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To cite this version:
Jean-Claude Souberbielle, Etienne Cavalier, Pierre Delanaye, Catherine Massart, Sylvie Brailly-Tabard, et al.. Serum calcitriol concentrations measured with a new direct automated assay in a large population of adult healthy subjects and in various clinical situations. Clinica Chimica Acta, Elsevier, 2015, 451 (PART B), pp.149-153. 10.1016/j.cca.2015.09.021 . hal-01207280

HAL Id: hal-01207280
https://hal-univ-rennes1.archives-ouvertes.fr/hal-01207280
Submitted on 13 Jan 2016

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Serum calcitriol concentrations measured with a new direct automated assay in a large population of adult healthy subjects and in various clinical situations.

Jean-Claude Souberbielle¹, Etienne Cavalier², Pierre Delanaye³, Catherine Massart⁴, Sylvie Brailly-Tabard⁵, Catherine Cormier⁶, Didier Borderie⁷, Alexandra Benachi⁸, and Philippe Chanson⁹.

¹: Service des explorations fonctionnelles, Necker-Enfants malades University hospital, Assistance Publique des Hôpitaux de Paris (AP-HP), Paris, France
²: Clinical Chemistry Department, Sart Tilman university hospital, Liège, Belgium
³: Nephrology and dialysis Department, Sart Tilman university hospital, Liège, Belgium
⁴: Hormonology Laboratory, Pontchaillou university hospital, Rennes, France
⁵: Hormonology Laboratory, Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Le Kremlin-Bicêtre, F94275, France; and Univ Paris-Sud, UMR S1185, Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre, F-94276, France; and INSERM U1185, Le Kremlin-Bicêtre, F-94276, France.
⁶: Rheumatology Department, Cochin university hospital, Assistance Publique des Hôpitaux de Paris (AP-HP), Paris, France
⁷: Clinical Chemistry Department, Cochin university hospital, Assistance Publique des Hôpitaux de Paris (AP-HP), Paris, France
⁸: Obstetrics, Gynecology and Reproductive Medicine Department. Antoine Beclere Hospital, Paris Sud University, Assistance Publique - Hôpitaux de Paris (AP-HP), Clamart, France
⁹: Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Service d'Endocrinologie et des Maladies de la Reproduction and Centre de Référence des Maladies Endocriniennes Rares de la Croissance, Le Kremlin-Bicêtre, F94275, France; and Univ Paris-Sud, UMR S1185, Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre, F-94276, France; and INSERM U1185, Le Kremlin-Bicêtre, F-94276, France.

Correspondence and person to whom reprint request should be addressed:

Jean-Claude Souberbielle
Laboratoire des explorations fonctionnelles
Hôpital Necker-Enfants malades
149 rue de Sèvres, 75015, Paris, France
Jean-claude.souberbielle@nck.aphp.fr
Tel: 33 1 44 38 17 43
Disclosure statement:

JCS reports lecture fees and/or travel/hotel expenses from DiaSorin, Roche Diagnostics, Abbott, Amgen, Shire, MSD, Lilly, and Rottapharm.; CM reports lecture fees and travel/hotel expenses from DiaSorin; EC is a consultant for IDS and DiaSorin and has received lecture fees from IDS, DiaSorin, Roche, Abbott and Amgen; PD is a consultant for IDS and has received lecture fees and/or travel expenses from DiaSorin, Amgen, Shire, Fresenius, Menarini, and Sanofi.; SBT, DB, CC, AB, and PC have nothing to disclose.
Abstract

