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ORIGINAL ARTICLE

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Comparison of two different methods for routine 25(OH)D measurement in paediatric serum samples

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ABSTRACT

Over the last decade interest in automated assays for 25-hydroxy-vitamin D measurement have greatly increased. The presence of different metabolites of vitamin D in the blood influences measurement of its concentration. In paediatric subjects the basic interference is due to the presence of 3-epi-25(OH)D_2/D_3, which despite their biological inactivity, influences the total concentration of 25(OH)D.

Aim: We assessed the analytical performance and usefulness of two different assays for measurement of total 25(OH)D in children.

Materials and Methods: The study was performed in blood samples taken from 100 school-children aged 9–11 years. In all serum samples 25(OH)D total concentration was measured with the use of chemiluminescent assay, which is known to show no cross-reactivity with 3-epi-25(OH)D, and with the use of a newly developed enzyme-immunosorbent method.

Results: The mean 25(OH)D concentration in children measured with enzyme-immunosorbent assay (EIA) was significantly higher, at 28.06 ng/mL, than with the chemiluminescent assay (CLIA), at 21.13 ng/mL; < 0.0001. In children with optimal weight the average 25(OH)D was 32.93 ng/ml (EIA) and 21.5 ng/mL (CLIA) ($p < 0.0001$), respectively, whereas in a subgroup with overweight/obesity the mean concentration of 25(OH)D was similar, at 23.2 ng/ml (EIA) and 20.76 ng/ml (CLIA) ($p = 0.15$). The nonparametric Spearman's rank correlation of two methods equalled 0.47; 95%CI (0.11 to 0.60) with a significance level $p < 0.0001$. The calculated concordance correlation coefficient between two methods in the whole group was 0.26; 95%CI (0.17 to 0.35). In a subgroup of children with optimal body mass ($N = 50$) the concordance correlation coefficient was 0.18; 95%CI (0.06 to 0.29), whereas in children with overweight/obesity ($N = 50$) it was 0.44; 95%CI (0.29 to 057). Mean bias for the enzyme-immunosorbent method equalled 18.7%; +/- 1.96 SD (101.3% to -64%).

Conclusions: With reference to 25(OH)D measurement in children, Spearman's correlation coefficient indicated "moderate correlation" between the two compared methods, whereas the strength of agreement (concordance) between both methods was characterised as "poor". The proper selection of assay for accurate assessment of vitamin D status in paediatric samples is necessary to avoid misdiagnosis.

Key words: Vitamin D, 25(OH)D concentration, CLIA, enzyme-immunosorbent method

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Abbreviations: VDR-vitamin D receptor, VDBP-vitamin D binding protein, CLIA-immunochemiluminescence (method), HPLC- *high-performance liquid chromatography,* LC-MS/MS-liquid chromatography with tandem mass spectrometry, CCC-concordance correlation coefficient.

Introduction

The positive role of vitamin D on skeletal system has been known for years, but studies over the last decade have discovered another dimension of interaction between vitamin D and non-skeletal systems. The

pleotropic effect of vitamin D mediated by vitamin D receptors localised on more than 60 types of cells affects more than 200 genes. The biological effects of interaction between vitamin D and VDR, besides calcium homeostasis, are inhibition of cell proliferation and promotion to maturation and regulation of the apoptosis [1–2].

Deficiency of vitamin D is recognised regardless of gender, age, or race but some circumstances particularly contribute. Obesity, especially among children, constitutes one of these factors, which promotes insufficiency of vitamin D. Studies from different countries highlight the common problem of vitamin D deficiency/insufficiency emerging among obese children [3–4]. An inverse correlation was found between fat mass and vitamin D status. Due to the increasing frequency of obesity and overweight among children and adolescents, the prevalence of parallel development of vitamin D insufficiency seems to be a real threat for health [5]. Vitamin D deficiency and obesity emerging in childhood have been classified as epidemics. Both of them contribute to the development of serious diseases and also have common risk factors, which are inappropriate diet and lack of activity. To avoid developing impaired bone mineralisation and also to reduce the risk of developing diabetes type 1 and 2 or cardiovascular diseases, it is important to maintain optimal concentration of vitamin D [5].

