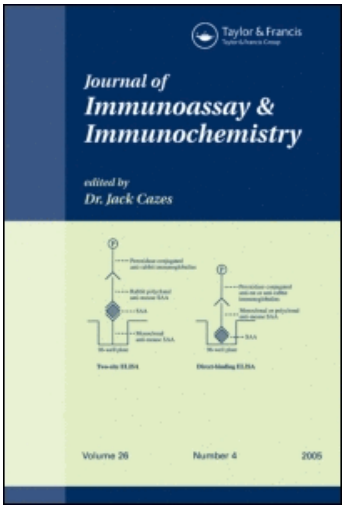


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L. A. Tortajada-Genaro[a]; C. Redal[b]
[a] Departamento Química, Universidad Politécnica Valencia, Valencia, Spain [b] Servicio de Medicina Nuclear, Hospital Clínico de Valencia, Valencia, Spain

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# Selection of Calibration Standard Concentrations for Determination of Intact-PTH by Immunoradiometric Assay

**L. A. Tortajada-Genaro[1] and C. Redal[2]**
[1]Departamento Química, Universidad Politécnica Valencia, Valencia, Spain
[2]Servicio de Medicina Nuclear, Hospital Clínico de Valencia, Valencia, Spain



**Abstract:** The routine determination of parathyroid hormone (PTH) by immunoradiometric assay (IRMA) has been studied. Concentrations of standards have been adequate to the clinical range in order to reduce errors. Proposed standards were tested by the calculation of different quality parameters. Recoveries of sample concentrations were estimated for different experimental alterations (methodological errors, reagent degradation, or changes in background response). Finally, inter-assays demonstrated that reproducibility of samples with concentrations in the critical clinical limits was improved. The results confirmed that the proposed selection provided a more robust method and it is possible to extrapolate to other clinical immunoassays.

**Keywords:** Calibration, Immunoradiometric assay, IRMA, Parathyroid hormone, PTH

## INTRODUCTION

Parathyroid hormone (PTH) is a linear polypeptide of 84 amino acids and a regulator of calcium homeostasis and other biological processes. Then, measurement of PTH levels in blood is prescribed to explore any disorder of calcium metabolism and in patients with a chronic renal

Address correspondence to Luis Antonio Tortajada Genaro, Departamento de Química, Universidad Politécnica Valencia, Camí de Vera s/n, 6022 Valencia, Spain. E-mail: luitorge@qim.upv.es

failure. The clinical applications are the diagnosis of hyperparathyroidism, hypoparathyroidism, and renal diseases.[1–3] For that, methods based on the immunoradiometric assay (IRMA) have been described for the routine detection of serum PTH.[3,4]

A precise determination of PTH has been difficult because of the heterogeneity of the forms present in blood (intact and truncated forms). The interference of PTH fragments by cross-reactivity has led to the development of new IRMA assays according to interaction region.[5] So, specificity for the complete hormone, that is the biologically active form, has been achieved.[6–9] For some assays, a polyclonal antibody coated to a plastic bead is directed against the 39–84 portion of the PTH molecule, whereas the 125-iodine labelled antibody recognises mainly the 13–24 portion of the PTH molecule.

Although the PTH analysis is routinely performed, the standardisation of commercial assays is still an outstanding matter.[9] Since PTH assays are usually calibrated against synthetic 1–84 PTH instead of a reference material, some problems can be described as matrix effect[10] or instability over extended storage periods.[11] Other methodological considerations are the result of the immunoreaction kinetic as influenced by some experimental conditions, i.e., ionic strength and temperature.[12]

Additionally, the relationship between radioactive response and intact-PTH concentration is non-linear. For this, the calibration step is especially critical and the selection of standard solutions is a key point.

