VITAMIN D AND ITS METABOLITES – SUPPLY OF PATIENTS WITH VARIOUS ENDOCRINE DISORDERS AND COMPARISON OF ANALYTICAL METHODS

MICHALA VOSATKOVA, PETRA HOSKOVCOVA, RADOVAN BÍLEK

Institute of Endocrinology, 116 94 Prague, Czech Republic
email: rbilek@endo.cz

Objective. Vitamin D is important not only for its effect on the homeostasis of calcium, but also for its anti-proliferative, pro-differentiation, pro-apoptotic and immuno-modulating effects in the tissues of living organisms. This work describes the results of serum 25(OH)D (n=2175) and 1α,25$(OH)_2$D (n=2271) radioimmunoassays (RIA) conducted at the Institute of Endocrinology in Prague (Czech Republic) during the period of 2004-2006.

Methods. Serum concentrations of vitamin D metabolites were determined by RIA kits from IDS Ltd., Boldon, UK. In a group of 20 healthy volunteers, results obtained using the RIA kit were compared with those obtained after serum extraction with acetonitrile, subsequent chromatographic (RP HPLC) separation of 25(OH)D and its detection in collected fractions using the same RIA kit from IDS.

Results. The mean concentrations ± S.D. in the samples studied were 76.4 ± 45.6 nmol/l for 25(OH)D and 88.9 ± 45.1 pmol/l for 1α,25$(OH)_2$D. The mean concentrations of both metabolites were higher in women than in men, but only the difference for 1α,25$(OH)_2$D) was statistically significant (p=0.0000). The lowest concentrations of 25(OH)D or 1α,25$(OH)_2$D were found in patients with hyperparathyroidism, the highest concentrations those treated with cholecalciferol for osteoporosis and vitamin D deficiency. The relationship between 25(OH)D and 1α,25$(OH)_2$D was expressed by the equation 1α,25$(OH)_2$D = 71.0845 + 0.1890 * 25(OH)D (n=1065, p=0.0000, R^2=0.0343). Based on the RIA results, only 4 % of individuals were inadequately supplied with 25(OH)D, 79 % were supplied adequately and almost 17 % were supplied more than adequately. Similarly, 1α,25$(OH)_2$D shows inadequate concentration in 15 % of the individuals tested, 59 % of these individuals were within the reference range and 26 % had values exceeding the upper limit of the reference range. The mean concentrations of 25(OH)D obtained by HPLC separation were in average by 8 % higher than those obtained using only the RIA kit.

Conclusion. Interpretation of studies dealing with vitamin D stores must be based on precise and correct analytical procedures. However, only a few information exists about the influence of other vitamin D metabolites and conjugates on the concentration of immunoanalytically measured serum 25(OH)D or 1α,25$(OH)_2$D.

Key words: 1α,25-dihydroxyvitamin D – 25-hydroxyvitamin D – radioimmunoassay – serum – chromatography – comparison of methods

Vitamin D is acquired by the organism exogenously in the form of vitamin D_2 (ergocalciferol) which is present in plants and fungi, but the main source of vitamin D is its endogenous biosynthesis from the precursor – 7-dehydrocholesterol. After the exposure of skin to sunlight at wavelengths of 290-315 nm, the precursor undergoes photolysis which results in the opening of ring B. Spontaneous isomeration of such inter-

