino-PTH even in hemolyzed, lipemic, and icteric samples.

MS-based PTH assays are still in developmental stages. The major limitations of MS are the complexity of the procedure right from sample preparation, enzymatic digestion, and further quantification of PTH fragments and sophisticated instrumentation and costs.

PTH Modifications and Immunoassays

Studies have revealed that posttranslational modifications can occur at different amino acids in PTH. Phosphorylation of serine 17-residue has been reported in human and bovine parathyroid glands. Another possible modification is oxidation of methionine present at positions 8 and 18 in PTH. In vitro studies using high-resolution MS have indeed identified that both methionine residues can be single or double oxidized to form sulfoxides and sulfones, respectively, (Pan et al. 2010; Chu et al. 2004). These oxidation products are biologically inactive because they lose their ability to bind to PTH1R. The oxidized PTH forms are identified in excess in CKD patients as these patients suffer with high oxidative stress (Nabuchi et al. 1995; Tepel et al. 2013). Classical PTH immunoassays do not differentiate between biologically active non-oxidized PTH (n-oxPTH) and non-active oxidized PTH (oxPTH). Hocher et al. (2013) recently generated monoclonal antibody against the oxPTH immobilized on CNBr-activated sepharose-4B beads and separated all oxidized forms of PTH (1–84) from plasma samples. These samples containing purified n-oxPTH can be further measured by high-resolution MS after tryptic

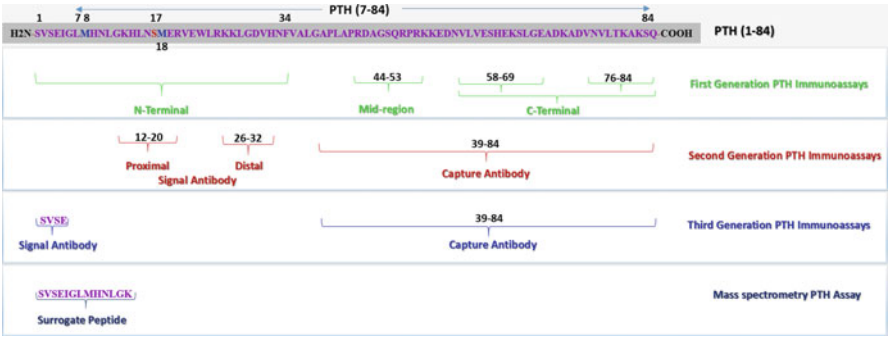

Fig. 2 PTH structure, modification sites, and epitopes selected for antibodies in different generation of PTH assays. Sequence (1–34) represents the amino-terminal, and sequence (35–84) represents carboxyl-terminal of PTH molecule. Sequence of PTH (7–84) is also depicted which starts from lysine amino acids at 7th position. Amino acid methionine, at 8th and 18th position, is a possible site for PTH oxidation. Oxidized PTH is biologically inactive. A newly described molecular form of PTH, amino-PTH, can be generated by phosphorylation of serine present at the 17th position. Different generations of PTH immunoassays were developed using antibodies directed against the specific epitopes on the PTH molecules. First-generation assays used single antibody, but second and third-generation assays used two antibodies directed against N-terminal region (signal antibody) and C-terminal region (capture antibody). Mass spectrometry-based methods are the latest to estimate PTH values using 13 amino acid tryptic peptide (SVSEIQLMHNLGK) as surrogate peptide to estimate only intact PTH (1–84) from the samples

digestion or by immunoassays using targeted antibodies. The sequence of PTH with its modification sites and epitopes used in different generations of assays are highlighted in Fig. 2. With growing evidences on various posttranslational and post-secretion modifications in PTH, it is imperative that immunoassays be constantly improved to enable detection of biologically active PTH in clinical samples. Recent literature suggests the requirement of a fourth-generation PTH assay for improving detection of bioactive and intact PTH with simultaneous detection of other fragments and forms of PTH.

Tissue-Specific PTH Expression

Ultrasound-assisted fine-needle aspiration (FNA) can be utilized to obtain a tissue biopsy from the potential tumor sites and can be used as localizing and diagnosis technique for severe and misdiagnosed primary hyperparathyroidism (Stephen et al. 2005). Cytological examination as well as PTH measurement can both be performed, simultaneously in the aspirates to establish the correct diagnosis of primary hyperparathyroidism. Biopsy can also be utilized for confirming tumor by analyzing the expression of PTH by immunohistochemistry. Gene and protein expression analysis of PTH by real-time PCR and western blotting, respectively, can also serve as adjunct tools in the diagnosis or prognosis of PTH-associated diseases.

