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COMPARISON OF THREE DIFFERENT METHODS FOR 25(OH)-VITAMIN D DETERMINATION AND VITAMIN D STATUS IN GENERAL POPULATION – SERBIAN EXPERIENCE

POREĐENJE TRI RAZLIČITE METODE ZA ODREĐIVANJE 25(OH)-VITAMINA D I STATUSA VITAMINA D U OPŠTOJ POPULACIJI – SRPSKO ISKUSTVO

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Summary: Determination of 25-hydroxyvitamin D [25(OH)D] represents a unique challenge, considering its lipophilic nature. Considering the widespread prevalence of vitamin D deficiency, which leads to increasing number of requests for 25(OH)D determination, immunoassay measurements adjusted to automated analyzers are being developed. Because of the variability among assays, it is often difficult to monitor vitamin D status and supplementation. The aim of this study was to compare the results of two immunoassays with high performance liquid chromatography with ultraviolet detection (HPLC-UV). Also, the aim was to estimate vitamin D status, since up to date the prevalence of vitamin D deficiency in Serbia was not examined. We have evaluated analytical characteristics of two automated immunoassays for 25(OH)D determination, from Roche (Cobas® e601) and Abbott (Architect). For comparison studies we used HPLC analysis of 25-(OH)- Vitamin D_3/D_2 from Chromsystems (Waters isocratic system). In order to estimate vitamin D status in general population, we have searched the database of the laboratory information system and analyzed the data from 533 patients whose 25(OH)D was determined together with intact parathyroid hormone (iPTH). For imprecision assessment, four serum pools were prepared with following 25(OH)D concentrations: 35 nmol/L, ~50 nmol/L, ~75 nmol/L and ~125 nmol/L. Obtained CVs for Roche method were 1.5–2.8% for within-run and 4.0–6.7% for between-run imprecision. For Abbott method, CVs were 0.7–4.4% for

Kratak sadr`aj: Određivanje 25-hidroksivitamina D [25(OH)D] predstavlja jedinstven izazov, s obzirom da je visoko lipofilno jedinjenje. Visoka prevalencija deficijencije vitamina D uzrok je povećanja broja zahteva za određivanjem 25(OH)D, zbog čega se razvijaju imunohemijske metode prilagođene automatizovanim sistemima. Često je teško pratiti status vitamina D i suplementaciju zbog varijabilnosti između testova. Cilj ove studije bio je da se uporede rezultati dve imunohemijske metode sa tečnom hromatografijom visoke efikasnosti sa detekcijom u ultraljubičastom delu spektra (HPLC-UV). Takođe, cilj je bio i procena statusa vitamina D, pošto do sada nije ispitivana prevalencija deficijencije vitamina D u Srbiji. Ispitivane su karakteristike dve imunohemijske metode za određivanje 25(OH)D, proizvođača Roche (analizator Cobas® e601) i Abbott (na analizatoru Architect). Metode su poređene sa rezultatima HPLC analize korišćenjem 25-(OH)-Vitamin D3/D2 reagenasa firme Chromsystems (Waters izokratski sistem). Da bi se proценio status vitamina D u opštoj populaciji, pretražena je baza podataka laboratorijskog informacionog sistema i analizirani su rezultati 533 pacijenata kojima je određen 25(OH)D zajedno sa intaktnim paratiroidnim hormonom (iPTH). Pripremljena su četiri serumska pool-a sa koncentracijama 25(OH)D ~ 35 nmol/L, ~50 nmol/L, ~75 nmol/L i ~125 nmol/L za procenu nepreciznosti imunohemijskih određivanja. Dobijeni koeficijenti varijacije za Roche metodu su se kretali u opsegu 1,5–2,8% u seriji i 4,0–6,7% između serija. Za Abbott metodu su koefi-

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within-run and 3.8–7.2% for between-run imprecision. Inaccuracy was analyzed with commercial control sera. Obtained deviations from target value were 2.1% for Roche assay and 1.3–1.5% for Abbott method, and were not statistically significant (P>0.05). Comparison of Roche and HPLC-UV methods using Passing-Bablok regression analysis gave the following equation for the regression line y=0.937x+9.518 (r=0.739; n=97) and the regression line equation from the comparison of Abbott and HPLC-UV methods was y=0.745x+10.343 (r=0.793; n=97). Mean difference and SD for Bland- Altman plot were –4.5 nmol/L and 21.75 nmo/L, respectively for Roche method and 6.4 nmol/L and 18.8 nmol/L, respectively for Abbott. Statistical analysis (Chi-square test) of frequency distribution among different vitamin D status categories (<25 nmol/L severe deficiency, 25–50 nmol/L deficiency, 50–75 nmol/L insufficiency and >75 nmol/L sufficiency) showed that the frequency distribution obtained with Abbott method was significantly different from the distribution of the HPLC results, in contrast to Roche results frequency distribution which did not differ significantly. Also, statistical analysis of the agreement between the three methods for each vitamin D status category showed that results of both Roche and Abbott methods were significantly higher than HPLC in the two deficiency categories (P=0.005 for Roche, P=0.0407 for Abbott), and in the sufficiency category Abbott method significantly underestimated concentration of 25(OH)D compared to HPLC results (P<0.0001). Median population values of 25(OH)D and iPTH were 41.8 nmol/L and 76.6 ng/L, respectively. ANOVA analyses showed significant (P<0.05) decrease in iPTH and Ca^{2+} concentrations across the 25(OH)D concentration categories. Stepwise multiple linear regression analysis indicated independent correlation of iPTH with 25(OH)D concentration (β=–0.290, P=0.0008). Also, one-way ANOVA with Student-Newman-Keuls test demonstrated that 25(OH)D concentrations measured in summer and autumn were significantly (P<0.001) higher compared to those determined in winter and spring. Despite acceptable imprecision and inaccuracy of both examined methods, results obtained with them did not correlate well with HPLC-UV (r<0.9), which was used as a reference. However, methods showed satisfactory ability to classify patients into vitamin D status categories, which is important for diagnosis of vitamin D deficiency and therapy follow-up. About two thirds (68.5%) of the examined population had vitamin D deficiency (25(OH)D<50 nmol/L) and only 8% had sufficient 25(OH)D concentration (>75 nmol/L).