mediate then results in a synthesis of vitamin D_3 (cholecalciferol) (WHITING and CALVO 2005). The average values of serum 25(OH)D_3 (20.5 ng/ml) determined by liquid chromatography/mass spectrometry exceeded by approximately 50 times the concentration of 25(OH)D_2 (0.4 ng/ml) (TSUGAWA et al. 2005). Important factors that play a role in supplying the organism by vitamin D_3 include, besides the sunlight, also the area of skin accessible to ultraviolet radiation, skin pigmentation and the age of the individuals (BOUILLON et al. 2005a). From the view of biological function, the question remains whether vitamin D_2 is equivalent to vitamin D_3. TRANG et al. (1998) stated that vitamin D_2 increases the level of 25-hydroxyvitamin D (25(OH)D) less effectively than D_3, and that the nutritional effect of both vitamins D is not equivalent. Vitamins D_2 and D_3 differ in the structure of the side chain of their molecule at the 17a-position of the secosteroid (vitamin D_2 – has one additional double bond and one additional methyl group). However, their subsequent metabolic pathway is identical (SHIMADA et al. 2003). Under the influence of hydroxylases from the cytochrome P-450 enzyme family, vitamin D is first hydroxylated in the C25 position via 25-hydroxylase (CYP27A) produced by the liver. This gives rise to 25(OH)D. The concentration of this biologically as yet inactive metabolite of vitamin D in blood reflects the amount of vitamin D stores in the organism from exogenous (D_2, D_3) as well as endogenous (D_3) sources (BOUILLON et al. 2005a; TURPEINEN et al. 2003). The biologically active and hormonally effective 1α,25-dihydroxyvitamin D (1α,25(OH)$_2$D) is formed in the kidney by the hormonally regulated 1α-hydroxylase (CYP27B) which is stimulated by parathyroid hormone (PTH), while 1α,25(OH)$_2$D has a negative feedback effect. The kidneys, as well as other tissues, contain the enzyme 24-hydroxylase (CYP24), which catalyses the synthesis of (24R)-24,25(OH)$_2$D from 25(OH)D, but mainly inactivates 1α,25(OH)$_2$D by transforming it to 1a,24,25(OH)$_3$D (SHIMADA et al. 2003). Overall, 37 various types of vitamin D_3 metabolites have been isolated and chemically characterized (http://vitamind.ucr.edu/biochem.html, access 7.1.2006).

1α,25(OH)$_2$D is a key regulator of calcium and phosphorus homeostasis, inducing the absorption of gastrointestinal calcium and mobilizing calcium from bone. It also exerts strong anti-proliferative, pro-differentiation, pro-apoptotic and immuno-modulatory (suppression of humoral response, activation of cellular response) effects (BOUILLON et al. 2005a; VAN ETTEN and MATHIEU 2005; BOUILLON et al. 2005b, STEWART et al. 2005). Long-term vitamin D deficiency and consequently decreased 1α,25(OH)$_2$D levels leads to rachitis in children and to osteomalacy in adults. Together with calcium deficiency and other metabolic changes related to increasing age, vitamin D deficiency plays a role in the pathiogenesis of osteoporosis (FRASER et al. 1997), alopecia (HSIEH et al. 2003), muscle weakness, development of secondary hyperparathyroidism in patients with chronic renal insufficiency.

Current research shows that vitamin D deficiency may be associated with hypertension, depression and immune system disorders that it also leads to multiple sclerosis, rheumatic arthritis and diabetes (Siu-Caldera et al. 1999). There is an increasing number of studies showing a relationship between the prevalence of cancer, especially of the colon, prostate and breast as well as other malignancies on one side and the amount of vitamin D body stores on the other (Bouillon et al. 2005a).

The concentration of 25(OH)D in blood is the best marker of vitamin D store (WHITING and CALVO 2005, BOUILLON et al. 2005a, QUESADA et al. 2004, MATA-GRANADOS et al. 2004). Within routine clinical examinations, serum 25(OH)D levels are usually determined using commercial immunoassays (radioimmunoassay (HOLLIS 2000), ELISA (LIND et al. 1997)) with or without chromatographic or extraction purification. The advantage of immunoassays is the large capacity and the analytical sensitivity in the order of pg per test tube. Immunoassays of the hydroxylated metabolites of vitamin D are also affected by a number of problems. One such problem involves the strong binding of vitamin D metabolites to the vitamin D binding protein (VDBP) and to a lesser extent to albumin during RIA (radioimmunoassay) quantification. Serum precipitation using organic solvents or acids may thus lead to losses due to co-precipitation of vitamin D metabolites with the binding proteins (QUESADA et al. 2004). Another difficulty involves the standardisation of the immunoanalytical kits used. Various types of assays yield various results from which various reference ranges are derived and clinical decisions created. These do not necessarily reflect the true vitamin D stores within a given population (GLENDENNING 2003). Methods based on the competition between specific binding proteins and a mixture of labeled (tracer) and unlabeled (assayed) ligands lack adequate specificity to differentiate between vitamin D_2 and vitamin D_3 metabolites. From this point of view the error in RIA determination of vitamin D metabolites can depend on the applied

system and on the proportion of individual types of vitamin D metabolites in the circulation.

