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Parathormone stability in hemodialyzed patients and healthy subjects: comparison on non-centrifuged EDTA and serum samples with second- and third-generation assays

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Abstract

Background: Parathyroid hormone (PTH) stability is important. Many studies have shown divergent results between EDTA and serum, which are mainly linked to differences in protocols or cut-offs used to determine whether or not PTH remained stable. No studies have yet compared PTH stability as measured by second- and third-generation assays on the same samples in hemodialyzed patients and healthy subjects.

Methods: Five pairs of samples (EDTA and gel tubes) were obtained in 10 hemodialyzed patients before a dialysis session and in 10 healthy subjects. One pair was centrifuged and run directly to define the "T0". Two pairs were kept at +4 °C and two pairs were kept at +25 °C. They were centrifuged after 4 and 18 h. Supernatant was kept at −80 °C for 1 week. All samples were measured in a single batch, on Roche Cobas and DiaSorin XL second- and third-generation PTH assays. We used three different approaches to evaluate PTH stability: Wilcoxon test, an Acceptable Change Limit (ACL) according to ISO Guide 5725-6 and a Total Change Limit (TCL) derived from the sum of biological and technical variability according to WHO.

Results: PTH decreased in all samples. Stability of PTH was mainly dependent on the way it was evaluated. Percentages of decrease were systematically lower in EDTA vs. serum. Wilcoxon and ACL showed that PTH was no more stable after 4 h at +4 °C in EDTA or serum gel tubes. None of the subjects presented a PTH decrease higher than the TCL with EDTA plasma. In serum gel tubes, PTH was unstable only when kept at 25 °C for 18 h.

Conclusions: PTH seems more stable in EDTA than in serum gel tubes but only when samples have to stay unprocessed for a long period (18 h) at room temperature (25 °C), which can happen when samples are delivered from external care centers. For all the other conditions, using serum gel tubes is recommended since calcium measurement, which is necessary for a good PTH results interpretation, can be achieved on the same tube.

Keywords: biological variation; parathyroid hormone; stability.

Introduction

Parathyroid hormone (PTH) is a major player in phosphocalcic metabolism, and its measurement is essential for the correct diagnosis and treatment of several diseases such as primary or secondary hyperparathyroidism and for the evaluation of chronic kidney disease-mineral and bone disorders (CKD-MBD) [1]. The pre-analytical phase is unfortunately a major issue for a correct evaluation of PTH levels. Indeed, PTH is quite unstable. Hence, many papers and a recent systematic review [2] have been published about PTH stability in EDTA and gel serum samples. Unfortunately, the results of these studies are discrepant, and the best sample to use for PTH determination, as well as sample handling and storage conditions, remains controversial [3]. These discrepancies may be explained by the variability of the pre- and intra-analytical process used in all the different studies on PTH stability. For example, the different populations studied, either healthy subjects or

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hemodialyzed patients [4–9], the various assays used for PTH determination [4–17], and the low number of samples analyzed in many studies may importantly impact the results. Moreover, PTH stability is not always studied in both EDTA and serum gel tubes in parallel [7, 9, 12, 16] and some studies have also evaluated PTH stability on samples that had directly been centrifuged and not on unprocessed samples like in "real life" [6, 8, 11, 14, 18, 19].

The cornerstone for PTH stability remains however, its definition. First, the definition of "T0" (the initial time-point for which PTH value is considered as the "true" value, before degradation) is not always the same, and second, PTH's stability cut-off is not homogenous. According to ISO Guide 30:2015, *"stability is the ability of a sample to maintain the initially measured value, within specified limits, of a constituent over a period of time under specified storage conditions. The instability is given as an absolute difference, as a quotient or as a percentage deviation of results obtained from measurements at initial time (T0) and after a given period of time (Tx)"* [20]. Regarding the "T0" some studies have used, as comparator, a sample that had been immediately frozen [7–9, 12–17, 19] and not an unfrozen sample, immediately assayed after centrifugation [4, 5, 11, 18, 21]. Concerning the the PTH's stability cut-off, a purely statistical approach is performed by most of the studies [5, 6, 9, 11–13, 15–17, 19]. However, this is unsatisfactory because a variation that may be significant from a statistical point of view may not be clinically relevant.