Keywords: 25-hydroxyvitamin D, immunoassay, HPLC, vitamin D status

cijenti varijacije iznosili 0,7–4,4% u seriji i 3,8–7,2% između serija. Netačnost je ispitivana pomoću komercijalnih kontrolnih uzoraka. Dobijena odstupanja od deklarisane vrednosti su iznosila 2,1% za Roche i 1,3–1,5% za Abbott, i nisu bila statistički značajna (P>0,05). Poređenjem Roche i HPLC-UV metoda pomoću Passing-Bablok regresione analize dobijena je sledeća regresiona jednačina y=0,937x+9,518 (r=0,739; n=97), dok regresiona jednačina dobijena poređenjem Abbott i HPLC-UV metoda glasi y=0,745x+10,343 (r=0,793; n=97). Srednja vrednost razlika na Bland-Altman dijagramu razlika i standardna devijacija su iznosile –4,5 nmol/L i 21,75 nmo/L, redom, za Roche metodu i 6,4 nmol/L i 18,8 nmol/L, redom, za Abbott metodu. Statistička analiza (Chi-kvadrat test) distribucije frekvencija među različitim kategorijama statusa vitamina D (<25 nmol/L teška deficijencija, 25–50 nmol/L deficijencija, 50–75 nmol/L insuficijencija i >75 nmol/L preporučena koncentracija) je pokazala da je distribucija frekvencija dobijena Abbott metodom značajno različita od distribucije HPLC rezultata, za razliku od raspodele frekvencija dobijene Roche metodom koja se nije značajno razlikovala. Takođe, statistička analiza slaganja između ispitivane tri metode u svakoj od kategorija statusa vitamina D je pokazala da su rezultati i Roche i Abbott metoda značajno veći od HPLC-UV u kategorijama deficijencije vitamina D (P=0,005 za Roche; P=0,0407 za Abbott), i u kategoriji sa preporučenom koncentracijom vitamina D Abbott metoda je značajno potcenjivala koncentraciju 25(OH)D u poređenju sa HPLC rezultatima (P<0,0001). Medijana za 25(OH)D u ispitivanoj populaciji bila je 41,8 nmol/L, i 76,6 za iPTH. ANOVA analiza je pokazala značajan pad (P<0,05) koncentracija iPTH i jonizovanog kalcijuma između kategorija koncentracija 25(OH)D. Multiplom linearnom regresionom analizom utvrđena je nezavisna korelacija između koncentracija iPTH i 25(OH)D (β =–0,290; P=0,0008). Takođe, ANOVA za jedan kriterijum klasifikacije sa Student-Newman-Keuls testom je pokazala da su koncentracije 25(OH)D određene u leto i jesen značajno više (P<0,001) u poređenju sa onima određenim u zimu ili proleće. Uprkos prihvatljivoj neprecisznosti i netačnosti obe ispitivane imunohemijske metode, dobijeni rezultati nisu u zadovoljavajućoj korelaciji sa HPLC-UV metodom (r<0,9), koja je korišćena kao referentna u ovom slučaju. Uprkos ovoj činjenici, metode su pokazale zadovoljavajuću sposobnost klasifikacije pacijenata u kategorije statusa vitamina D, što je važno za dijagnozu deficijencije vitamina D i praćenje terapije. Oko dve trećine (68,5%) ispitivane populacije je imalo deficijenciju vitamina D (25(OH)D<50 nmol/L) i samo 8% je imalo preporučenu koncentraciju 25(OH)D (>75 nmol/L).

Klju~nere~i: 25-hidroksivitamin D, imunohemijska određivanja, HPLC, status vitamina D

Introduction

For years, vitamin D was considered essential only for bone health in children and adults. However, with the discovery of presence of vitamin D receptor in most tissues and cells throughout the body, it became clear that vitamin D had an important role not only in the prevention of osteoporosis and osteomalacia, but also in the prevention of many chronic ilnesses, like cancers, autoimmune, infectious and cardiovascular diseases (1). These findings have caused the considerable increase in number of requests for vitamin D status estimation, which initiated the development of automated assays capable to cope with this rise in laboratory workload.

The best biomarker of vitamin D status is its circulating form, 25-hydroxyvitamin D [25-(OH)D]. There are two major vitamin D metabolites in the circulation, endogenosly synthesized 25-hydroxyvitamin D_3 (cholecalciferol, 25(OH)D_3), and 25-hydroxyvitamin D_2 (ergocalciferol, 25(OH)D_2) derived from the plants. Therefore, assays used to determine vitamin D status and nutritional deficency should measure both

forms, 25(OH)D_2 and 25(OH)D_3. In circulation, 95% of circulating 25(OH)D represents 25(OH)D_3, while 25(OH)D_2 is usually a minor fraction, unless vitamin D_2 supplements are used by the patient. Measurement of 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], metabolicaly active form of vitamin D, should not be used to determine vitamin D status, since patients with vitamin D deficiency and secondary hyperparathyroidism most of the time have normal or even increased concentrations of 1,25(OH)$_2$D (2).