In most commercial kits, calibration concentrations are selected in order to obtain the same distance between consecutive standards in the double logarithm calibration curve. This distribution achieves a regression model with homogenous confidence intervals for a wide range of concentrations (0.7–2,000 μg/L) useful in research studies. However, this working range of concentrations disagrees with the critical clinical values (<400 μg/L) presented in routine analysis.[8,13–16]

For other methods, it has been demonstrated that a non-optimal selection of the regression model provides bad quality coefficients.[17] In fact, if the unknown samples are concentrated in a narrow interval of concentrations, the interpolation is improved by selecting an appropriated distribution of standard concentrations.[18] For this, we propose an alternative procedure based on recalculating standard concentrations to increase the similarity between standards and critical samples. In the determination of a clinical analyte, such as intact-PTH, the best prediction should be close to cut-off values (normal-disease).[9] The objective is to check the performance of this statistically based methodology, evaluating advantages and limitations for the routine determination in a clinical laboratory. Moreover, the results of this study can be extrapolated to other clinical immunoassays in order to reduce calibration errors in similar routine determinations.

## EXPERIMENTAL

### Patients

Samples of 1,100 patients were collected and sent to the Radioimmunanalysis Laboratory of Hospital Clínico of Valencia. The patients (559 females and 541 males) were between 16 and 91 years of age (median 68 years).

The blood samples were aseptically collected and aliquots were frozen (–20°C) after centrifugation (15 minutes, 760 g). In order to avoid degradation, storage time ranged between 10–15 days obtaining 30 analysis series. Control samples were prepared from different vials containing human serum with PTH 1–84 (Diasorin, Italy).

A subset of serum samples (n = 40) was selected for evaluating the influence of the working interval in the critical region. The patients were classified according to PTH level (0.7–55 μg/L and 55–100 μg/L) and a dilution protocol (1:2) was performed. Different sets of calibration solutions were prepared: 0–200, 0–280, 0–400, 0–550, 0–800, 0–1100, 0–1600, and 0–2200 μg/L. The prediction error, considering the dilution factor between samples and diluted samples, was calculated as a function of the working interval of each calibration curve.

The influence of experimental conditions on prediction abilities of models was studied with a subset of serum samples (randomly chosen) and controls. For this, the reference concentration of PTH was established as the average of the concentrations obtained from five determinations using different sets of immunoreagents and measured by two instruments.

A selection of patients was chosen for the inter- and intra-assay variability study. Retrospectively, patients have been followed up for 8 to 48 months. The sample S1 corresponds to normal patient with normal intact-PTH and calcium levels. The sample S2 is from a patient with chronic renal failure not drafted rejected hemodialysis. The sample S3 is from a patient with a chronic secondary renal failure in situation of pre-dialysis. The sample S4 was obtained from a patient with a clinical history of renal failure with conservative treatment (non dialysis). Table 1 summarises the average concentrations of intact-PTH and calcium obtained from previous analysis during the treatment period.

### Methods

For the semiautomatic procedure, a shaker (bioMerieux, France), a washing system (Nichols Institute Diagnostics, USA), and a gamma counter LKB (Wallac, Finland) were employed. For the automatic procedure, a Stratec SR300 (Birkenfeld, Germany) that incorporates a

**Table 1.** Retrospective concentrations of calcium and intact-PTH of selected patients

| Patient | Number of analyses | Calcium (mg/dL)[a] | Intact-PTH (μg/L)[b] | |
|---|---|---|---|---|
| S1 | 3 | $9.3 \pm 0.3$ | $26 \pm 4$ | [23,28] |
| S2 | 6 | $9.4 \pm 0.6$ | $35 \pm 10$ | [20,49] |
| S3 | 5 | $8.7 \pm 0.4$ | $121 \pm 25$ | [81,149] |
| S4 | 5 | $9.6 \pm 0.3$ | $279 \pm 103$ | [160,411] |

[a]Mean concentration ± standard deviation.
[b]Mean concentration ± standard deviation [minimal value, maximum value].

pipetting module for automatic addition of tracer reagent, standards, controls and samples, shaking, and incubating module, a washing module, and a gamma counter was used.