Taking all this information into consideration, deciding on PTH stability and the best type of sample to use for PTH determination is difficult. In this perspective, we aimed to conduct a study to evaluate the effect of the sample type and the effects of different storage temperatures and storage times on the PTH levels of samples from healthy subjects and hemodialyzed patients that were kept unprocessed with second- and third-generation PTH assays. As "T0" point, we chose an unfrozen sample, immediately assayed after centrifugation, and for monitoring changes in concentrations due to the pre-analytical instability of PTH, we used three different approaches described in the literature [22]. For that purpose, we established in our own population, the intra-individual variation of PTH when measured with second- and third-generation assays, in healthy subjects.

Materials and methods

Samples

Blood samples, EDTA, and serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA) were obtained from 10 healthy laboratory staff members, with age ranging from 20 to 60 years and from 10 end-stage renal disease patients undergoing hemodialysis three times a week (hemodialyzed patients). All the subjects and patients signed informed consent, and the study was approved by the Ethics Committee of the CHU de Liege.

Pre-analytical handling of samples

All the blood samples were collected in the morning (between 8 and 9 a.m.) after an 8-h fast and before the dialysis session for the hemodialyzed patients. The blood was drawn at the Blood Collection Center and at the Haemodialysis Service of our hospital.

Five pairs of samples (EDTA and gel tubes) were obtained from each subject and were all completely filled. Immediately after the blood collection, they were sent to the clinical chemistry laboratory, where EDTA and gel tubes of each subject were processed differently as described in Table 1. After 4 and 18 h of storage time at +4 °C and +25 °C, the samples were centrifuged and the supernatant was kept at −80 °C for 1 week.

PTH assays

PTH was measured on two automated platforms, Roche Cobas and DiaSorin Liaison XL, with the second- and third-generation PTH assays in our ISO 15189 compliant laboratory. The inter-assay coefficients of variation (CVa) range from 2.8% to 4.2% and from 4.1% to 5.5% on EDTA samples according to DiaSorin for the second- and third-generation PTH assays on Liaison XL, respectively. They are < 2% on controls and range from 3.1% to 9.4% on serum for the second- and third-generation assays according to Roche on Cobas E411, respectively.

All samples were measured in duplicate, in a single batch, on each instrument. One pair of samples was, however, run directly after centrifugation and immediately frozen at −80 °C after determination to be run in batch with the other pairs.

Table 1: Sample processing and storage conditions.

Tube type	First pair		Second pair		Third pair		Fourth pair		Fifth pair	
	EDTA	Gel tube	EDTA	Gel tube	EDTA	Gel tube	EDTA	Gel tube	EDTA	Gel tube
Storage condition	Centrifuged directly and immediately run: T0		Kept at +4 °C – 4 h		Kept at +4 °C – 18 h		Kept at +25 °C – 4 h		Kept at +25 °C – 18 h	
	Centrifugation → supernatant kept at −80 °C/1 week → run in batch									

Statistics

Basically, there are three approaches to define PTH's stability cut-off.

The first one is the purely statistical approach in which a Wilcoxon test is performed to see if there is a difference before/after storage.

The second one compares the mean percentage deviation with an Acceptable Change Limit (ACL) according to ISO Guide 5725-6 [23]. The calculation of the ACL is based on the CVa of the methods calculated with an ANOVA1 on the duplicates and is expressed as $ACL = 2.77 \times CVa$. The factor 2.77 is derived from $Z \times \sqrt{2}$, where Z is the statistical factor with a value of 1.96 that corresponds to the 95% probability of a bi-directional change, and $\sqrt{2}$ is used because two samples are used. A mean percentage deviation > $2.77 \times CVa$ thus represents a probable difference in analyte concentration between two measurements, with a probability of 95%.

The third one is the "biological" approach that defines, according to the World Health Organization document "*Use of anticoagulants in diagnostic laboratory investigations and stability of blood, plasma and serum samples*," the "maximum permissible instability" based on the total error derived from the sum of biological and technical variability [22]. This approach is interesting because a statistically significant change is not always clinically relevant. Hence, we decided to choose a standardized criterion, the Total Change Limit (TCL), defined by Oddoze et al. [4], which takes CVa and intra-individual coefficient of variation (CVi) coefficients of correlation into consideration.

$$TCL = \sqrt{(1.96 \times \sqrt{2} \times CVa)^2 + (0.5 \times CVi)^2}$$

The different CVas for each PTH generation, method, population, and sample used were calculated from the duplicates. The CVi was established in our healthy population (see below), whereas we used the CVi for hemodialyzed patients published by Gardham et al. [24].