Determination of 25(OH)D represents a unique challenge, considering its lipophilic nature and strong binding to vitamin D-binding protein (DBP). Traditionally, assays for 25(OH)D contain pretreatment steps, like deproteinization, extraction and purification, followed by quantification. Deproteinization or extraction frees metabolite from DBP and may partially purify it. Purification steps, most often column chromatography, separate the various forms of vitamin D, lipid and interfering substances (3). Pretreatment is part of the two non-immunological assays, high performance liquid chromatography (HPLC) linked to ultra-violet (UV) detector and linked to mass detectors. The later procedure is commonly termed LC-MS/MS or tandem mass spectrometry (4). The two candidate reference methods for 25(OH)D determination are the LC-MS/MS method by Tai et al. (5), recognized by the Joint Committee for Traceability in Laboratory Medicine, and LC-MS/MS method published by the Laboratory for Analytical Chemistry at Ghent University (6). Unfortunately, these methods are rather time consuming and laborious so that nowadays there is a tendency for the development of automated 25(OH)D methods. Current commercial immunoassays are founded on two major principles – competitive protein binding assays that use DBP as the binder, and immunoassays that employ polyclonal or monoclonal antibodies directed against 25(OH)D. However, manufacturers of 25(OH)D immunoassay methods had to replace pretreatment extraction step with blocking agent in order to include these immunoassays on automated platforms, which led to greater imprecision when compared to chromatographic methods. Also, these methods are often limited in equipotent quantification of both forms of 25(OH)D – 25(OH)D_3 and 25(OH)D_2, compared with HPLC and LC-MS/MS methods which quantitatively measure circulating concentrations of both forms (7, 8).

For the reasons mentioned above, and because of the lack of harmonization of all assays, it is not a rare case that a physician obtain different results of 25(OH)D measurement for an individual patient from different laboratories that use assays based on different measuring principles or from different manufacturers (9). Therefore, the aim of this study was to compare two automated immunoassays with HPLC-UV method for the measurement of 25(OH)D that are commonly used and available in laboratories of the Center for Medical Biochemistry of the Clinical Centre of Serbia. Since numerous studies have revealed that the prevalence of vitamin D deficiency and insuficiency is >50% in the general population, and considering uncovered associations with increased risks for hypertension, type II diabetes, colorectal and breast cancers, myocardial infarction, strokes, and peripheral vascular disease (1, 10), the aim was also to estimate vitamin D status among Serbian population, since up to date the prevalence of vitamin D deficiency in Serbia was not examined.

Material and Methods

Comparison Studies

For method comparison we used 100 serum samples from routine 25(OH)D assay requests processed in the laboratory of Department for Poliklinic Laboratory Diagnostics in the Center for Medical Biochemistry of Clinical Centre of Serbia in Belgrade. Remained amounts of samples, after the completion of analysis requested by attending physicians, were aliquoted, stored at –70 °C and analyzed in batch with all three methods. For three samples, the collected volume was <500 μL, which was not enough for analysis with the HPLC-UV method, why we used the results of 25(OH)D determination in 97 samples for statistical analysis. We compared results of 25(OH)D determination obtained with electrochemiluminescent immunoassay, Elecsys® Vitamin D total (Roche Diagnostics GmbH, Mannheim, Germany) performed on Cobas® e601 analyzer, and of chemiluminescent microparticle immunoassay, ARCHITECT 25-(OH) vitamin D (Abbott Diagnostics, Wiesbaden, Germany) performed on Architect® ci8200 analyzer, with the results of HPLC analysis of 25-(OH)- Vitamin D_3/D_2 (Chromsystems Instruments & Chemicals GmbH, Munich, Germany) using Waters isocratic HPLC-UV system. The Roche method uses competitive protein binding principle and the Abbott method employs sheep polyclonal antibodies directed against 25(OH)D. HPLC-UV method was used as the reference for comparison because, by means of efficient protein precipitation and selective solid phase extraction, interfering components are removed and the analytes are concentrated, which increases the sensitivity and specificity of this method of determination.

For imprecision assessment of chemiluminescent immunoassays, four serum pools were prepared. Samples were mixed to obtain the following 25(OH)D concentrations: ~35 nmoL/L, ~50 nmol/L, ~75 nmol/L and ~125 nmol/L. Each pool was aliquoted and stored at –70 °C until use. For within-run imprecision 25(OH)D was determined in each serum pool with 10 replicates per run. Between-run imprecision was evaluated by analyzing every pool on 10 successive days on the basis of a single calibration. Inaccuracy was analyzed with commercial control sera for corresponding methods (PreciControl Bone, Roche Diagnostics

GmbH, Mannheim, Germany, and ARCHITECT 25-(OH) Vitamin D Controls, Abbott Diagnostics, Wiesbaden, Germany). 25(OH)D concentration in each control serum was measured 20 times to determine bias from expected values.

The concentrations of 25(OH)D in 97 serum samples measured with the two chemiluminescent immunoassays and the HPLC-UV method were statistically analyzed by Passing-Bablok regression and Bland-Altman plots (11, 12).

In order to evaluate concordance between the methods more accurately, their agreement in the ability to diagnose vitamin D deficiency was examined. 25(OH)D concentrations measured in 97 serum samples were classified into categories according to the vitamin D status. The categories were defined on the basis of established cut-off values for severe deficiency (<25 nmol/L), deficiency (25–50 nmol/L), insufficiency (50–75 nmol/L) and sufficiency (>75 nmol/L) (1). The frequency distribution of results of 25(OH)D determinations with HPLC-UV method according to these categories was compared with frequency distributions of 25(OH)D results obtained with both chemiluminescent immunoassays. We used Chi-square test on frequency table to determine whether the frequency distributions of immunoassay results were significantly different from the distribution of HPLC-UV values. Statistical analysis of mean difference of 25(OH)D concentrations obtained with HPLC-UV and immunoassays for each vitamin D status category was also performed. Category classification was based on values obtained by HPLC-UV method. Student t-test was used to determine whether there were differences between means in corresponding categories obtained with HPLC-UV method and examined immunoassays.