The measurement of calcitriol [1,25(OH)2D], is important for the differential diagnosis of several disorders of calcium/phosphorus metabolism but is time-consuming and tricky. We measured serum calcitriol with a new automated direct assay on the Liaison XL platform in 888 healthy French Caucasian subjects aged 18-89 years, 32 patients with a surgically-proven PHPT, 32 pregnant women at the end of the first and at the end of the third trimester, and 24 Dialysis patients before and after one year of supplementation with vitamin D3 or placebo. The mean calcitriol concentration (±SD) in the healthy population was 52.9±14.5 ng/L with a 95 % CI interval of 29-83.6 ng/L. In PHPT patients, calcitriol concentration was 81.6±29.0 ng/L, 15 of them (46.9%) having a concentration >83.6 ng/L. In pregnant women, calcitriol was 80.4±26.4 ng/L at the end of the first trimester, and 113.1±33.0 ng/L at the end of the third trimester, 12 (37.5%) and 26 (81.3%) of them having a calcitriol >83.6 ng/L at first and third trimester respectively. In 14 dialysis patients, calcitriol was 9.5 ± 7.7 ng/L and rose to 19.3 ng/L after one year of supplementation with 50,000 IU vitamin D3/month. In 10 other dialysis patients, calcitriol was 9.9±2.9 ng/L and remained stable (12.4±3.7 ng/L) after one year of placebo. In conclusion, this new automated calcitriol assay, in addition to presenting excellent analytical performances, gives the expected variations in patients compared to “normal” values obtained in an extensive reference population.

Key words: calcitriol; vitamin D; reference values; primary hyperparathyroidism; dialysis ; pregnancy
Hoghlights

- A new, more sensitive, assays for 1,25(OH)2 vitamin D is now available on the market
- This assay allows performing new basic and clinical studies
- Reference ranges for 1,25(OH)2D have been established on a 888 healthy individuals cohort
- Well characterized clinical populations have been studied and compared with these results
- 25OHD concentration is the most closely correlated to 1,25(OH)2D serum concentration in healthy subjects, both in univariate and multivariate analysis
1. Introduction

The active vitamin D metabolite, 1,25 dihydroxyvitamin D \([1,25(\text{OH}_2)\text{D}]\), also called calcitriol, is secreted into the bloodstream by the cells of the renal proximal tubule and binds to the vitamin D receptor (VDR) in several distant tissues to exert genomic effects. It must thus be considered as a real hormone [1]. Renal \(1\alpha\)-hydroxylation of 25-hydroxyvitamin D (25OHD), the precursor of calcitriol produced by the liver, is tightly regulated, mainly stimulated by parathyroid hormone (PTH) and inhibited by Fibroblast Growth Factor 23 (FGF23). Calcitriol is also produced by numerous tissues where it acts in an intracrine manner [2]. The measurement of calcitriol in serum must not be used to evaluate the vitamin D status which is consensually assessed through the measurement of 25OHD [3]. However, it is important for the differential diagnosis of several disorders of calcium/phosphorus metabolism, especially in case of hypercalcemia, hypercalciuria, and low PTH level [4], or in case of rickets/osteomalacia which persists after vitamin D supplementation [5]. Calcitriol serum levels are modified in many clinical situations, increased for example during pregnancy [6], or primary hyperparathyroidism (PHPT) [7], and decreased in chronic kidney disease (CKD) [8] or hypoparathyroidism [9].

The measurement of \(1,25(\text{OH}_2)\text{D}\) concentration in serum is not an easy task due to its hydrophobic nature and because it circulates at picomolar levels compared to 25OHD, which has one less hydroxyl residue and circulates at a 1000-fold higher concentration. Currently available \(1,25(\text{OH}_2)\text{D}\) assays are either immunoassays [10; 11] or HPLC/LC-MSMS methods [12; 13] that require quite a large amount of serum (most often 0.5 mL) and a complicated and time-consuming extraction step to eliminate potentially interfering compounds. Very recently, a new automated rapid (65 minutes to obtain the first result), and with a high throughput (90 tests/hour) \(1,25(\text{OH}_2)\text{D}\) assay that does not require an extraction step became available on the LIAISON XL automated platform [14]. Another potential advantage of this new assay is that
it requires a much smaller sample volume (75 µL + adjustable dead volume of 50 µL at least) than the other 1,25(OH)₂D assays, a point that may be especially interesting in paediatric settings. A recently published evaluation of this new kit reported excellent analytical performances [14].