Another key issue is the antibody specificity when determining 1α,25$(OH)_2$D, whose concentration is lower than that of 25(OH)D by approximately an order of 3. Inadequately effective processes of separation of 1α,25$(OH)_2$D from 25(OH)D, or the use of an antibody with even minimum cross-reactivity with 25(OH)D may lead to false high results related to 1α,25$(OH)_2$D. The specificity of used antibodies against the conjugates (sulphate, glucuronide) of hydroxylated vitamin D metabolites is not being discussed at all. However, the determination of plasma 25$(OH)D_3$ and 25$(OH)D_3$-3-sulphate using reverse-phase high-pressure liquid chromatography (RP HPLC) and ultraviolet (UV) detection at 265 nm has shown that the plasma of healthy persons contains a nearly equivalent amount of both substances (n=15, 15 ng/ml 25$(OH)D_3$-3-sulphate, 14 ng/ml 25$(OH)D_3$) (Shimada et al. 1997, Shimada et al. 1995). Similar concentrations 16.7 ng/ml for 25$(OH)D_3$-3-sulphate were obtained by gas chromatography/mass spectrometry (Axelson 1985).

This work describes the results obtained by radioimmunoassays of two main metabolites of vitamin D, i.e. 25(OH)D and 1α,25$(OH)_2$D in a group of patients of the Institute of Endocrinology (Prague, Czech Republic) in 2004-2006. We developed a simple RP HPLC separation of 25(OH)D following prior extraction of 25(OH)D from serum with acetonitrile. Parallel determination of 25(OH)D by commercially available RIA kit or following RP HPLC separation of 25(OH)D from serum with the same RIA detection served to comparison of results. The RP HPLC procedure is also suitable for the determination levels of other lipophilic vitamins (e.g. A, E, K1) assuming that more effective detection is used than that involving a UV detector at 254 nm or 290 nm.

Materials and Methods

Chemicals. All chemicals used were of analytical grade. Vitamins D_3, D_2 and their metabolites 25$(OH)D_3$, 25$(OH)D_2$, 1α,25$(OH)_2$D, as well as ((±)-(-tocopherol (vit. E), all trans-retinal (vit. A) and vitamin K_1 were purchased from Sigma-Aldrich (Stenheim, Germany), labeled ^{125}I-25$(OH)D_3$ from IDS (Boldon, UK); labeled ^{3}H-25$(OH)D_3$ from Perkin Elmer Life Sciences (Boston, USA). We used methanol and acetonitrile from Merck (Darmstadt, Germany); diethylether, dichloromethane and n-hexane from Lachema (Brno, Czech Republic). Deionised water was prepared by a water-treatment apparatus from Millipore AFS-18D (Pittsburgh, USA).

Stock solutions of vitamins and their metabolites at concentrations of 10 μg /100 μl were prepared by dissolving the given analyte in acetonitrile. These solutions were stored in darkness at –18 °C.

For the conversion of units following equations were used: 1α,25$(OH)_2D_3$(pmol/l) x 0,417 = 1α,25$(OH)_2D_3$(pg/ml); 25$(OH)D_3$(nmol/l) x 0,401 = 25$(OH)D_3$(ng/ml); 1α,25$(OH)_2D_2$(pmol/l) x 0,429 = 1α,25$(OH)_2D_2$(pg/ ml); 25$(OH)D_2$(nmol/l) x 0,417 = 25$(OH)D_2$(ng/ml).

Patients. During the period of 2004 – 2006, serum concentrations of 25(OH)D (n=2175, 1937 women, 238 men) and 1α,25$(OH)_2$D (n=2271, 2024 women, 247 men) were determined by RIA kits in the Institute of Endocrinology. The subjects were 1-93 years old (mean ± SD = 56 ± 18) (Table 1). Hyperparathyroidism was diagnosed in 259 patients, osteoporosis or vitamin D deficiency in 336 patients, defective function of thyroid gland in 2332 patients. Various endocrine disturbances were found in 454 patients. Parallel determination of 25(OH)D together with 1α,25$(OH)_2$D was made in 1065 patients (928 women, 137 men).