Vitamin D status estimation

To estimate vitamin D status in Serbian population, we searched the database of the laboratory information system (LabOnLine Ver. 2 Rel. 2.03; Omnilab, Milan, Italy) in the Department of Polyclinic Laboratory Diagnostics of the Center for Medical Biochemistry in Clinical Centre of Serbia. From over 2000 determinations of 25(OH)D_3, using Elecsys® Vitamin D_3 (25-OH) assay, in period from 4 November 2008. to 26 May 2010., 533 patients had also the values for intact parathyroid hormone (iPTH), total and ionized calcium and inorganic phosphorus, and this group of results was included in statistical analysis of vitamin D status in Serbian population.

Normally distributed continuous variables are presented as the mean and standard deviation. To determine whether the distribution was Gaussian Kolomogorov-Smirnoff test was applied. Continuous variables with non-Gaussian distribution are presented as geometric mean and 95% confidence interval (CI) for mean, and in consecutive statistical analysis have been log transformed. The serum 25(OH)D_3 concentrations were categorized into four vitamin D status groups. Comparison of mean values of continuous variables between categories was performed by one-way ANOVA. Chi-square test for contingency tables was used for the analysis of categorical variables. Multiple linear regression analysis was conducted to determine independent contribution of examined variables to the change in concentration of 25(OH)D_3.

We analyzed the seasonal variation of 25(OH)D in the examined population. Time period for individual season was defined according to the calendar as spring from 21 March to 21 June, summer from 22 June to 22 September, autumn from 23 September to 21 December, and winter from 22 December to 20 March. Average concentrations in each season were compared using one-way ANOVA. Also, we examined the frequency distribution of each vitamin D status category depending on the season using Chi-square test.

Statistical significance was assumed at $P<0.05$. We performed all statistical calculations using MedCalc® Ver. 12.1.3 (MedCalc software, Mariakerke, Belgium), CB Stat Ver. 4.3 (Kristian Linnet, Risskov, Denmark) and SPSS for Windows 11.5 (Chicago, Illinois, USA) computer softwares.

Results

Comparison Studies

Results of the imprecision assessment of the two examined chemiluminescent immunoassys are presented in *Table I*. Analysis of four serum pools with wide range of 25(OH)D concentrations obtained within-run imprecision (CV) in the range 0.7–4.4% for Abbott and 1.5–2.8% for Roche immunoassay, and between-run CVs were 3.8–7.2% and 4.0–6.7% for Abbott and Roche, respectively.

Results of Student t-test showed that there were no statistically significant differences between means of results of repeated measurements of 25(OH)D and expected values of analyzed control sera, with bias around 2.1% for Roche and between 1.3 and 1.5% for Abbott assay (*Table II*).

The correlation of results of 25(OH)D determination obtained using chemiluminescent immuno assays and HPLC-UV results was examined with Passing-Bablok regression analysis. Spearman's correlation coefficients for Abbott and Roche methods were 0.793 and 0.739, respectively. Regression equations showed proportional bias significantly different from 0 for Abbott method, which was 10.343 nmol/L (95% CI=3.531–15.485 nmol/L, $P<0.05$), but not for the

Table I Within-run and between-run imprecision assessment for 25(OH)D determination by Roche and Abbott immunoassays.

pool	Within-run						Between-run					
	Roche			Abbott			Roche			Abbot		
	$\bar{x}$ (nmol/L)	SD (nmol/L)	CV (%)	$\bar{x}$ (nmol/L)	SD (nmol/L)	CV (%)	$\bar{x}$ (nmol/L)	SD (nmol/L)	CV (%)	$\bar{x}$ (nmol/L)	SD (nmol/L)	CV (%)
1	49.0	1.35	2.8	49.2	1.18	2.4	49.5	2.00	4.0	48.8	1.85	3.8
2	78.8	2.12	2.7	79.8	1.32	1.7	81.0	4.02	5.0	79.2	4.25	5.4
3	126.0	1.88	1.5	125.8	0.88	0.7	127.5	5.38	4.2	125.8	7.30	5.8
4	37.2	1.05	2.8	35.5	1.55	4.4	36.5	2.42	6.7	34.5	2.48	7.2

Table II Inaccuracy assessment for 25(OH)D determination by Roche and Abbott immunoassay (N=20; $t_{0.05}$=2.101).

Control	Expected value (nmol/L)	$\bar{x}$ (nmol/L)	SD (nmol/L)	$t_{0.05}$	P*	Bias (%)
Roche						
PC Bone 1	35.2	36.0	2.28	1.936	0.0612	2.1
PC Bone 2	71.8	73.2	6.05	1.603	0.1169	2.1
Abbott						
Control L	50.0	49.2	1.18	1.751	0.1138	1.3
Control M	100.0	101.5	3.98	1.234	0.2484	1.5
Control H	187.5	184.8	5.70	1.472	0.1752	1.5

*P>0.05 – no statistically significant difference

Roche method, where it was 9.518 nmol/L (95% CI=–1.748–16.727, P>0.05). The constant bias was 0.745 for the Abbott method, which was also significantly different from 1 (95% CI=0.641–0.857, P<0.05), and not for the Roche method with the value of 0.939 (95% CI=0.791–1.129, P>0.05) (*Figures 1A and 1B*).