Nowadays many laboratories routinely analyse the concentration of 25(OH)D in blood samples. Most assays allow measurement of total 25(OH)D, including both forms: 25(OH)D_2 and 25(OH)D_3. This biomarker of vitamin D status is characterised by high stability under different storage conditions and also some pre-analytical impacts [6]. One should be aware that in some medical conditions, such as kidney failure, it is recommended to assess both metabolites: 25(OH)D and an active form 1,25$(OH)_2$D. Measurements of 25(OH)D are burdened with certain difficulties. The "matrix effect", related to interference with the serum components, is considered as one of the greatest analytical difficulties associated with the analysis of 25(OH)D. It results from the specific structure of 25(OH)D, which is characterised by high hydrophobicity [7]. Another kind of interference with measurement of 25(OH)D in the blood is the presence of different metabolites of vitamin D. There are more than 50 different metabolites of vitamin D, and some of them, such as 3-epi-25(OH)D_2/D_3, despite its biological inactivity, influence the total concentration of 25(OH)D [8–9]. A recent study reported that the concentration of 3-epi-25(OH)D_3 is strictly related to the concentration of 25(OH)D_3, and individuals with lower concentrations of total 25(OH)D_3 consequently showed reduced levels of this metabolite [9]. Significantly elevated content of 3-epi-25(OH)D_3 has been found in the paediatric population, especially in children below one year old [10]. The content of 3-epi-25(OH)D_3 in infants can reach 9 to 61.1 % of the total 25(OH)D [9]. The presence of different metabolites of vitamin D can contribute to misclassification between sufficiency and deficiency of vitamin D by inclusion of these metabolites into the total concentration of 25(OH)D [10].

Currently, a wide spectrum of different methods used for evaluation of vitamin D status is available. There are manual and automated immunoassays, methods of direct detection such as HPLC and LC-MS/MS, and recently a new developed enzyme-immunosorbent assay was adapted on general chemistry analysers [11]. Most commercially available automated immunoassays for 25(OH)D measurement use immunochemiluminescence technology. Identified differences between various assays basically result from the specificity of applied antibodies, which are characterised by lower or higher cross-reactivity with different metabolites of vitamin D [7, 9].

Although most widely used commercially available immunoassays for analysis of 25(OH)D have comparable correlation coefficient values to the reference method LC-MS/MS, an overall significant bias was found when compared to LC-MS/MS, especially at lower concentrations of 25(OH)D (< 30 ng/mL) [12]. Most automated assays give reasonably accurate measurements, but certain pitfalls occur, which, in fact, are difficult to eliminate. The cross-reactivity with 24,25$(OH)_2$ or 3-epimers in some immunoassays can contribute to overestimation of 25(OH)D and consequently lead to misclassification of vitamin D status. The percentage of 3-epi metabolite was estimated by Bailey et al. to be 11% in pregnant women and up to 25% in infants [9,13]. The "gold standard" for adequate separation of 3-epimers and 24,25(OH)D metabolites, which is the function of LC-MS/MS, cannot be commonly implemented because of economical and organisational reasons. That is why methods used for vitamin D analysis should be standardised with comparable reference material. The National Institutes of Standard and Technology has developed standardised Calibration Solutions - NIST SRM 2972 (which include both forms: 25(OH)D_2 and 25(OH)D_3), which allows standardisation of different available methods used for the measurement of 25(OH)D and also allows harmonisation of results of vitamin D concentration obtained from different laboratories [14].

The growing need for 25(OH)D testing forced most of the laboratories to implement fast and accurate tests for routine evaluation of vitamin D status. The Federal Drug Administration from early the 2000s approved the first fully automated immunoassay method for 25(OH)D measurement [11]. Lately a new homogenous assay measuring 25(OH)D was adapted on general chemistry analysers. This new approach is based on the principle of α-complementation of the β-galactosidase enzyme and reaction of the competition between an enzyme donor-25(OH)D conjugate, an anti-vitamin D antibody

and the 25(OH)D in the blood [11]. This method was fully validated for the use on several chemistry analysers [11]. Our study aimed to assess the performance of two different methods, one available on an immunoassay automated system and the other on a general chemistry system, for measurement of 25(OH)D in children. We compared IDS-iSYS 25-hydroxy vitamin D^S assay performed on an IDS-iSYS (Immunodiagnostic Systems) and Diazyme 25(OH)D assay (Pointe Scientific) performed on a Pentra 400 general chemistry analyser (Horiba). Comparison was performed on 100 serum samples collected from school-children.