Establishment of the intra-individual coefficient of variation of PTH in healthy subjects

We established the CVi of PTH in a population of 22 healthy volunteers patients that gave informed consent to participate. Blood samples were obtained at 08:00 h and 12:00 h on the Mondays, Wednesdays and Fridays of two consecutive weeks. They were fasting at 08h00 and received the same standardized breakfast after the sampling. They were allowed to drink 500 mL of poorly mineralized water and were not allowed to eat until the second sampling. They were asked not to change their habits during the 2 weeks of the study. PTH determination was performed in duplicate with the Roche Cobas second-generation assay and with the DiaSorin Liaison third-generation PTH assays. The CVi was calculated according to Fraser and Harris [25].

Results

At T0, no difference was observed between EDTA and serum gels tubes for any of the methods ($p < 0.05$). There was neither any difference between the samples processed and run immediately after centrifugation and the T0 samples kept 1 week at – 80 °C either for EDTA or serum gel tubes, with any of the methods. The medians (IQR) obtained at all the time points are presented in Table 2.

The mean percentages of decrease, according to the method, patient, and sample type are presented in Table 3. In serum gel tubes, there was no significant difference between percentages of degradation observed in healthy subjects vs. hemodialyzed patients. On the contrary, in tubes containing EDTA, the percentages of decrease were significantly ($p < 0.05$) higher in hemodialyzed patients when compared to healthy individuals with both DiaSorin assays, but not with Roche assays. When we compared the percentages of degradation in the different tubes at the same time points, there was no difference between serum and EDTA tubes when samples were kept at + 4 °C, whereas keeping samples at + 25 °C induced a significantly higher degradation of PTH in healthy individuals already after 4 h of storage but only after 18 h of storage in hemodialyzed patients.

The CVa of all the methods, calculated on the duplicates, the CVi calculated on our healthy population with the third- and second-generation PTH assays according to Fraser and Harris [25], the CVi obtained by Gardham et al. [24] in hemodialyzed patients with the second- and third-generation PTH assays, the ACL and the TCL are presented in Table 4.

The Wilcoxon test showed that, compared to baseline, PTH concentrations decreased significantly ($p < 0.05$) in all samples and with all methods in hemodialyzed patients over time. The same observation was also true for healthy subjects except for Liaison third-generation on serum samples kept 4 h at + 4 °C, for Liaison third-generation and Cobas second-generation on EDTA samples kept 4 h at room temperature, and for Cobas third-generation on EDTA samples kept 4 h at + 4 °C, which did not show a significant decrease compared to T0.

Using the ACL limit, PTH in serum gel tubes was stable in healthy subjects for only 4 h at + 4 °C with second- and third-generation assays form DiaSorin and second-generation assay from Roche; otherwise, it was considered as unstable. In hemodialyzed patients, PTH was only stable for 4 h at + 4 °C with third-generation PTH assays from DiaSorin and Roche. In EDTA whole blood, PTH was considered as stable in healthy subjects whatever the storage conditions with second-generation assay from Roche, whereas it was stable 4 h, at + 4 °C or + 25 °C, with the other methods. In hemodialyzed patients, PTH was considered as stable whatever the storage conditions

Table 2: Medians (pg/mL) and interquartile range (pg/mL) of PTH according to the subjects (healthy or hemodialyzed), the sample type (serum or EDTA tubes), the method used (second- and third-generation PTH on Liaison XL and Cobas) and the storage conditions (4 h at +4 °C and +25 °C and 18 h at +4 °C and +25 °C).