The agreement between HPLC-UV and immunoassays was also examined using Bland-Altman difference plots. The absolute difference plot showed the mean difference between HPLC and Abbott method of 6.4 nmol/L (95% CI=–31.4–44.1 nmol/L) and between HPLC and Roche method of –4.5 nmol/L (95% CI=–48.0–39.0 nmol/L) (*Figures 1C* and *1D*). The correlation coefficients between the numerical value of the difference between HPLC and Abbott and the mean value of these two determinations was r=0.342, which was significantly different from 0 (P<0.001), and for HPLC and Roche was r=0.203 (0.02<P<0.05), meaning that the scatter of differences tends to increase proportionally with the measurement level. This was confirmed on the relative difference plot, where the mean relative difference between HPLC and Abbott was 6.9% (95% CI=–66.8–80.6%), with r=–0.23 significantly different from 0 (0.01<P<0.05), and –10.7% (95% CI=–93.3–72.0%) with r=–0.424 (P<0.001) for HPLC and Roche, showing that the scatter of relative differences was not constant for either methods (*Figures 1E and 1F*).

The frequency distribution in vitamin D status categories obtained according to results of 25(OH)D determinations with HPLC-UV method was used as a reference in comparison with frequency distributions based on results of chemiluminescent immunoassays (data not shown). Results of Chi-square test showed statistically significant difference between the distributions of concentrations obtained with HPLC and Abbott methods (P=0.0293), but the difference between HPLC and Roche distributions was not significantly different (P=0.1858).

Statistical analysis of difference between means of 25(OH)D concentrations obtained with HPLC-UV and immunoassays for each vitamin D status category is presented in *Table III*. The results showed statistically significant difference for mean values obtained with the Abbott method from HPLC results in the category of severe deficiency (<25 nmol/L) and sufficiency (>75 nmol/L), while the difference had borderline significance (P=0.0521) in the group of persons with insufficiency (50–75 nmol/L). The mean concentrations of 25(OH)D determined using the Roche