Subjects and materials

One hundred presumably healthy children aged 9–11 years (45 boys and 55 girls) were included in the study. Blood samples were taken in the fasting state. Anthropometric measurements (height and weight) were conducted and body mass index percentiles were calculated with an online BMI calculator (based on the "OLAF" project). The BMI percentiles classification was accepted according to the International Obesity Task Force (IOTF); optimal weight: BMI ≥ 5 and < 85 percentile; overweight: BMI ≥ 85 and < 95; obesity: BMI ≥ 95 percentile. Subjects were divided based on BMI percentiles into two groups: with optimal weight (N = 50) and with overweight and obesity (N = 50).

This study was approved by the Bioethics Committee of the Collegium Medicum in Bydgoszcz and Nicolaus Copernicus University in Toruń.

Methods

IDS-iSYS 25(OH)D^S assay (Immunodiagnostic Systems Ltd, Boldon, United Kingdom).

This assay of the quantitative determination of 25-hydroxyvitamin D [(25(OH)D] and other hydroxylated metabolites in human serum was performed on an IDS-iSYS automated analyser. The method used in this assay is based on immunochemiluminescence technology (CLIA) and is aligned to NIST SRM 2972. The assay is traceable to the isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LCMS/MS). Cross-reactivity with 3-epi-25(OH)D2 and with 3-epi-25(OH)D3 is 1% at 25(OH)D concentration 100 ng/mL (according to manufacturer's insert).

25-hydroxy vitamin D assay (Pointe Scientific)

Assay for quantitative determination of 25(OH)D by Pointe Scientific was performed on a clinical chemistry analyser — Pentra 400 (Horiba). The principle of the assay is based on α-complementation of the β-galactosidase and competition between enzyme donor-25-(OH)D conjugate, antibody of vitamin D, and the 25(OH)D in the serum sample (EIA). The concentration of vitamin D in serum is proportional to the activity of β-galactosidase with maximum absorbance at 415 nm [11]. Cross-reactivity with 3-epi-25(OH)D2 is 55.1% and with 3-epi-25(OH)D3 is 66.7% at 25(OH)D concentration 4 ng/mL (according to manufacturer's insert).

Statistical analysis

Results of this study were analysed with Bland-Altman regression. Correlations were assessed by Spearman's coefficient (rho) and concordance correlation coefficient (CCC). Basic statistics were performed by using the D'Agostino-Pearson test. For considering statistical significance a p value was established at the level of < 0.05. MedCalc Statistical Software trial version 16.2.1 (MedCalc Software BVBA, Ostend, Belgium; https://www.medcalc.org; 2016) was used for statistical analysis.

Results

Subjects taking part in this study were divided into two groups: children with optimal weight (N = 50) and children with overweight/obesity (N = 50). The average BMI percentile in a subgroup with optimal weigh was 45, and with overweight/obesity it was 93. Mean serum 25(OH)D concentration measured by EIA in all children was significantly higher than with CLIA, 28.06 ng/mL; 95%CI (25.37 to 30.75) vs. 21.13 ng/mL; 95%CI (20.15 to 22.11) (p<0,0001). Comparison of vitamin D concentrations distribution performed on Pentra 400 and IDS-iSYS systems are shown in Figure 1 A–B. The average serum 25(OH)D concentrations in the subgroup of children with optimal weight was significantly higher with EIA (32.93 ng/ml; 95%CI [28.85 to 37.00]) than with CLIA (21.50 ng/mL; 95%CI [22.03 to 22.98]) (p < 0.0001), whereas in the subgroup with overweight/obesity both means were similar (23.20 ng/ml [20.11–26.29] and 20.76 ng/ml [19.42–22.09]); p = 0.15.