	Healthy subjects				Hemodialyzed patients			
	4 h, +4 °C	4 h, +25 °C	18 h, +4 °C	18 h, +25 °C	4 h, +4 °C	4 h, +25 °C	18 h, +4 °C	18 h, +25 °C
Serum gel tubes								
Liaison XL third-generation	25.9 (21.6–29.5)	22.4 (19.9–26.9)	23.9 (21.7–28.8)	18.7 (16.8–22.3)	183.0 (58.3–222.0)	163.8 (51.0–209.5)	175.5 (57.9–219.0)	138.0 (43.5–180.5)
Cobas third-generation	37.6 (33.9–45.5)	35.5 (34.1–42.1)	35.2 (32.0–44.7)	30.8 (28.4–38.1)	203.5 (78.0–255.2)	192.4 (69.0–240.2)	201.5 (76.2–254.6)	171.7 (59.8–213.9)
Liaison XL second-generation	49.0 (41.2–57.8)	45.2 (38.8–52.7)	45.6 (40.0–58.7)	35.7 (32.3–40.7)	514.5 (140.0–683.5)	474.0 (134.0–652.0)	489.8 (135.0–648.0)	405.5 (107.5–556.5)
Cobas second-generation	48.1 (43.8–59.3)	45.3 (42.0–54.6)	45.3 (42.6–57.6)	38.3 (35.7–48.3)	347.6 (130.6–490.2)	335.8 (126.6–462.7)	330.6 (129.5–482.4)	295.9 (107.1–405.1)
EDTA tubes								
Liaison XL third-generation	25.5 (23.8–30.1)	25.6 (23.8–30.2)	25.5 (22.7–29.3)	25.8 (24.1–30.3)	178.5 (60.5–225.5)	168.5 (60.7–224.0)	180.5 (61.9–216.5)	182.3 (61.3–213.0)
Cobas third-generation	41.9 (39.0–50.0)	42.2 (39.6–49.8)	41.4 (39.2–49.0)	41.7 (38.2–49.1)	207.5 (82.2–256.4)	200.5 (82.2–253.3)	204.5 (81.5–246.2)	207.0 (82.5–247.0)
Liaison XL second-generation	49.2 (43.5–61.7)	49.3 (45.0–62.5)	48.9 (41.4–59.5)	48.1 (44.2–60.1)	476.5 (140.0–654.0)	472.0 (143.0–654.0)	471.8 (138.5–618.5)	495.0 (148.0–625.0)
Cobas second-generation	51.1 (46.4–61.6)	50.2 (47.5–61.8)	50.4 (45.8–59.6)	50.3 (46.7–61.5)	335.1 (128.2–481.2)	336.5 (131.1–471.1)	328.2 (131.1–465.2)	332.8 (130.8–460.4)

Table 3: Percentages of degradation of PTH compared to the baseline according to the subjects (healthy or hemodialyzed), the sample type (serum or EDTA tubes) and the storage conditions (4 h at +4 °C and +25 °C and 18 h at +4 °C and +25 °C).