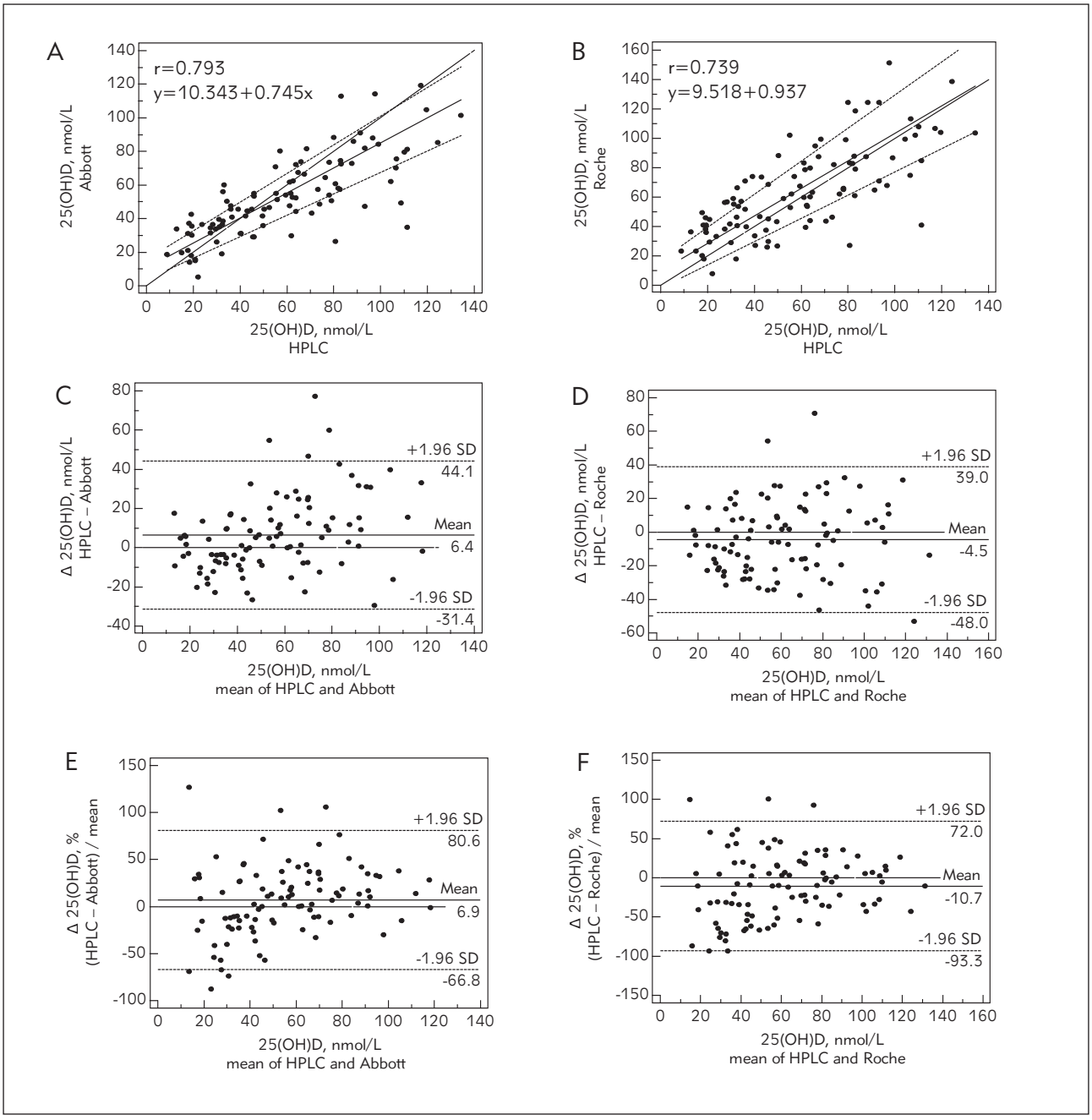

Figure 1 Comparison of Abbott and Roche immunoassays against HPLC-UV by Passing-Bablok regression analysis (panels A and B, respectively) and Bland-Altman plots (panels C–F). On Passing-Bablok plots the full line represents the regression line and the dotted line represents the line of unity y=x. Panels C and D represent absolute difference plots, and panels E and F show relative difference plots for Abbott and Roche immunoassays against HPLC, respectively. On Bland-Altman plots, the mean difference in the absolute difference plot and the mean relative difference in the relative difference plot are displayed as dotted lines, and the 95%-confidence limits for individual absolute and relative differences are displayed as dashed lines.

method were significantly different from those obtained with HPLC in both deficiency categories (values obtained with the Roche method were higher than those obtained with HPLC), while they were not significantly different from the HPLC results in insufficiency and sufficiency categories.

Vitamin D status estamination

Median $25(OH)D_3$ concentration in the examined population was 41.8 nmol/L. Among selected results from the laboratory information system data base, 134 individuals had $25(OH)D_3 < 25$ nmol/L (12

Table III Statistical analysis of method differences by vitamin D status categories.

25(OH)D (nmol/L)	$\bar{x}$, nmol/L			HPLC-Abbott			HPLC-Roche		
	HPLC	Abbott	Roche	Mean difference[a] (nmol/L)	SD of difference, (nmo/L)	P[b]	Mean difference[a] (nmol/L)	SD of difference, (nmol/L)	P[b]
<25	18.38	24.72	32.28	–6.34	11.575	0.0407	–13.90	11.775	0.0005
25–50	36.95	39.80	47.45	–2.85	10.650	0.1497	–10.50	17.275	0.0115
50–75	61.90	56.30	66.58	5.60	13.575	0.0521	–4.68	20.675	0.2688
>75	95.88	74.72	90.48	21.15	22.375	<0.0001	5.40	27.325	0.2914

[a] Vitamin D status assignment of samples was based on their HPLC r esults of 25(OH)D.
[b] A t-test was performed to determine whether means of 25(OH)D values obtained from Abbott and Roche methods were statistically significantly different from those obtained by the HPLC-UV method.

Table IV Characteristics of the examined population accor ding to four groups of 25(OH)D_3 levels.

	25(OH)D_3, nmol/L				P[a]
	<25 n=134 (25.1%)	25–50 n=231 (43.3%)	50–75 n=124 (23.3%)	>75 n=44 (8.3%)	
Age[b, c] (years)	56.9 (54.3–59.6)	54.8 (52.7–56.9)	57.3 (54.8–59.8)	52.4 (48.1–57.4)	0.181
Gender (male/female)	12/122	42/189	14/110	4/40	**0.047**
Ca[d] (mmol/L)	2.37±0.150	2.42±0.167	2.35±0.164	2.38±0.119	0.058
Ca^{2+} [b, c] (mmol/)L	1.28 (1.24–0.31)	1.28 (1.26–1.30)	1.23 (1.21–1.26)	1.29 (1.26–1.33)	**0.016**
P[d] (mmol/L)	1.16±0.175	1.12±0.211	1.16±0.164	1.16±0.119	0.504
iPTH[b, c] (ng/L)	74.4 (67.0–82.7)	64.1 (59.9–68.8)	63.2 (57.4–69.7)	59.1 (50.6–69.0)	**0.027**
25(OH)D_3[b, c] (nmo/L)	14.2 (13.0–15.5)	37.0 (36.0–38.0)	59.5 (58.2–60.5)	89.2 (84.5–94.2)	<0.0001

[a] One-way ANOVA across the four 25(OH)D level groups for continuous variables and chi-square test for categorical variables.
[b] Statistical tests on $\log_{10}$-transformed values.
[c] Geometric mean and 95% confidence interval (CI) for mean.
[d] Arithmetic mean ± SD.
Ca, calcium; Ca^{2+}, ionized calcium; P, inorganic phosphorus; iPTH, intact parathyroid hormone; 25(OH)D_3, 25-hydroxyvitamin D_3.

males, 122 females), in 231 individuals (42 males, 189 females) 25(OH)D_3 was 25–50 nmol/L, 124 persons (14 males, 110 females) had 25(OH)D_3 between 50 and 75 nmol/L and in the category of 25(OH)D_3> 75 nmol/L 44 persons were classified (4 females, 40 males). Characteristics according to the four vitamin D status groups are summarized in *Table IV*.

ANOVA analysis indicated significant (P<0.05) changes in ionized calcium concentration (P=0.016) and significant decr ease in iPTH concentraction (P=0.027) with the incr ease of 25(OH) concen - tration, while the changes in concentrations of total calcium showed bor derline significance (P=0.058). Chi-square test r evealed significant differ ence in the percent of gender distribution among vitamin D status categories (P=0.047). Consequently, a stepwise multiple linear regression model showed that the concentration of 25(OH)D_3 correlated significantly only with iPTH (β=–0.290, P=0.0008). It explained 6.5% of the variance in the values of 25(OH)D_3 (P=0.001).

Average concentrations of 25(OH)D_3 in different seasons are compared with one-way ANO VA and the results are presented in *Table V*. The results showed statistically significant differ ence in the average 25(OH)D_3 concentration depending on the season. Concentrations were significantly higher in summer and autumn when compar ed with those measured in winter or spring.