Potential Applications to Prognosis, Other Diseases, or Conditions

Primary Hyperparathyroidism: PHPT is mainly characterized by hypercalcemia and inappropriately elevated PTH levels. Therefore, measurement of PTH is a primary diagnostic criterion for PHPT patients. Second-generation PTH assays are widely in use and have good sensitivity and reproducibility in PHPT patients. These assays can also detect subnormal levels of PTH and discriminate hypoparathyroidism from a mild form of PHPT or hypercalcemia due to malignancy. However, the second-generation PTH assays also detect the (7–84) PTH fragment, which was later proved to have antagonist functions to PTH. Hence, third-generation assays were developed that detect only the active iPTH. Several studies have compared the sensitivity and specificity of second and third-generation assays in PTH measurement. Direct comparison between second- and third-generation PTH assays showed that both assays provide good sensitivity with minimal differences in PTH levels of PHPT patients (Boudou et al. 2005; Silverberg et al. 2003). The recently held Fourth International Workshop on Asymptomatic PHPT also concluded that diagnostic sensitivity of both the assays was similar (Eastell et al. 2014). However, more studies are required to assess the superiority of third-generation assays over second-generation assays in the diagnosis of PHPT.

Intraoperative PTH Measurement: Rapid measurement of intraoperative PTH (IO-PTH) is a procedure to confirm the successful removal of a parathyroid adenoma during minimally invasive parathyroidectomy after preoperative localization of putative adenoma. Assessment of IO-PTH is becoming a useful tool in guiding operative decisions in PHPT (Irvin et al. 2004; Chen et al. 2005). According to the well-accepted *Miami criterion* (Carneiro and Irvin 2002), $\geq$ 50% decrease in PTH levels compared to the highest pre-incision or pre-excision PTH levels at 10 min. after removal of hyperfunctioning gland represents successful parathyroidectomy. Since iPTH is degraded immediately after release, third-generation PTH assays show faster decrease in PTH levels than second-generation PTH assays, when PTH is measured at different time intervals of 5, 10, and 15 min post-excision of parathyroid adenoma. At 5 min., PTH levels dropped more than 50% in all the patients after parathyroidectomy as determined by third-generation assays but only in 92% of patients when second-generation assays were used (Yamashita et al. 2002). Therefore, third-generation assays appear to be better in assessing IO-PTH levels. Sohn et al. (2015) have demonstrated that in PHPT patients with CKD, decrease in IO-PTH is slower as after 10 min only 89% of patients with chronic renal insufficiency (CRI) met intraoperative cure criteria, and at 15 min only 95% met the criteria. The site of blood collection (peripheral or central) doesn't influence the success of parathyroidectomy and predictive accuracy of IO-PTH levels (Abdel-Misih et al. 2011).

Parathyroid Carcinoma: With differences in the size of PTH fragments analyzed, the ratio of PTH levels detected in third-generation assays over the second-generation assays can be utilized for differentiating parathyroid carcinoma from benign parathyroid tumors. The third-/second-generation PTH ratio is normally less than 1.0 which increases in parathyroid carcinoma (Cavalier et al. 2010; Caron et al. 2011). In

parathyroid carcinoma and severe PHPT, amino-PTH is secreted which is only detected by second-generation assays with a signal antibody in the distal N-terminal region and all third-generation PTH assays (Rakel et al. 2005; Rubin et al. 2007). In healthy individuals, amino-PTH makes 2–4% of total PTH in circulation, but in severe PHPT and CKD patients, it increases up to 15–20%. The third-/second-generation PTH ratio has the sensitivity of 81.8% and specificity of 97.3% in detecting parathyroid carcinoma among the PHPT patients (Cavalier et al. 2014).

In context of the recent observations, these assays also need to be standardized and validated in symptomatic PHPT, where levels of PTH remain very high in parathyroid carcinoma as well as in benign tumors.

Secondary Hyperparathyroidism: Secondary hyperparathyroidism (SHPT) is a complex entity comprising of the bone and mineral metabolism-related complications that have a direct effect on the kidney, such as in CKD. SHPT is associated with hyperphosphatemia, vitamin D deficiency, and skeletal resistance to PTH. CKD patients at stage 3, stage 4, and stage 5 may develop or already have developed SHPT. Metabolic bone disorders associated with CKD are referred to as renal osteodystrophy, two major types being osteitis fibrosa cystica and adynamic bone disease. Osteitis fibrosa cystica is a high-turnover bone disease with elevated PTH levels that stimulate osteoclast activity and bone resorption. Adynamic bone disease is a low-turnover disease with normal mineralization and may result from low PTH levels. The use of vitamin D agents and/or calcimimetics increases the incidence of adynamic bone diseases due to misdiagnosis of PTH levels and further suppression of PTH secretion in CKD patients. Iliac crest bone biopsy with double tetracycline labeling and bone histomorphometry analysis is a gold standard for the diagnosis of skeletal complications in the CKD patients although it is difficult to obtain bone biopsies from the patients.