Method comparison requires that specimens are assayed by both methods and the results are compared to assess the bias that is used to express the closeness of agreement between the average value obtained from a series of measurements and the true value [15]. Estimation of mean bias was performed after Bland-Altman plots were calculated (Figure 2 A–C). The highest mean bias for enzymatic method (EIA) was identified for the subgroup of children with optimal weight, and equalled 35.3. In children with overweight/obesity the mean bias for the EIA method was the lowest, and equalled

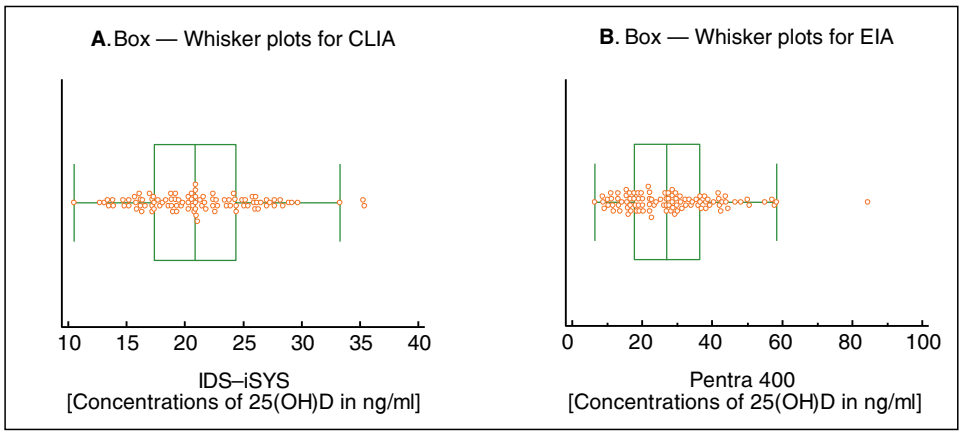

Figure 1 A-B. Distribution of 25(OH)D concentrations measured in the whole group of children by using two different methods.

2.0. Measurements by enzymatic method in the whole group showed a positive mean bias of 18.7.

Next, a nonparametric Spearman's coefficient of rank correlation (rho) was calculated for all participants and two subgroups. Spearman's correlation for all subjects showed rho=0.47 with 95%CI (0.30 to 0.61) and significance level $p < 0.0001$, which indicated "moderate correlation". The concentration of 25(OH)D in (N = 50) when comparing two methods characterised with Spearman's rho was 0.38 with 95%CI (0.11 to 0.60) in children with optimal mass, indicating "weak correlation" [16]. Positive correlation on "moderate level" was found in a subgroup of overweight/obese children (rho=0.59 with 95%CI [0.37 to 0.75]).

Concordance correlation between the two methods was evaluated, and the concordance correlation coefficient was calculated for assessment of the strength of the agreement. The concordance correlation coefficient r_c contains a measurement of precision ρ and accuracy $C_{b.}$

Concordance for all paediatric samples was poor: p_c=0.26 with 95%CI (0.17 to 0.35); p_c=p(0.50) xC_b(0.52). For samples of children with optimal body mass the concordance coefficient was even lower $p_{c=}$ 0.18 with 95%CI (0.06 to 0.29); p_c=p(0.43)xC_b(0.41). Poor concordance between two methods was also found in a subgroup with overweight and obesity: pc was only 0.44 with 95%CI (0.29 to 0.57); p_c=p(0.63) xC_b(0.70) (Figure 3 A–C).

Discussion

Requests for vitamin D measurements have escalated immensely during the past 10 years. Such a big workload of requested tests has forced clinical laboratories to replace highly specific but simultaneously expensive and demanding methods such as HPLC or LC-MS/MS with fully automated techniques adapted on routine analysers. Although direct methods like isotope dilution LC-MS/MS are still considered as the "gold standard" for evaluation of 25(OH)D concentration, a wide variety of assays launched on automated analysers has become an available alternative for routine vitamin D assessment [17–18]. The new automated assays for vitamin D, which have recently emerged on the *in vitro* diagnostic market, are burdened with unsatisfactory analytical accuracy and precision as well as a lack of standardisation [19]. Analytical difficulties result from the use of unspecific antibodies against 25(OH)D, interference of different vitamin D metabolites in the assay, and ineffective separation of 25(OH)D from VDBP [20].