Table V One-way ANOVA of differences in 25(OH)D concentration between seasons.

	Season				P
	spring	summer	autumn	winter	
25(OH)D[1a] (nmo/L)	31.2[2, 3] (28.68–34.05)	58.4[1,3,4] (52.68–64.75)	37.8[1,2,4] (34.00–42.12)	30.2[2,3] (37.02–33.72)	<0.001

[a]Geometric mean and 95% confidence interval (CI) for mean
[1]significantly different from the first group (spring) – Student-Newman-Keuls test
[2]significantly different from the second group (summer) – Student-Newman-Keuls test
[3]significantly different from the third group (autumn) – Student-Newman-Keuls test
[4]significantly different from the fourth group (winter) – Student-Newman-Keuls test

Table VI Frequency distribution in vitamin D status categories in different seasons.

		season				total	P*
		spring	summer	autumn	winter		
25(OH)D (nmo/L)	<25	54	5	24	51	134	<0.0001
	25–50	82	12	62	75	231	<0.0001
	50–75	32	28	28	36	124	0.7010
	>75	7	18	13	6	44	0.036
total		175	63	127	168	533	<0.0001

*Chi-square test on tabulated data.

Also, we examined the frequency distribution of individual vitamin D status categories in different seasons. Chi-square test showed that the differences in the distribution are statistically significant (P<0.0001), where the percentage of persons with 25(OH)D_3 concentration <25 nmol/L varied significantly between seasons, and the highest was in winter (38.1%) and spring (40.3%). Also, the percentage of persons with 25(OH)D_3 concentration in the range 25–50 nmol/L changed significantly (P<0.0001) and the highest was in spring, 46.9%. The percentage of persons with 25(OH)D_3 concentration between 50 and 75 nmol/L was relatively constant and there was no significant difference between seasons (P=0.7010), while, as expected, the percentage of persons with 25(OH)D_3 values >75 nmol/L changed significantly (P=0.036) and the highest was in summer (28.6%). Cross-classification of vitamin D status frequency distribution among seasons is represented in *Table VI*.

Discussion

Because of the great differences between the determination principles of various 25(OH)D methods, the variation of results among laboratories using different methods exists. In Serbia, most of the laboratories use several immunoassays for 25(OH)D determination, depending on the automated platform available. In this study, we compared the two immunoassays used in the laboratory of Department for Polyclinic Laboratory Diagnostics in the Center for Medical Biochemistry of Clinical Centre of Serbia in Belgrade with the commercial HPLC method with UV detection. Immunoassays used different principles – competitive protein binding (Roche) and polyclonal sheep antibody employment (Abbott). For both methods is stated that they determine both 25(OH)D_2 and 25(OH)D_3, though the determination of 25(OH)D_2 is of little importance in Serbia, since the registered supplements are only vitamin D_3 preparations. We used the HPLC-UV method as a reference because it uses completely different principle and requests sample pretreatment which removes interferences.

Imprecision assessment of the two immunoassays was conducted using four serum pools which encompassed a wide range of 25(OH)D concentrations (*Table I*) and inaccuracy was examined using corresponding commercial control sera for each method (*Table II*). The obtained results were within the defined limits according to specifications for trueness and precision for 25(OH)D analysis for routine testing, where imprecision and bias should be ≤10% and ≤5%, respectively (13, 14).

However, the comparison of the two immunoassays with HPLC -UV method as a reference using Passing-Bablok regression analysis gave correlation coeficients <0.9, which showed that the agreement and correlation between methods was not acceptable. Besides, obtained slope and intercept together with the Bland-Altman analysis showed the statistically significant difference between the paired determinations for both HPLC–Roche and HPLC–Abbott.

These differences were not constant, but rather increased with the 25(OH)D concentration. The lack of correlation between immunoassays and HPLC reflected on their agreement in the diagnosis of vitamin D deficiency. The results of Chi-square test showed significant difference between the frequency distribution across vitamin D status categories only between Abbott and HPLC but not for Roche. However, examination of differences in means of 25(OH)D concentrations obtained with HPLC -UV and immunoassays for each vitamin D status category (*Table III*) revealed significant differences for both immunoassays, with the Roche method giving higher values in the severe deficiency and deficiency category, and the higher values were obtained in the severe deficiency category and lower results in the sufficiency category with the Abbott method.

First of all, the lack of satisfactory analytical correlation between the examined immunoassays and HPLC-UV could be explained with the different calibrator traceability. For calibration of the HPLC method National Institute for Standards and Technology standard reference material (NIST SRM 2972) was used as the primary reference material, which is also used as the calibrator for the reference LC-MS/MS method. Roche method can also be traceable to the same primary reference material through standardization against LC-MS/MS calibrated with NIST SRM 2972. This might be the reason for somewhat better agreement of the Roche method with HPLC, compared with the Abbott method, which was standardized against internal reference material. Also, poor method comparability may be related to the specificity of different methods and the possibility to cross-react with other metabolites of 25(OH)D. Most immunoassays, for example, show significant cross-reactivity with dihydroxy and other vitamin D metabolites – 24,25(OH)$_2$D$_3$, 25,26(OH)$_2$D$_3$, 25(OH)D$_3$-26,23-lactone. These metabolites, especially 24,25-dihydroxyvitamin D metabolites, circulate at up to 10–15% of the 25(OH)D concentration and their presence could increase the 25(OH)D concentration measured by immunoassays (4). This may explain higher values obtained with immunoassays when determining values of 25(OH)D below 50 nmol/L, which is the border between deficiency and insufficiency. Also, spuriously high results may be caused by matrix effects, which occur between the matrix in calibrators and patient samples in the presence of other lipids in serum or plasma sample, that change the ability of the binding agent to associate with 25(OH)D in the sample and the standard in equal fashion (4). Hopefully, these issues with the examined and other immunoassays will be minimized when the results of the ongoing Vitamin D Standardization Program (VDSP) are published, which should introduce SRM 972a, the human serum panel for calibration of immunoassays (15, 16).