The aim of the present study was to use a large, well-defined cohort of French healthy subjects to establish serum 1,25(OH)₂D reference values with this new assay. We also measured 1,25(OH)₂D in several well-characterized groups of patients.

2. Subjects and methods

2.1. Healthy subjects and patients

We enrolled healthy volunteers who participated in the VARIETE study, a population-based cross-sectional study designed to recruit a reference population in order to harmonize normal serum IGF-I values in adults (ClinicalTrials.gov identifier: NCT01831648). They were recruited between January 2011 and February 2012 by the clinical research units of 10 university hospitals distributed throughout France. To be included in the study, subjects had to have a normal physical work-up (weight, height, blood pressure, nutritional status and gonadal/sexual status), normal laboratory values determined after an overnight fast (plasma sodium, potassium, calcium, phosphate, creatinine, glycemia, total cholesterol, liver enzymes, TSH, blood cell counts, albuminemia, prothrombin time, and HIV and HCV serology), age 18-89 years and BMI between 19 and 28 kg/m², and to give their written informed consent to participate in the study. The exclusion criteria were a medical history of thyroid, renal, hepatic, cardiovascular, pulmonary, intestinal or psychiatric disorders, cancer, epilepsy, intercurrent illness occurring during the week preceding inclusion, current consumption of tobacco or other toxics, and treatment potentially modifying IGF-I or calcium/phosphorus metabolism (antiandrogens or antiestrogens, loop diuretics, hydrochlorothiazide, CYP-inducing drugs). In addition to the blood samples necessary for the screening biological
evaluation, 50 mL of whole blood and 30 mL of EDTA blood was obtained from each subject. Blood was promptly centrifuged (3000 g at 4°C), and serum or plasma was aliquoted in polypropylene tubes that were immediately stored at -80°C. This study was funded by Programme Hospitalier de Recherche Clinique, French Ministry of Health, No P081216 / IDRCB 2009-A00892-55, and was approved by the Paris-Sud Ethics Committee in November 2009.

We also studied serum samples from 32 patients with PHPT before parathyroid surgery. In these patients, the diagnosis of PHPT was ascertained by a calcium load test showing an insufficient fall in serum PTH when ionized calcemia rose well above the upper normal limit. In those with either normal total calcemia or normal ionized calcemia (n=13), an IV calcium load test was performed as described in [15]. These patients were osteoporotic (low bone mineral density [BMD] and/or low-trauma fracture) and were initially referred to our tertiary care center for etiological diagnosis of abnormal calcium/phosphorus or related hormone levels detected during a screening biological evaluation aimed at ruling out secondary causes of osteoporosis. We requested that the physician who referred the patient prescribe vitamin D if the 25OHD concentration was low, before investigations in our unit. All patients in this group underwent successful parathyroidectomy, as confirmed by pathological examination of parathyroid tissue removed during surgery.

We also obtained serum samples of 32 pregnant women participating in the FEPED study, an ongoing prospective observational study of the association between serum 25OHD levels and the risk of preeclampsia (ClinicalTrials.gov identifier: NCT01648842). In these 32 women, blood was drawn at the end of the first trimester and at the end of the third trimester of pregnancy. They received a 100,000 IU vitamin D3 dose at the beginning of the third trimester of pregnancy as it is the rule in France for the prevention of neonatal hypocalcemia. The FEPED study was funded by Programme Hospitalier de Recherche Clinique, French
Ministry of Health, N° NI10024-ID RCB 2011-A00355-36 and was approved by the CPP Ile de France IV Ethics Committee in April 2011.

Finally, we included sera from 24 haemodialysis patients who participated in a pilot randomized study of vitamin D supplementation, registered with a Belgian clinical trial number (B70720084117) [16]. In these patients, blood was drawn before and after one year of vitamin D supplementation (50,000IU vitamin D3 per month; n=14) or placebo (n=10) just before a dialysis session.