PTH measurement with bone turnover markers (alkaline phosphatase, tartrate-resistant acid phosphatase) is essential for the diagnosis of CKD patients as well as for monitoring the patient's response to therapy. According to Kidney Disease Outcomes and Quality Initiative (KDOQI) clinical practice guidelines for metabolic bone diseases in CKD patients, PTH levels should be maintained between 150 and 300 pg/ml in CKD patients. PTH measurement also acts as a screening tool for differentiating osteitis fibrosa cystica from adynamic bone disease. Usually, second-generation PTH assays provide good sensitivity and a high correlation with bone histomorphometry studies and biochemical bone turnover markers in CKD patients. However, PTH fragments mainly PTH (7–84) accumulate in the kidney and further released into circulation after glomerular filtration and make up to 45–50% of total PTH in CKD patients. This leads to the overestimation of active PTH by second-generation assays that finally influence the diagnosis and management in CKD patients (D'Amour et al. 2006; D'Amour and Brossard 2005). Several studies had also found a strong correlation of PTH levels between second- and third-generation assays in CKD patients. However, the PTH levels measured by third-generation assays were 30–50% lower than measured by second-generation assays (John et al. 1999; Savoca et al. 2004; Souberbielle et al. 2006). Monier-Faugere et al. (2001) have suggested that PTH (1–84)/PTH (7–84) ratio can be helpful in

differentiating normal or high bone turnover disease (osteitis fibrosa cystica) with low bone turnover disease (adynamic bone disease) in CKD patients. They have reported that PTH (1–84) /PTH (7–84) ratio can be less than 1 in adynamic bone disease and more than 1 in normal or high bone turnover diseases. However, other studies were unable to confirm these findings (Coen et al. 2002; Salusky et al. 2003). Therefore, more focused studies are required to evaluate the third- generation assays and relevance of PTH (7–84) in the diagnosis of CKD.

Pseudohypoparathyroidism: Pseudohypoparathyroidism (PHP) is a group of rare diseases where resistance to various hormones that activates G-protein coupled receptors, primarily PTH is observed. PHP is mainly of two types, PHP-I and PHP-II. PHP-I is a major type and further subdivided into two major subtypes – PHP-Ia and PHP-Ib. PHP-Ia is caused by inactivating mutations in exons of GNAS gene which encode G stimulatory protein (Gsα) that affect the maternal allele (Aldred and Trembath 2000; Ahmed et al. 1998). PHP-Ib is mainly caused by loss of genomic imprinting at the exon A/B in the GNAS gene (Bastepe and Juppner 2005). Clinically, PHP-I patients show resistance to PTH in renal proximal tubules that lead to hypocalcemia and hyperphosphatemia and increase circulatory PTH levels (Weinstein et al. 2001; Bastepe and Juppner 2000). It has been shown that besides elevated PTH, PTH (7–84)/PTH (1–84) ratios were also higher in PHP-Ia and Ib patients (Hatakeyama et al. 2003).

Osteoporosis: PTH is essential for normal bone function, and elevated PTH levels increase the risk of low bone mineral density, bone resorption, osteoporosis, and fractures in elderly persons, whereas intermittent doses of PTH increase bone formation and bone mass and is used as an anabolic agent [teriparatide (PTH (1–34)), recombinant PTH (1–84)] for the treatment of osteoporosis (Canalis et al. 2007). PTH treatment also has some adverse effects like mild hypercalcemia, hypercalciuria, and possibly increased uric acid concentrations (Canalis et al. 2007; Greenspan et al. 2007). Currently, PTH is the only approved anabolic agent available for treatment of osteoporosis treatments. Hence, measurement of accurate PTH levels in osteoporotic patients is required for deciding the correct dose of PTH. As the third-generation assays only detect the biologically active PTH (1–84), so, they have an advantage over the second-generation assays in the accurate assessment of PTH levels in osteoporosis patients.

Other Diseases: Elevated PTH levels as in PHPT patients also affect the cardiovascular and gastrointestinal systems, and these patients show improvement in symptoms after successful parathyroidectomy (Agarwal et al. 2013; Abboud et al. 2011; Shah et al. 2014; Barletta et al. 2000; Walker et al. 2013). In case of the cardiovascular system, recent reviews suggest that chronic PTH level increases the risk of hypertension, cardiac hypertrophy, and myocardial dysfunction. PTH receptor is also present in myocardium and leads the hypertrophic function. Recent report from Atherosclerosis Risk in Communities (ARIC) group demonstrated that PTH is elevated, but not an independent risk factor for cardiovascular diseases (Folsom et al. 2014). It is suggested that hypocalcemia may induce inappropriate rise in PTH levels in pancreatic patients. Levels of PTH are variable in acute to a chronic form of pancreatitis, but higher levels were observed in patients with GI complications (McKay et al. 1994). The European