	Healthy subjects				Hemodialyzed patients			
	4 h, +4 °C	4 h, +25 °C	18 h +4 °C	18 h, +25 °C	4 h, +4 °C	4 h, +25 °C	18 h, +4 °C	18 h, +25 °C
Serum gel tubes								
Liaison XL third-generation	3.3 ± 6.1$^{W, ACL, TCL}$	11.8 ± 5.0$^{W, acl, TCL}$	7.4 ± 7.3$^{W, acl, TCL}$	28.3 ± 6.1$^{W, acl, tcl}$	7.0 ± 3.2$^{W, acl, TCL}$	13.7 ± 4.4$^{W, acl, TCL}$	9.3 ± 3.0$^{W, acl, TCL}$	27.5 ± 6.8$^{W, acl, tcl}$
Cobas third-generation	7.0 ± 4.6$^{W, ACL, TCL}$	10.8 ± 3.7$^{W, acl, TCL}$	10.7 ± 8.4$^{W, acl, TCL}$	23.3 ± 3.9$^{W, acl, tcl}$	5.7 ± 3.6$^{W, acl, TCL}$	10.4 ± 4.4$^{W, acl, TCL}$	7.1 ± 3.2$^{W, acl, TCL}$	22.9 ± 6.2$^{W, acl, tcl}$
Liaison XL second-generation	4.3 ± 6.4$^{W, ACL, TCL}$	11.1 ± 4.9$^{W, acl, TCL}$	9.1 ± 7.8$^{W, acl, TCL}$	29.5 ± 5.0$^{W, acl, tcl}$	5.7 ± 4.3$^{W, acl, TCL}$	11.6 ± 6.0$^{W, acl, TCL}$	8.5 ± 4.1$^{W, acl, TCL}$	26.3 ± 8.2$^{W, acl, tcl}$
Cobas second-generation	3.5 ± 5.7$^{W, acl, TCL}$	8.4 ± 4.4$^{W, acl, TCL}$	7.1 ± 6.8$^{W, acl, TCL}$	23.2 ± 4.0$^{W, acl, tcl}$	2.2 ± 5.4$^{W, acl, TCL}$	7.3 ± 5.5$^{W, acl, TCL}$	4.7 ± 5.2$^{W, acl, TCL}$	19.6 ± 7.2$^{W, acl, tcl}$
EDTA tubes								
Liaison XL third-generation	3.2 ± 3.9$^{W, ACL, TCL}$	3.2 ± 4.4$^{W, ACL, TCL}$	5.1 ± 5.6$^{W, acl, TCL}$	4.4 ± 4.1$^{W, acl, TCL}$	8.0 ± 3.1$^{W, acl, TCL}$	9.0 ± 3.9$^{W, acl, TCL}$	8.4 ± 2.6$^{W, acl, TCL}$	8.5 ± 2.3$^{W, acl, TCL}$
Cobas third-generation	3.1 ± 4.6$^{W, ACL, TCL}$	3.7 ± 3.4$^{W, ACL, TCL}$	4.4 ± 4.9$^{W, acl, TCL}$	5.8 ± 2.3$^{W, acl, TCL}$	5.4 ± 4.8$^{W, ACL, TCL}$	5.8 ± 2.7$^{W, ACL, TCL}$	6.2 ± 4.5$^{W, ACL, TCL}$	6.5 ± 2.8$^{W, ACL, TCL}$
Liaison XL second-generation	3.5 ± 4.3$^{W, ACL, TCL}$	3.4 ± 3.5$^{W, ACL, TCL}$	6.1 ± 5.5$^{W, acl, TCL}$	4.6 ± 3.8$^{W, acl, TCL}$	10.0 ± 4.6$^{W, acl, TCL}$	11.2 ± 4.9$^{W, acl, TCL}$	11.1 ± 5.0$^{W, acl, TCL}$	10.3 ± 4.0$^{W, acl, TCL}$
Cobas second-generation	2.0 ± 2.9$^{W, ACL, TCL}$	1.3 ± 3.2$^{W, ACL, TCL}$	3.1 ± 4.2$^{W, ACL, TCL}$	3.4 ± 2.3$^{W, ACL, TCL}$	4.1 ± 3.5$^{W, ACL, TCL}$	4.8 ± 3.8$^{W, acl, TCL}$	4.9 ± 4.5$^{W, acl, TCL}$	5.5 ± 3.2$^{W, acl, TCL}$

w, significant ($p < 0.05$) decrease observed with the Wilcoxon test, compared to T0; W, no significant decrease observed with the Wilcoxon test; acl, decrease higher than the Acceptbale Change Limit compared to T0; ACL, decrease lower than the Acceptable Change Limit compared to T0; tcl, decrease higher than the Total Change Limit compared to T0; TCL, decrease lower than the Total Change Limit compared to T0.

with Roche third-generation assay and 4 h at +4 °C with Roche second-generation assay. The other storage conditions did lead to a percentage of decrease higher than the ACL.

The CVi were found to be 19% for Roche Cobas second-generation PTH and 24% for DiaSorin third-generation PTH assays. With these data, we calculated the TCL, and taking the results in consideration, PTH in serum gel tubes was considered as stable except when samples were kept for 18 h at +25 °C, either in healthy subjects or hemodialyzed patients. In whole EDTA blood, PTH was stable whatever the storage conditions, in healthy subjects or hemodialyzed patients.

Discussion

PTH stability is of paramount importance for correct interpretation of results, and many papers have been published on this topic. The best sample (EDTA or gel tubes) to use for its determination, as well as sample handling and storage, remains controversial. The main advantages of using EDTA tubes are that clotting time before centrifugation is bypassed, thus reducing the turnaround time, and that the stability of the peptide may be increased due to inactivation of metalloproteases by chelation of divalent ions [9]. Another explanation for the higher apparent stability in EDTA is that the clotting process releases thrombin in serum which, in turn, can cleave the peptide between the Arg in position 44 and the Asp in position 45, making it invisible to the antibodies used in immunoassays [26]. This has also recently been shown with bovine thrombin contained in Rapid Separator Tubes (RST) from Becton Dickinson [27]. The major advantage of using serum over EDTA plasma relates to the fact that clinical interpretation of PTH concentration must be performed together with calcium concentrations. Since calcium cannot be determined in EDTA plasma, another (serum) sample would be needed if EDTA was used.