### Procedure

The commercial kit N-tact® PTH SP (Diasorin), with two different polyclonal antibodies, was employed. The first antibody, specific for PTH 39–84, is bound to a solid phase (polystyrene beads). The second antibody is specific for PTH 1–34 and is labelled with iodine-125.

Each vial of lyophilised human PTH 1–84 was reconstituted with human serum and 0.1% sodium azide. Proposed standards were prepared by dilution with the same reagent.

In borosilicate glass tubes, one polystyrene bead with the first antibody, 200 μL of standards, or sample and 100 μL of $^{125}$I-labelled antibody were added. Following the incubation period (22 hours), each bead was washed (3 cycles) to remove any unbound-labelled antibody. Finally, the radioactivity was measured using a gamma counter.

### Statistical Analysis

The calibration curve obtained for each series of analyses was calculated as the double-logarithm relationship between counts-per-minute (cpm) corrected by background signal and the concentration of standard. Statistical tests were calculated for a 95% confidence level.

## RESULTS AND DISCUSSION

Serum samples of 1,100 patients were analysed and the patient histogram was calculated (Figure 1). The range of PTH values was from detection

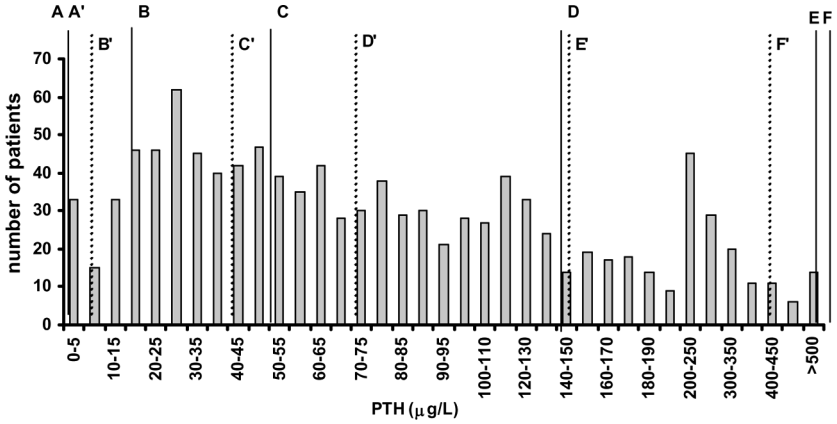



**Figure 1.** Histogram of levels of parathyroid hormone measured in serum (1100 patients). A–F concentrations of commercial standards, A'–F': concentrations of proposed standards.



limit 0.7 µg/L to 1,200 µg/L with a median 67 µg/L. This histogram was representative of a population of patients with PTH related diseases and comparable to others registered.[19] The interval of normal intact parathyroid hormone in serum was defined as 10–55 µg/L. In this study, the percentage of patients with concentrations near to inferior level of normality was 8.7% and near to the higher level was 11.2%. These values indicated the clinical importance of the accurate and precise determination of intact-PTH.

The distribution of patient values was compared to commercial interval of standard concentrations, see Figure 1 (A–F standards). This distribution of standards divided the working range into concentration intervals and Figure 2 represents the percentage of clinical samples included for each interval. The distribution was heterogeneous because of the normal and "gray zone" intervals (70% of the patients) which ranged between only three standards (labelled by B, C and D). It made necessary the optimisation of standard concentrations to match those clinically presented by patients.

For establishing the influence of the calibration interval on the prediction, patients with normal and high values were selected (n = 40). Serum samples and diluted samples were interpolated on calibration curves obtained from sets of PTH standards with different working intervals. Figure 3 shows that the prediction error of samples decreased if the working interval of calibration curve was closer to the critical region. These results confirmed the importance of an adequate calibration interval on the prediction in the region with high clinical significance.