We evaluated the performance of two different routine methods for 25(OH)D quantification in a set of paediatric samples. The newly developed enzyme-immunosorbent method performed on a general chemistry analyser was compared to the recalibrated IDS-iSYS 25(OH)D^s immunochemiluminescence method. The mean concentration of 25(OH)D assayed with EIA method was significantly higher than the one measured with CLIA. With the use of EIA for 25(OH)D measurement, 33% of all tested paediatric samples were identified as vitamin D deficient (< 20 ng/ml) whereas with the use of CLIA 43% of samples were identified as vitamin D deficient. Differences in the assessment of the 25(OH) D status with the use of these two methods may result from the cross-reactivity reactions of antibodies used with C3-epimers present in the serum. Because of the high percentage (51-62%) of cross-reactivity with 3-epi-25-hydroxyvitamin D, the enzymatic method seems to be unsuitable for measurements of vitamin D in paediatric samples [11]. A recent study by Stepman et al. showed that 3-epi metabolites could be identified not only in children but also in adults, reaching up to

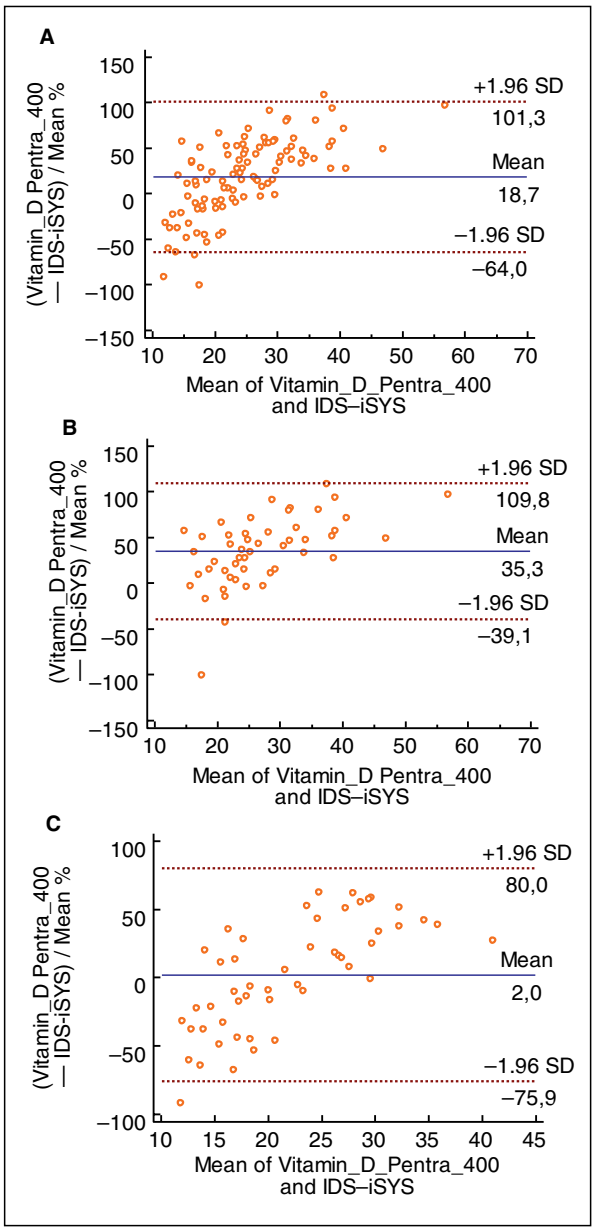

Figure 2 A-C. Bland-Altman plots illustrated bias in the whole group of participants (A), subgroup with optimal weight (B) and overweight/obesity(C).