Although the correlation between HPLC-UV and immunoassays was not satisfactory, mean values of immunoassay results in each vitamin D status category defined according to HPLC did not exceed the category limit (*Table III*). This implies that the examined immunoassays have acceptable ability to classify patients into appropriate vitamin D status categories, which is important for diagnosis of vitamin D deficiency and monitoring supplementation therapy. Both methods overestimate the values in the severe deficiency category, with Roche means exceeding the category limit of 25 nmol/L, but the mean values remain within the deficiency category (25–50 nmol/L) where therapy is indicated. The potential problem might be the underestimation of results obtained with Abbott assay in the sufficiency category, where mean value remain below the sufficiency limit of 75 nmol/L. This might draw the wrong conclusion that the patient is not responding to supplementation treatment.

The limitation of this study was that HPLC-UV is not a reference method for 25(OH)D determination. For definitive assessment of analytical characteristics of two examined immunoassays, they should be compared with the reference LC-MS/MS method.

Numerous studies have assessed the prevalence of vitamin D deficiency in general population (17–20). Up to date the prevalence of vitamin D deficiency in Serbia was not examined. If we use the 50 nmol/L values as the cut off between the deficiency and relative insufficiency, 68.5% of examined population lack vitamin D, while 25.1% is already under severe deficiency, with 25(OH)D concentration <25 nmol/L (*Table IV*). Only about 8% of population have sufficient 25(OH)D concentration. The situation is similar in USA, where the prevalence of low 25(OH)D concentrations (<50 nmol/L) is around 36% in healthy young persons 18–29 years old, 42% in African-American women age between 15 and 49, 41% in outpatients 43–83 years of age and up to 57% of hospital patients. In Europe, the prevalence is even higher, with 28–100% in healthy persons and 70–100% in hospital patients (21).

The low vitamin D levels in examined population was accompanied by increase in iPTH concentration (*Table IV*), which was showed to be statistically significant with one-way ANOVA and confirmed its independent influence in the change of 25(OH)D concentration using multiple linear regression analysis. These results are in accordance with other studies which found negative correlation between 25(OH)D and iPTH levels (17, 19).

Concentration of 25(OH)D significantly varied with the change of seasons, reaching maximum concentrations in summer and minimum in winter and spring (*Table V*). Percentage of persons with 25(OH)D concentration <25 nmol/L was the highest in winter (38.1%) and in spring (40.3%), while the percentage

of people with 25(OH)D concentration >75 nmol/L was the highest in summer, when it was 28.6% (*Table VI*). There are several potential explanations for high prevalence of vitamin D deficiency in Serbian population. It is considered that 90% of necessary amount of vitamin D is synthesized in the skin after the exposure to sunlight. Anything that disturbs the penetration of UV radiation into the skin will reduce vitamin D_3 production in skin, including clothes and sunscreens. Also, solar UV radiation exposure is influenced by altitude, season, pollution and clouds (22). On latitudes above 37° N and below 37° S, the intensity of solar radiation is not enough to induce vitamin D_3 synthesis during winter months (1, 21). Reasons that promote vitamin D deficiency in older persons include decreased food intake, decreased sun exposure, but also the decreased ability of the skin to synthesize vitamin D_3. In the skin of 70 year old person, skin's ability to synthesize vitamin D_3 is four times less compared with the 20-year old (23, 24). Dietary sources of vitamin D are limited and intake of adequate amounts often represents a problem for most people whose diet does not include rare natural sources. Also, patients with malabsorption are under particular risk of vitamin D deficiency.

Serbia is located at the 44° N, which is above the mentioned 37° N and where the sunlight intensity is not enough to induce vitamin D_3 synthesis in the skin during the winter. For this reason, concentrations achieved during summer should be high enough to provide the amounts of vitamin D throughout the year. Since even in the period of the highest synthesis 25(OH)D concentration does not reach the cutoff for sufficiency of 75 nmol/L, in the examined population we have median iPTH concentration of 65 ng/L, which is at the very upper limit of the reference interval (15–65 ng/L), and represents an increased risk for secondary hyperparathyroidism. This is contributed by the diet, traditionally poor in fatty sea fish and fish oil, natural sources of vitamin D, while there is no national program of food fortification.

This study of vitamin D deficiency prevalence have certain limitations since the examination was not controlled and other information about participants and their medical history weren't available, except for gender and age. Also, other parameters which would be significant for vitamin D status assessment, like creatinin, as an indicator of kidney function, and lipid status, weren't known.

In conclusion, even though the lack of standardization of immunoassays and chromatographic methods for 25(OH)D status determination exists, why most probably we have obtained poor method comparison results, examined immunoassays showed satisfactory results in classifying patients into appropriate vitamin D status categories compared with HPLC-UV method. Although the results for vitamin D deficiency prevalence showed significant number of vitamin D deficient individuals, these findings must be considered as preliminary, because the analyzed results belonged to persons whose medical history was not available and the sample size was too small to draw conclusions regarding the general population. Even with these limitations, high prevalence of vitamin D deficiency in the examined population should alarm health services to initiate vitamin D food fortification program, because this is known to be the most successful way of preventing health complications caused with vitamin D deficiency.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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