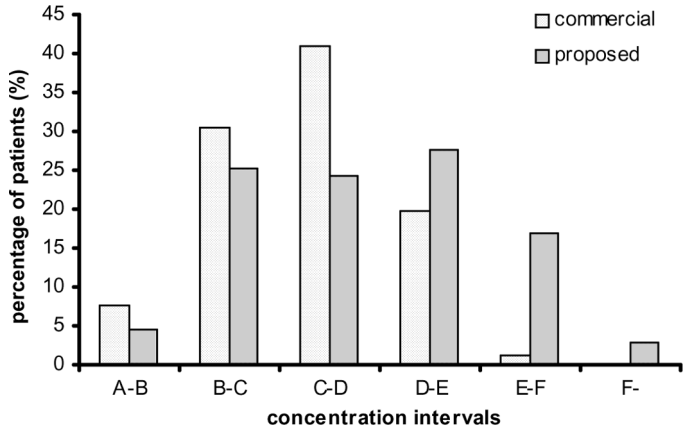



**Figure 2.** Distribution of measured PTH values for the concentration intervals defined by commercial standards and proposed standards.



In the selected the working interval (0.7–400 μg/L), the distribution of standard concentration was optimised. For this, standard concentrations were calculated from the patient frequencies and general assumptions of the non-linear regression model based on a homogeneous

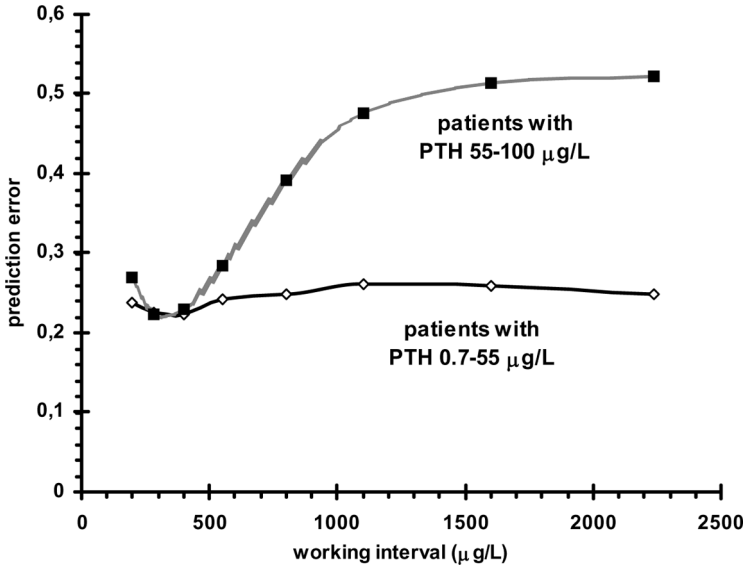



**Figure 3.** Prediction error of serum samples (n = 40) for different working intervals of calibration curve. Abscissa indicates the upper limit of working interval.

distribution (A'–F' standards). So, standard concentrations were the quartile values of the highly significant intact-PTH levels and two extreme values (see Figures 1 and 2). This selection, with progressive difference of consecutive standards, provides a homogeneous prediction error in a double logarithm calibration curve. Moreover, the values with higher clinical significance ranged between four standards (labelled by B', C', D', and E').

A disadvantage of the proposal is a reduction of the applicability interval (0.7–400 μg/L) compared to the commercial kit (0.7–2,000 μg/L), because there are some clinical samples with intact-PTH concentrations higher than 400 μg/L. However, the number of these serum samples is low (3%) and, in these cases, a previous dilution with human serum is recommended. Even the determination by extrapolation (or reporting a >400 μg/L value) is also possible, because the clinical significance is similar. In fact, official guidelines for chronic renal failure recommended maintaining intact-PTH in a dialysed patient at a concentration ranging between 150 and 300 μg/L.[9] Therefore, the specificity of this IRMA method remains unaltered using the proposed distribution.