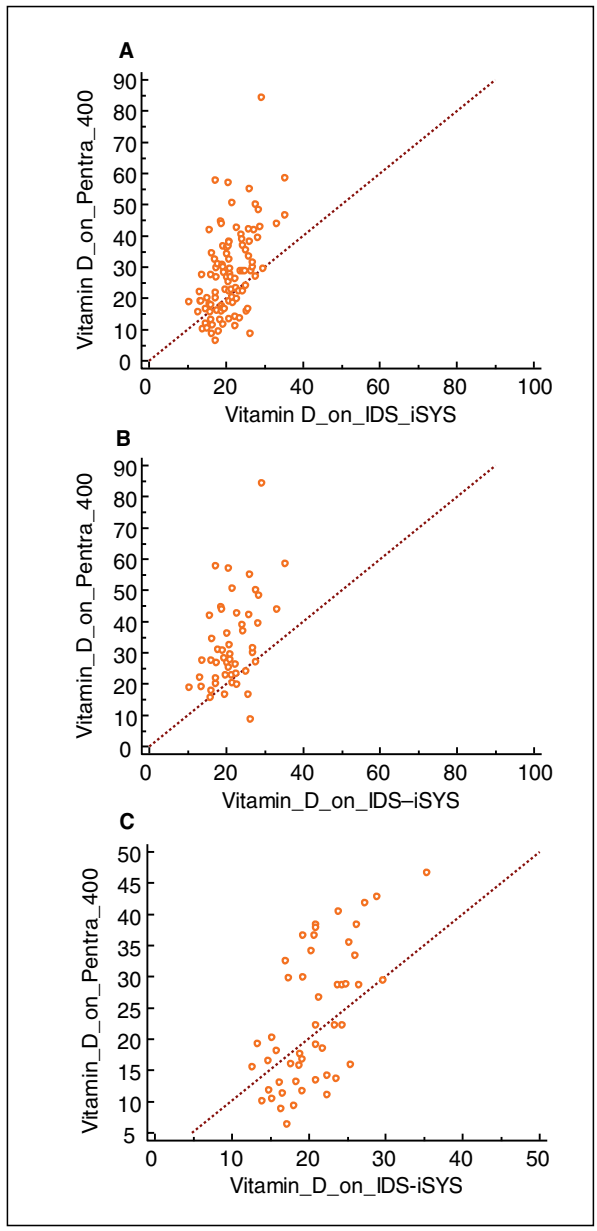

Figure 3 A-C. Scatter diagrams for concordance correlation coefficient (CCC). A- the whole group, B- subgroup with optimum weight, C- subgroup with overweight/obesity

17% of total 25(OH)D [13,17]. Evaluation of mean bias was achieved by Bland-Altman calculations. According to the Endocrine Society suggestion, the maximum acceptable performance goals for mean bias of assays measuring 25(OH)D is 15.8% [21]. In our study the mean bias for enzymatic compared to CLIA method in all paediatric samples was 18.7. In samples from overweight/obese children the mean bias was only 2.0% (LoA: 80% to -75.9%), which could result from the lower arithmetic mean of 25(OH)D concentration in this set of samples. For the purpose of our study we used two statistical parameters for comparisons between enzymatic and CLIA methods, which were: Spearman's and concordance correlation. The Spearman's rank correlation has shown positive "moderate correlation" (rho=0.47) between EIA and CLIA. Differences in correlation were identified in a set of samples from children with optimal mass (rho=0.38) and overweight/obesity (rho=0.59) with stronger positive correlation but on the "moderate" level in children with BMI ≥85 percentile. According to McBride descriptive scale the strength of agreement between enzymatic and CLIA methods

indicated a "poor correlation" (p_c=0.26) [16]. Slightly better concordance correlation was observed in overweight/obese children (p_c=0.44) but still an even "moderate" level of coefficient correlation was not achieved. Based on correlation factors calculated for 25(OH)D concentrations for enzymatic and CLIA methods, it is not possible to recommend the use of these techniques interchangeably for routine diagnostic purposes. The evaluated EIA method for 25(OH)D measurement in paediatric samples showed excessive mean bias and also unacceptable concordance correlation. Blood samples used in this study were taken from children aged 9–11 years, and we assume that the differences between 25(OH)D values obtained with the use of two chosen methods are due to cross-reactivity with 3-epimers.

Conclusions

The two compared technologies for 25(OH)D measurement, enzyme-immunosorbent and immunochemiluminescent, showed moderate correlation and poor concordance. The proper selection of assay for accurate assessment of vitamin D status in paediatric samples is necessary to avoid misdiagnosis.

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