Table 4 Circulating levels of PTH and its molecular forms in different disease conditions

Disease	Levels of PTH and its molecular forms
Primary hyperparathyroidism	Increased or inappropriate normal levels of **PTH (1–84),** Increased percentage of **PTH (7–84)** Increased percentage of **amino-PTH**, particularly in parathyroid carcinoma and severe PHPT
Secondary hyperparathyroidism	Increased **PTH (1–84)** levels Increased percentage of **PTH (7–84)** in high bone turnover disease, which decreases in adynamic bone disease Increased percentage of **amino-PTH**, particularly in CKD.
Hypoparathyroidism	Decreased **PTH (1–84)** levels
Pseudohypoparathyroidism	Increased **PTH (1–84)** levels with increased percentage of **PTH (7–84)**
Coronary heart disease	Increased **PTH (1–84)** levels
Acute pancreatitis	Increased **PTH (1–84)** levels in severe acute pancreatitis
Anemia	Increased **PTH (1–84)** levels
Colorectal cancer	Increased **PTH (1–84)** levels particularly in males

Prospective Investigation into Cancer and Nutrition (EPIC) group reported that high PTH levels may be associated with the incidence of sporadic colorectal cancer, particularly in males (Fedirko et al. 2011). High levels of PTH also inhibit the erythropoiesis that leads to the genesis of anemia. This pathway is also associated with patients of PHPT and SHPT (Bhadada et al. 2009; Brancaccio et al. 2004; Chutia et al. 2013). Therefore, with growing knowledge about the extra-osseous roles of PTH, the utility of accurate assessment of PTH particularly iPTH becomes important. Variations in circulating levels of PTH and its molecular forms in different disease conditions have been summarized in Table 4.

Current Challenges and Future Prospectives

PTH measurements from currently available second- and third-generation assays correlate well, but differ sometimes. Besides, the difference in the N-terminal antibody, another reason for this inter-assay variability, is calibration of the assays using synthetic PTH (1–84) from different origins and measurement of different PTH forms. Souberbielle et al. (2006) had suggested the use of correction factors for each assay considering allegro intact PTH assay as a reference to overcome this problem. However, the use of correction factors cannot be the final solution. For this, all manufacturers need to calibrate the assays against the currently available WHO International Standard IS PTH 95/646 for uniformity. Heterogeneity of the PTH molecules and the potential physiological functions of the different molecular forms of PTH have made our understanding of calcium homeostasis complex. The scientific community needs to work on structure–function relationship of PTH and its molecular forms in different pathophysiological conditions and to develop more sensitive assays. The MS-based PTH assay is an accurate method for quantification

of iPTH as it identifies the molecules on the basis of the mass-to-charge ratio and fragmentation property and is not interfered by different forms of PTH cross-reacting with the antibody. But as discussed earlier, MS is technically challenging, is costly, and requires sophisticated instrumentation. Also, currently used assays still do not entirely differentiate iPTH from its modified forms [phosphorylation at serine (17) and oxidation at methionines (8 & 18)].

In a clinical set up, PTH is highly associated with vitamin D status of the individuals and has an inverse relationship with PTH secretion. Populations targeted for PTH measurements mostly have vitamin D insufficiency and should be avoided in generation of reference data. Nonetheless, PTH still has a long way to go before it is used as a surrogate marker for bone turnover. Extensive studies are thus required to address the pathophysiology of PTH and its different molecular forms and their diagnostic applicability.

Summary Points

- Accurate assessment of PTH in clinical samples is essential for diagnosis of diseases associated with parathyroid glands, bone, and kidney.
- First-generation radioimmunoassays, based on the use of a single antibody, were the first to quantify PTH in vitro and were used till the late 1980s.
- Second-generation PTH assays measure intact PTH (1–84), but also detect PTH (7–84) fragment and are widely used in clinical laboratories worldwide.
- PTH measured by second-generation assays correlates well with bone histomorphometric studies of metabolic bone disorders (diagnostic gold standard) in CKD patients.
- Third-generation PTH assays detect bioactive PTH (1–84) and do not show cross-reactivity with PTH (7–84) fragment.
- The ratio of PTH levels obtained from the third-generation over the second-generation assays can be used for reliable diagnosis of parathyroid carcinoma and severe primary hyperparathyroidism.
- Liquid chromatography-assisted mass spectrometry (LC-MS/MS)-based assays are recent addition to the PTH assays, provide accurate assessment of PTH, and can distinguish different fragments of PTH.
- Commercially available second- and third-generation assays have a lot of variability in measured PTH values and need to be calibrated against WHO International Standard PTH IS 95/646.

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