The performance of the proposed methodology was evaluated by calculating quality parameters and the influence of experimental conditions in the calibration step. For this, the prediction capability, i.e., residuals between an estimated value and a real value, was selected as a statistical quality parameter.

Immunoassays are subjected to different errors arising from the instability of reagents, manipulation, alterations of the conditions of immunological reaction, or counting measurement. Several variations can also affect the background signal as errors due to pipetting, mixing, irregular yield of immune reaction, or incomplete separation of the bound and free fractions. These factors can introduce inaccuracy by modifying calibration curve and/or sample interpolation. Therefore, the evaluation of method robustness due to the presence of experimental errors was included in the list of checked parameters. Their influence was evaluated by obtaining the percentage of error tolerated without introducing a significant variation in the concentration.

A set of representative patient samples was randomly selected, based on clinical values distributed along the working interval. Sample concentrations were calculated by interpolation (commercial and proposed standards) under the following conditions.

A first simulation was performed, including different percentages of random error in the radioactive signal (counts-per-minute, cpm) of standards (0–20%). Calibration curves were calculated and the radioactive measurements of samples were interpolated. The prediction error calculated for the different conditions was lower for the proposed distribution (see Table 2).

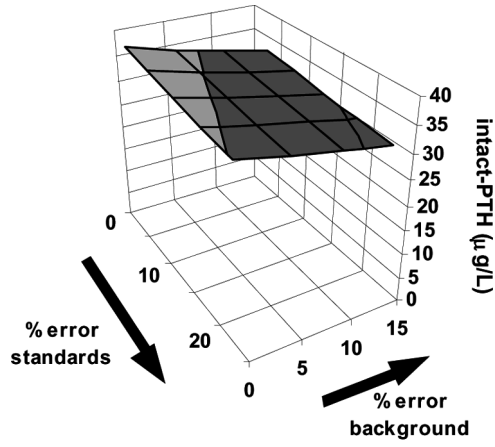
**Table 2.** Values of prediction errors obtained in the PTH measurement of clinical samples using different calibration curves

| | Error in standard curve (%) | | | | |
|---|---|---|---|---|---|
| | 0% | 5% | 10% | 15% | 20% |
| Commercial | 4.02 | 5.31 | 6.55 | 7.76 | 8.93 |
| Proposed | 3.79 | 5.08 | 6.33 | 7.54 | 8.72 |

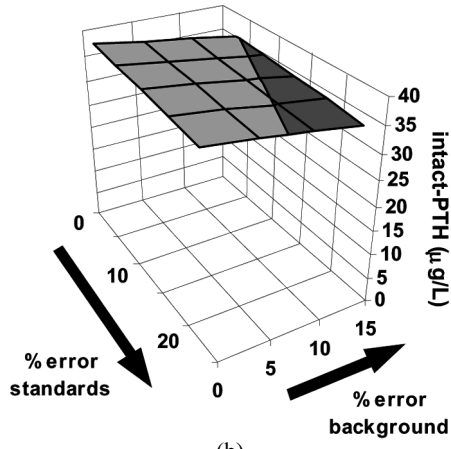
A second simulation was performed, including different percentages of random errors in the radioactive signal of standards (0–20%) and background signal (0–15%). Calibration curves were calculated and the radioactive measurements of a control sample were interpolated. Figure 4 shows the predicted concentration of sample for the different conditions. The area with high error (>10%) with respect to reference concentration of PTH was broader for the commercial distribution.

In order to get a high performance of laboratory conditions, the reduction of these error sources is critical, especially in samples with similar concentrations than cut-off values of PTH normal levels. Replicates of control samples (25–45 μg/L) were analysed in different days ($n = 10$) and compared to specified limits. A value outside of limits was observed for concentrations calculated using commercial distribution (1/10) with respect to the proposed distribution (0/10). According to control quality protocols, this difference indicates an error in the assay and involves a repetition of the analysis. The problems are consumption of serum sample, changes in programmed tasks, economical cost of kits and staff, losses of time and increasing of biological and radioactive waste.