As a consequence, many studies have been published on PTH stability in EDTA and gel serum samples at different temperatures and times of storage. Unfortunately, the results of the different studies on the topic are quite contradictory. Indeed, as an example, in the papers published by Morales García et al. [12], and Jane Ellis [7], it was showed that using EDTA tubes could maintain PTH stability during a longer period without the necessity of freezing the samples immediately (even if there was no serum "control group" in these studies). On the contrary, the results published by Joly et al. [14] led the authors to recommend serum over EDTA (and citrate) plasma, while

Table 4: Intra-assay coefficient of variation (CVa), intra-individual coefficient of variation (CVi), Acceptable Change Limit (ACL) and Total Change Limit (TCL) obtained in serum gel and EDTA tubes, in healthy and hemodialyzed patients.

	Healthy subjects				Hemodialyzed patients			
	CVa (%)	CVi (%)	ACL (%)	TCL (%)	CVa (%)	CVi (%)	ACL (%)	TCL (%)
Serum gel tubes								
Liaison XL third-generation	2.0	24	5.5	13.2	3.1	30	8.6	17.3
Cobas third-generation	2.0		5.5	13.2	2.1		5.8	16.1
Liaison XL second-generation	2.0	19	5.5	11.0	1.9	26	5.3	14.0
Cobas second-generation	1.3		3.6	10.1	1.5		4.2	13.7
EDTA tubes								
Liaison XL third-generation	1.5	24	4.2	12.7	2.5	30	6.9	16.5
Cobas third-generation	1.5		4.2	12.7	2.5		6.9	16.5
Liaison XL second-generation	1.7	19	4.7	10.6	2.4	26	6.6	14.6
Cobas second-generation	1.6		4.4	10.5	1.6		4.4	13.7

Parent et al. [13] showed that PTH determination was compatible with an indifferent use of serum or EDTA plasma, if the samples were quickly processed, and Ratcliffe et al. [16], after having tested the stability of PTH in whole blood and serum, showed that PTH concentrations were not significantly decreased when blood or serum was left for up to 6 h at room temperature.

Unfortunately, none of the studies on PTH stability are free from criticisms, mainly because of their designs. As an example, only one type of tube was analyzed [7, 9, 12, 16] or the populations studied were often healthy subjects [4–9], which does not reflect real-life laboratory conditions. Also, most of the studies have used a second-generation of PTH assay, which can detect large fragments that could be more stable than the active peptide itself and little is really known on the 1–84 PTH as measured by a third-generation PTH assay.

Many papers have also studied PTH stability in serum or plasma on directly centrifuged samples [6, 11, 14, 18, 19] and not on whole or coagulated blood. Another point of criticism is the definition of the "T0" point since many studies have used as comparator a sample that had been immediately frozen at – 20 °C [7, 8, 12–16, 19] or at – 80 °C [9, 17] in order to run all the samples in a single batch to reduce the CV. However, it remains unclear if prolonged storage at – 20 °C or – 80 °C could have consequences on the final results. Few studies have really used an unfrozen sample, immediately assayed after centrifugation, as T0 [4, 5, 11, 18, 21].

Even more important is the cut-off below which it is decided whether PTH remains stable or not. In some studies, the observed variations were mainly analyzed by a purely statistical approach [5, 6, 9, 11–13, 15, 16, 19]. Also, an arbitrary cut-off of 10% [7, 8, 17] or 20% [18] relative to baseline concentrations has been used. This statistical way of estimating the stability is questionable since a statistical significant change is not always clinically significant. Very few studies have used the "biological" approach, as recommended by the WHO guideline, which defines the "maximum permissible instability", which should be smaller than half of the total error derived from the sum of biological and technical variability [22]. In our study, we decided to choose a standardized criterion which takes both analytical (CVa) and biological (CVi) variability into consideration.

In this paper, we tested the stability of PTH (measured with second- and third-generation assays from two different manufacturers) in whole EDTA and clotted blood samples obtained in healthy subjects and hemodialyzed patients, and kept at + 4 °C and + 25 °C during 4 and 18 h. All the samples have been run in duplicate. We used a "true" T0 and three different approaches to evaluate the stability: a purely statistical method, the ACL as suggested by the ISO Guide 5725-6 and the WHO guideline, which take into account the biological variation. For that purpose, we established the biological variability of second- and third-generation PTHs in a group of 22 healthy subjects according to Fraser and Harris' methodology [25] and we used the CVi published by Gardham et al. [24] to evaluate the stability in hemodialyzed patients.