Radioimmunoassay of 25(OH)D and 1α,25$(OH)_2$D. RIA IDS Gamma-B25-D-hydroxy-vitamin D immunodiagnostic kit from IDS (Boldon,UK) was used for the determination of 25(OH)D in serum aliquots of 50 μl. In accordance with the information provided by manufacturer, the reference range for such kit is 23-113 nmol/l, an analytical sensitivity less than 3 nmol/l and a specificity (cross-reactions in %) of sheep polyclonal antibody 100 % for 25$(OH)D_3$, 75 % for 25$(OH)D_2$, more than 100 % for 24,25$(OH)_2D_3$, and less than 0.01 % or 0.3 % for D_3, and D_2 respectively.

1α,25$(OH)_2$D was determined in serum aliquots of 500 μl using the immuno-extraction Gamma-B1,25-Dihydroxy Vitamin D RIA kit from the same company. In this case the immuno-extraction is conducted with the aid of monoclonal antibodies immobilized to solid particles. The RIA itself uses a polyclonal sheep antibody whose analytical sensitivity is 8 pmol/l and which shows 100 % specificity for 1α,25$(OH)_2D_3$, 91 % for 1α,25$(OH)_2D_2$, less than 0.01 % for 24,25$(OH)_2D_3$ and less than 0.001 % for 25$(OH)D_3$. The reference range of 1α,25$(OH)_2$D is 48-110 pmol/l.

RP HPLC. A. Extraction of vitamin D metabolites from serum. 2000 μl of acetonitrile were added to 500 μl of serum. After vortexing for 10 s the mixture was centrifuged in a cooled centrifuge (4 °C) for 10

min at 2000 g. The supernatant was evaporated using a vacuum centrifuge (2 h at 40 °C) and the vaporized material was stored at –18 °C in polypropylene test tubes until analysis. Before the determination, the vaporized material was reconstituted in acetonitrile. The effectiveness of extraction was evaluated by the addition of ^{3}H-25(OH)D_3 to the analyzed serum and the measurement of radioactivity in the extract.

B. Analysis with UV detection of vitamin D metabolites and RIA detection of 25(OH)D. A liquid chromatograph was used (Laboratorní přístroje Prague, Czech Republic) including: 2 x HPP 5001 pump, GP2 gradient controls, LCI 30 dispenser, UV filter detector 254 nm LCD 2563, UV grid detector 290 nm LCD 2040, steel column (250 x 4,6 mm i.d.) filled with the stationary phase Separon SGX RPC18 (dp7 μm) from Tessek (Czech Republic). The Apex Integrator 3.1 (DataApex, Czech Republic) served to evaluate the chromatograms.

The chromatography column was washed prior to each analysis with methanol and acetonitrile, than was equilibrated for 5 minutes with the mobile phase. Vitamin D metabolites (injection of 100 μl vaporized serum extract reconstituted in acetonitrile) were separated isocratically by the mobile phase composed of methanol-acetonitrile = 95 – 5, at a flow rate of 1.5 ml/min. The detection was performed by UV detectors working at wavelengths of 254 nm and 290 nm.

The individual fractions of the eluate corresponding to the retention time of 25(OH)D (t_r = 3.0 – 4.5 min.) were collected in 0.5 minute intervals (3 x 750 μl), drained off and vaporised within one hour at 40 °C using the vacuum centrifuge RC 10.10 (Jouan, France). The vaporized material was reconstituted in 800 μl of acetonitrile and 50 μl of the solution was used for the IDS immunoassay of 25(OH)D.

Statistical evaluation. All statistical procedures were conducted using the NCSS 2001 program (Hintze 2006) at a probability level of 0.05. In view of the non-normal distribution, we used non-parametric tests (Mann-Whitney U test and Wilcoxon rank-sum test for difference in medians, Kolgomorov-Smirnov test for normal distributions) as well as parametric tests (t-test for equivalent and non-equivalent distribution) to evaluate the statistical significance of value differences.