2.2. Laboratory methods

The biological parameters of the healthy volunteer screening evaluation were determined locally by the laboratories attached to the clinical research units, using standard chemistry. The CKDepi formula was used to estimate glomerular filtration rate (eGFR). PTH, 25OHD, and 1,25(OH)₂D measurements were centralized and done in batches by means of immunochemiluminescent assays on the LIAISON XL automated platform (DiaSorin, Stillwater, Mn, USA), using serum samples that had never been thawed. The new 1,25(OH)₂D assay is a sandwich assay that uses the ligand binding domain (LBD) of the VDR as a 1,25(OH)₂D capture molecule. Reaction conditions were chosen at which 1,25(OH)₂D binds the VDR with an approximately 200-fold higher affinity than 25OHD, while the vitamin D binding protein binds 1,25(OH)₂D with a 10 to 100-fold lower affinity than 25OHD, leaving thus a 2,000-20,000 fold differential favouring the transfer of 1,25(OH)₂D from the VDBP with binding to the recombinant VDR-LBD. The assay then exploits the conformational change of the LBD induced by 1,25(OH)₂D binding with the use of paramagnetic microparticles coated with a specific monoclonal antibody which recognizes this induced 1,25(OH)₂D-bound LBD conformation. After a washing step aimed at eliminating unbound LBD, a second isoluminol-labelled anti-LBD antibody is added and binds to the 1,25(OH)₂D-LBD complex already bound to the solid-phase. After eliminating the excess (unbound)
second antibody, luminescence is counted and is proportional to the 1,25(OH)₂D present in the serum sample. Analytical performances of the new 1,25(OH)₂D assay have been reported in [14].

2.3. Statistical analysis
Quantitative variables are reported as means ± SD. The median, quartile (Q) 1 (25th percentile), Q3 (75th percentile), and interquartile range (IQR) of 1,25(OH)₂D values are reported for the healthy subjects. We used the Horn algorithm to determine the 1,25(OH)₂D reference range [17]. We first detected outliers, defined as 1,25(OH)₂D concentrations below Q1-1.5 IQR and above Q3+1.5 IQR after log transformation of the raw values. We then calculated the 95% confidence interval in the remaining subjects after eliminating the outliers. Paired data were compared with the Wilcoxon test. Unpaired data were compared with the Mann-Whitey U-test. A p value <0.05 was considered significant.

3. Results
3.1. Healthy subjects
Nine hundred seventy-two Caucasian subjects were initially recruited. Two were excluded because their informed consent was not available, and another 60 were excluded because of abnormal values in the screening evaluation. Among the remaining 910 subjects, no serum sample was available for 1,25(OH)₂D testing in 22 cases. The study population thus consisted of 888 subjects, whose main characteristics are summarized in Table 1. The mean 1,25(OH)₂D value in these 888 subjects was 52.9 ± 14.5 ng/L (median: 51.6 ng/L; Q1: 42.6 ng/L; Q3: 61.8 ng/L; IQR: 19.2 ng/L). After exclusion of twelve outliers (two high values, ten low values), the range of 1,25(OH)₂D concentrations (2.5th-97.5th percentile) was 29.0-83.6 ng/L. Mean 1,25(OH)₂D level was slightly higher in women than in men (54.0 ± 15.1 ng/L and 51.8 ± 13.9 ng/L respectively; p=0.026) but without any difference in the upper and lower limit of normal.
3.2. Patients

The main characteristics of the 32 PHPT patients are presented in table 2. Their mean 1,25(OH)$_2$D concentration (81.6 ± 29.0 ng/L) was significantly higher than in the healthy population (p<0.001). Fifteen of them (46.9%) had a 1,25(OH)$_2$D concentration above our upper limit of normal of 83.6 ng/L.