The first approach was the pure statistical evaluation of the stability via a simple Wilcoxon test. Using this approach, we showed that, basically, PTH could not be considered as a stable peptide since a significant decrease was systematically observed with all the methods even when the samples were kept untreated for 4 h at + 4 °C. This is, of course, purely statistical and does not really reflect the different variations inherent to the analytical

phase. That is why we also used the ISO Guide 5725-6 approach that defines ACL according the CVa of the different methods and two factors, 1.96 for a bidirectional 95% of a significant change and √2 because two results are taken into consideration. This approach showed that, if serum samples are used, they need to be treated and centrifuged rapidly since PTH in stable only for 4 h at +4 °C. PTH in EDTA samples seems more stable, but not with all the combinations population/method/storage conditions. Since some of these conditions did not show stability >4 h at +4 °C, treatment of EDTA samples should thus be the same as treatment of serum gel tubes and these samples should also be centrifuged and treated immediately. These two approaches lack, however, a biological dimension since a decrease of the peptide might be observed, but whether this decrease is biologically significant or not remains to be determined. So, we used a third approach based on a WHO guideline that takes both biological and analytical variation to decipher whether a decrease of PTH is biologically significant or not [22]. For that purpose, we determined the CVi of PTH in a population of healthy young adults, very close from the population used to test PTH stability in order to calculate the TCL. The CVi we found (24% and 19% for PTH run with third- and second-generation PTH assays, respectively) was in total accordance with Gardham et al. [24] who found a CVi of 23.8% and 19.2% for third- and second-generation PTH assays, respectively. We thus used the CVi published by these authors for hemodialyzed patients (30.2% and 25.6% for third- and second-generation PTH assays, respectively), and not the one we published previously for hemodialyzed patients (14.9% and 13.8%) [28]. The reasons why we observed such difference in hemodialyzed patients need further investigations and are beyond the scope of this manuscript. According to this approach, we show that PTH is stable in EDTA samples whatever the conditions tested, the population, and the generation of PTH assays. In whole clotted blood, PTH is also stable, except when samples need to be stored unprocessed for a long period (18 h) at room temperature.

In conclusion, the concept of PTH stability mainly relies on the way that this stability is evaluated. Our results show that PTH seems more stable in EDTA than in serum gel tubes but only when samples have to stay unprocessed for a long period of time (18 h) at room temperature (25 °C), which can happen when samples are delivered from external care centers. For all the other conditions, using serum gel tubes is recommended since calcium measurement, necessary for a good PTH results interpretation, can be achieved on the same sample.

References

1. Delanaye P, Souberbielle J-C, Lafage-Proust MH, Jean G, Cavalier E. Can we use circulating biomarkers to monitor bone turnover in CKD haemodialysis patients? Hypotheses and facts. Nephrol Dial Transplant 2014;29:997–1004.
2. Hanon EA, Sturgeon CM, Lamb EJ. Sampling and storage conditions influencing the measurement of parathyroid hormone in blood samples: a systematic review. Clin Chem Lab Med 2013;51:1925–41.
3. Cavalier E, Plebani M, Delanaye P, Souberbielle J-C. Considerations in parathyroid hormone testing. Clin Chem Lab Med 2015;53:1913–9. Available at: www.degruyter.com/view/j/cclm.ahead-of-print/cclm-2015-0314/cclm-2015-0314.xml.
4. Oddoze C, Lombard E, Portugal H. Stability study of 81 analytes in human whole blood, in serum and in plasma. Clin Biochem 2012;45:464–9. Available at: http://dx.doi.org/10.1016/j.clinbiochem.2012.01.012.
5. Scharnhornst V, Valkenburg J, Vosters C, Vader H. Influence of preanalytical factors on the immulite intact parathyroid hormone assay. Clin Chem 2004;50:974–5.
6. Glendenning P, Laffer LL, Weber HK, Musk AA, Vasikaran SD. Parathyroid hormone is more stable in EDTA plasma than in serum. Clin Chem 2002;48:766–7.
7. Jane Ellis M, Livesey JH, Evans MJ. Hormone stability in human whole blood. Clin Biochem 2003;36:109–12.
8. Evans MJ, Livesey JH, Ellis MJ, Yandle TG. Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. Clin Biochem 2001;34:107–12.
9. Zwart SR, Wolf M, Rogers A, Rodgers S, Gillman PL, Hitchcox K, et al. Stability of analytes related to clinical chemistry and bone metabolism in blood specimens after delayed processing. Clin Biochem 2009;42:907–10. Available at: http://dx.doi.org/10.1016/j.clinbiochem.2009.02.010.
10. Gutierrez O, Isakova T, Rhee E, Shah A, Holmes J, Collerone G, et al. Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. J Am Soc Nephrol 2005;16:2205–15.
11. Holmes DT, Levin A, Forer B, Rosenberg F. Preanalytical influences on DPC IMMULITE 2000 intact PTH assays of plasma and serum from dialysis patients. Clin Chem 2005;51:913–5.
12. Morales García AI, Górriz Teruel JL, Plancha Mansanet MC, Escudero Quesada V, Pallardó Mateu LM. Analysis of variability in determining intact parathyroid hormone (iPTH) according to the method used to process the sample. Nefrologia 2009;29:331–5.
13. Parent X, Alenabi F, Brignon P, Souberbielle J-C. Delayed measurement of PTH in patients with CKD: storage of the primary tube in the dialysis unit, which temperature? Which kind of tube? Nephrol Ther 2009;5:34–40.
14. Joly D, Drueke TB, Alberti C, Houillier P, Lawson-Body E, Martin KJ, et al. Variation in serum and plasma PTH levels in second-generation assays in hemodialysis patients: a cross-sectional study. Am J Kidney Dis. 2008;51:987–95.
15. Parent X, Alenabi F, Etienne E, Brignon P, Chantrel F, Meynaud-Kraemer L. Variabilité pré-analytique du dosage de la parathormone chez le patient dialysé; application à l'automate Elecsys 2010 (Roche). Ann Biol Clin (Paris) 2008;66:53–8.