Analysis reproducibility of selected patients is another interesting quality parameter. A lower dispersion of laboratory results can improve the discrimination and the monitoring of patients, since the concentration variations could be related to changes in the patient status. The concentrations of serum samples of selected patients (see Table 1) was determined using different assays ($n = 6$). Figure 5 shows a boxplot of concentrations using both distributions of standards. Statistical tests were applied in order to verify the differences observed in the representation. A T-test of paired samples confirmed differences between averages between both strategies ($t_{calc} = 3.4$). An F-test for comparison of variances reported that the intra-assay variability was similar, but statistically significative differences were obtained for inter-day determinations. Therefore, the use of the proposed calibration curve also reduced the inter-assay variability in 26%.




(a)




(b)



**Figure 4.** Concentration of PTH (control sample) for different error percentage in the signal of standards and background signal: (a) commercial calibration, (b) proposed calibration. The dark region indicates a measured error of PTH concentration higher to 10%.

These results have an important relevance for the diagnosis of some disorders. For instance, recent studies have demonstrated that this diagnosis may be evoked in a hypercalcemic patient whose PTH value is close to upper limit of the normal range.[9,20] Then, determining serum concentrations with better precision can contribute to increase the diagnostic sensitivity of primary hyperparathyroism, the third most common endocrinopathology.

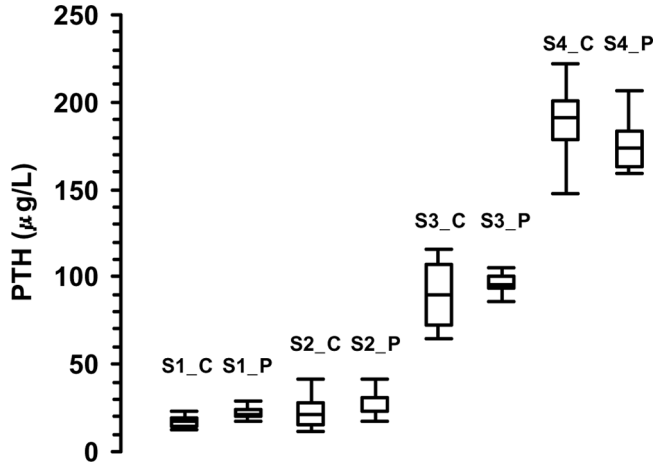



**Figure 5.** Boxplot of estimated concentrations for serum samples: commercial calibration (C) and proposed calibration (P).



## CONCLUSIONS

The most important contribution of this study is the adaptation of standard concentrations to intact-PTH levels clinically presented by patients. The importance of this variation in setting the standards has been demonstrated. Routine analysis of serum samples is improved by obtaining more accurate and precise results and fewer repetitions of assays due to out-range of control samples. This optimised distribution provides better regression models and improves the reliability of analytical results, especially for the samples containing intact-PTH in the critical clinical limits. Then, applications in the diagnosis and monitoring of hyperparathyroidism, hypoparathyroidism, and renal diseases could be benefited.

These results with parathyroid hormone can be extrapolated to determination of other clinical analytes by immunoassays (IRMA, ELISA, . . .). For this, a strategy has been proposed in order to ease that each laboratory check the suitability of standards and how to evaluate the quality of predictions using a systematic analytical and statistical methodology. The first step is to compare the histogram of analyte to the distribution of standards. If it is necessary, the new standard concentrations are selected based on a homogeneous distribution, non-linear response and cut-off value criteria. For checking the improvement, prediction errors and method robustness have to be calculated considering experimental errors, control samples, and representative samples. Finally, intra- and inter-assays allow the evaluation of reproducibility of analyte determinations in clinical samples.

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