In the 32 pregnant women, the mean 1,25(OH)$_2$D concentration at the end of the first trimester (80.4 ± 26.4 ng/L) was significantly higher than in the healthy population (p<0.001), and significantly increased to 113.1 ± 33.0 ng/L at the end of the third trimester (p<0.001) as did their 25OHD serum concentration which increased significantly from 18.5 ± 9.9 ng/mL to 39.8 ± 11.8 ng/mL. Twelve (37.5%), and 26 (81.3 %) of them had a 1,25(OH)$_2$D concentration above 83.6 ng/L at the end of the first and third trimester respectively. Calcitriol concentrations of these 3 groups of patients are summarized in figure 1.

Eight of the 24 dialysis patients (5 in the vitamin D group, and 3 in the placebo group) had a 1,25(OH)$_2$D concentration below the LOQ of 5 ng/L before vitamin D supplementation, while 2 patients in the placebo group and none in the vitamin D group had such a value after one year of supplementation. They were attributed an arbitrary 1,25(OH)$_2$D concentration of 4 ng/L. In the 14 HD patients who received vitamin D, serum 25OHD increased from 11.9 ± 4.9 ng/mL to 36.9 ± 9.3 ng/mL (p=0.001), and serum 1,25(OH)$_2$D increased from 9.5 ± 7.7 ng/L to 19.3 ± 9.7 ng/L (p=0.001) after one year of supplementation. Three of them (21.4 %) reached a normal 1,25(OH)$_2$D concentration (>29 ng/L) at one year (Figure 2). By contrast 25OHD (9.9 ± 2.9 ng/mL before, and 12.4 ± 3.7 ng/mL after supplementation) and 1,25(OH)$_2$D (7.4 ± 3.9 before, and 10.7 ± 7.1 ng/L after supplementation) concentration did not change in the placebo group, and none of the subjects from this group had a normal 1,25(OH)$_2$D level at any time-point.

4. Discussion
In the present study, we measured serum 1,25(OH)₂D concentration with a new automated, direct immunoassay in a large cohort of healthy French adult subjects. Using a recommended statistical method [17], we propose an adult reference range of 29-83.6 ng/L for serum 1,25(OH)₂D concentration with this new assay. In several well-characterized groups of patients, we found the expected percentages of either high (PHPT patients and pregnant women) or low (dialysis patients) calcitriol concentrations.

Indeed calcitriol concentration was elevated (i.e. >83.6 ng/L) in approximately one of two PHPT patients, a finding consistent with previous studies [7; 18-23]. In these patients, increased 1,25(OH)₂D levels are believed to be due to increased PTH secretion which is the main stimulator of renal 1α-hydroxylase [8; 22]. Clinical associations of increased 1,25(OH)₂D concentration in PHPT differed among studies. In one study, elevated 1,25(OH)₂D was present in PHPT patients with renal stone disease but not in those with low bone mass [7]. In contrary, elevated 1,25(OH)₂D level was associated with increased bone turnover and low BMD in PHPT patients before surgery [21; 24], and weakly but significantly with the change in spine BMD one year after parathyroid surgery [25].

Similarly, two of five and four of five pregnant women had a 1,25(OH)₂D serum concentration >83.6 ng/L at the end of the first and the third trimester of pregnancy respectively. Here again, this is in accordance with previously published studies [10; 26-28]. This increase in 1,25(OH)₂D throughout pregnancy seems largely independent of PTH which has been found to be decreased during pregnancy in some studies [28], seems to be of both renal and placental origin [29], and is probably related to various factors such as an increase in the circulating level of vitamin D-binding protein (VDBP), and stimulation by prolactin, IGF-I, and PTHrP. One extremely remarkable point is that pregnant women remain normocalcemic despite having 1,25(OH)₂D concentrations that are able to induce a frank hypercalcemia in some clinical conditions such as sarcoidosis [4].
Calcitriol concentration was low, but often detectable, in our CKD patients treated by chronic haemodialysis, and significantly increased after vitamin D supplementation. An increase in serum 1,25(OH)_{2}D after supplementation with 25OHD [30; 31] or vitamin D3 [32] in dialysis patients has previously been reported, and is clearly of extra-renal origin as evidenced by studies that shown an increase in 1,25(OH)_{2}D serum level during vitamin D supplementation in anephric patients [33; 34]. To our knowledge, it remains to be shown whether the well-known decrease in PTH concentration during vitamin D or 25OHD supplementation in dialysis patients [16; 32; 35] is tightly correlated with the increase in 1,25(OH)_{2}D concentration.