16. Ratcliffe W, Heath D, Ryan M, Jones SR. Performance and diagnostic application of a two-site immunoradiometric assay for parathyrin in serum. Clin Chem 1989;35:1957–61.
17. Stokes FJ, Ivanov P, Bailey LM, Fraser WD. The effects of sampling procedures and storage conditions on short-term stability of blood-based biochemical markers of bone metabolism. Clin Chem 2011;57:138–40.
18. Cavalier E, Delanaye P, Hubert P, Krzesinski JM, Chapelle JP, Rozet E. Estimation of the stability of parathyroid hormone when stored at −80°C for a long period. Clin J Am Soc Nephrol 2009;4:1988–92.
19. Teal TK, Wood JL, Stevens PE, Lamb EJ. Stability of bio-intact (1-84) parathyroid hormone ex vivo in serum and EDTA plasma from hemodialysis patients. Clin Chem 2004;50:1713–4.
20. ISO Guide 30:2015. Terms and conditions used in connection with reference materials.
21. Cavalier E, Carlisi A, Bekaert A-C, Rousselle O, Chapelle JP, Delanaye P. New insights on the stability of the parathyroid hormone as assayed by an automated 3rd generation PTH assay. Clin Chim Acta 2012;413:353–4. Available at: http://dx.doi.org/10.1016/j.cca.2011.09.034.
22. World Health Organization. Use of anticoagulants in diagnostic laboratory investigations. 2002.
23. ISO Guide 5725-6:1994. Accuracy (trueness and precision) of measurement methods and results–part 6: use in practice of accuracy values.
24. Gardham C, Stevens PE, Delaney MP, LeRoux M, Coleman A, Lamb EJ. Variability of parathyroid hormone and other markers of bone mineral metabolism in patients receiving hemodialysis. Clin J Am Soc Nephrol 2010;5:1261–7.
25. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 1989;27:409–37.
26. Forsberg G, Brobjer M, Holmgren E, Bergdahl K, Persson P, Gautvik KM, et al. Thrombin and H64A subtilisin cleavage of fusion proteins for preparation of human recombinant parathyroid hormone. J Protein Chem 1991;10:517–26.
27. La'ulu SL, Straseski JA, Schmidt RL, Genzen JR. Thrombin-mediated degradation of parathyroid hormone in serum tubes. Clin Chim Acta 2014;437:191–6. Available at: http://linkinghub.elsevier.com/retrieve/pii/S000989811400326X.
28. Cavalier E, Delanaye P, Moranne O. Variability of new bone mineral metabolism markers in patients treated with maintenance hemodialysis: implications for clinical decision making. Am J Kidney Dis 2013;61:847–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23357107.