In conclusion, this new 1,25(OH)_{2}D assay, in addition to presenting excellent analytical performances, detects the expected variations in selected groups of patients. As it requires much less sample than the other currently available assays and is extremely rapid and easy to use, it should become the method of choice for measuring serum 1,25(OH)_{2}D in routine practice. It should be also of great interest in clinical research, specially for measuring 1,25(OH)_{2}D in large cohorts of healthy or diseased persons.
References


Table 1. Characteristics of the healthy subjects participating in the VARIETE study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ±/SD (range)</th>
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<tbody>
<tr>
<td>Gender: men/women</td>
<td>466/432</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.7 ± 18.6 (18-89)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.0 ± 2.4 (18.5-28)</td>
</tr>
<tr>
<td>Serum 25OHD (ng/mL)</td>
<td>23.8 ± 8.1 (5.2-59.4)</td>
</tr>
<tr>
<td>Serum PTH (ng/L)</td>
<td>20.6 ± 8.0 (7.4-79.0)</td>
</tr>
<tr>
<td>Serum calcium (mmol/L)</td>
<td>2.30 ± 0.10 (2.10-2.60)</td>
</tr>
<tr>
<td>Serum phosphate (mmol/L)</td>
<td>1.09 ± 0.18 (0.75-1.51)</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>43.0 ± 3.9 (32.6-50)</td>
</tr>
<tr>
<td>eGFR (CKDepi) (mL/min/1.73 m²)</td>
<td>100 ± 7 (60-144)</td>
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</table>
Table 2: Main characteristics of the 32 patients with surgically-proven PHPT before parathyroidectomy.

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<table>
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<tbody>
<tr>
<td>Gender : men/women</td>
<td>2/30</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.6 ± 10.9 (min : 43-max: 89)</td>
</tr>
<tr>
<td>Serum total calcium (mmol/L)</td>
<td>2.63 ± 0.14</td>
</tr>
<tr>
<td>Serum ionized calcium (mmol/L)</td>
<td>1.37 ± 0.07</td>
</tr>
<tr>
<td>Serum 25OHD (ng/mL)</td>
<td>32.0 ± 9.5</td>
</tr>
<tr>
<td>Serum 3rd-generation PTH (ng/L)</td>
<td>48.9 ± 23.3</td>
</tr>
<tr>
<td>Serum 1,25OH2D (ng/L)</td>
<td>81.0 ± 29.0</td>
</tr>
</tbody>
</table>
Figure 1: Calcitriol concentration in patients with primary hyperparathyroidism (PHPT), pregnant women at the end of the first trimester, and at the end of the third trimester of pregnancy. The mean calcitriol concentration of these 3 groups is significantly higher than the mean level in the normal population. The two horizontal lines are the lower (29 ng/L) and upper (83.6 ng/L) normal limit that we have determined in the VARIETE population.
Figure 2: Evolution of serum calcitriol concentration over a one year period of supplementation with vitamin D3, 50,000 IU/month (A) or placebo (B). The solid horizontal lines are the lower limit of normal (29 ng/L) as determined in the VARIETE population.
Highlights

- A new, more sensitive, assays for 1.25(OH)2 vitamin D is now available on the market
- This assay allows performing new basic and clinical studies
- Reference ranges for 1.25(OH)2D have been established on a 888 healthy individuals cohort
- Well characterized clinical populations have been studied and compared with these results
- 25OHD concentration is the most closely correlated to 1,25(OH)2D serum concentration in healthy subjects, both in univariate and multivariate analysis