Results

Radioimmunoassay data. The average values of RIA assays of serum 25(OH)D, 1α,25$(OH)_2$D are listed in Table 1. The mean and median serum levels of 25(OH)D (73.8 nmol/l in men, 76.8 nmol/l in women) and 1α,25$(OH)_2$D (76.0 pmol/l in men, 90.5 pmol/l in women) were higher in women than in men (Table 1). But only in the case of 1α,25$(OH)_2$D the difference was statistically significant using both parametric and non-parametric tests ($p<0.0000$). The distribution of 25(OH)D and 1α,25$(OH)_2$D values is shown in Fig. 1 and the relationship between 25(OH)D and 1α,25$(OH)_2$D is shown in Fig. 2. This relationship is described on the basis of linear regression by the equation 1α,25$(OH)_2$D = 71.0845+0.1890*25(OH)D, and is highly significant ($p<0.0000$) with the coefficient of determination R^2 = 0.0343, Pearson's correlation coefficient of 0.1853 and Spearman's correlation coefficient of 0.2223. Fig. 3 represents seasonal changes of 25(OH)D and 1α,25$(OH)_2$D concentrations as average values obtained in the individual months. Based on the

Table 1
Mean concentrations of serum 25(OH)D and 1a,25$(OH)_2$D.

	n	age (years) ± SD	mean	stds	median	range	reference range
25(OH)D (nmol/l)							
Total	2175	57.5 +/- 16.9	76.4	45.6	67.7	7.5 – 406.2	23-113
Men	238	47.3 +/- 21.5	73.8	51.8	60.5	8.8 – 406.2	23-113
Women	1937	58.7 +/- 15.8	76.8	44.8	68.3	7.5 – 380.2	23-113
1α,25$(OH)_2$D (pmol/l)							
Total	2271	55.4 +/- 18.9	88.9	45.1	85.7	1.3 – 514.8	48-110
Men	247	51.4 +/- 19.8	76.0	49.1	75.3	2 – 258.1	48-110
Women	2024	55.9 +/- 18.7	90.5	44.3	87.4	1.3 – 514.8	48-110

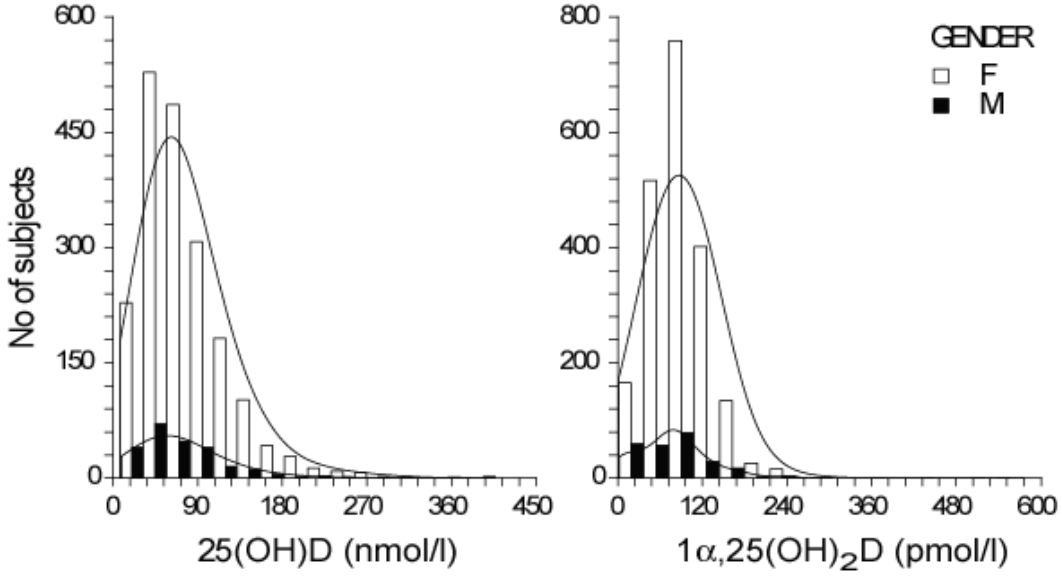

Fig 1 The distribution of serum 25(OH)D and 1α,25(OH)$_2$D (F= female, M= male).

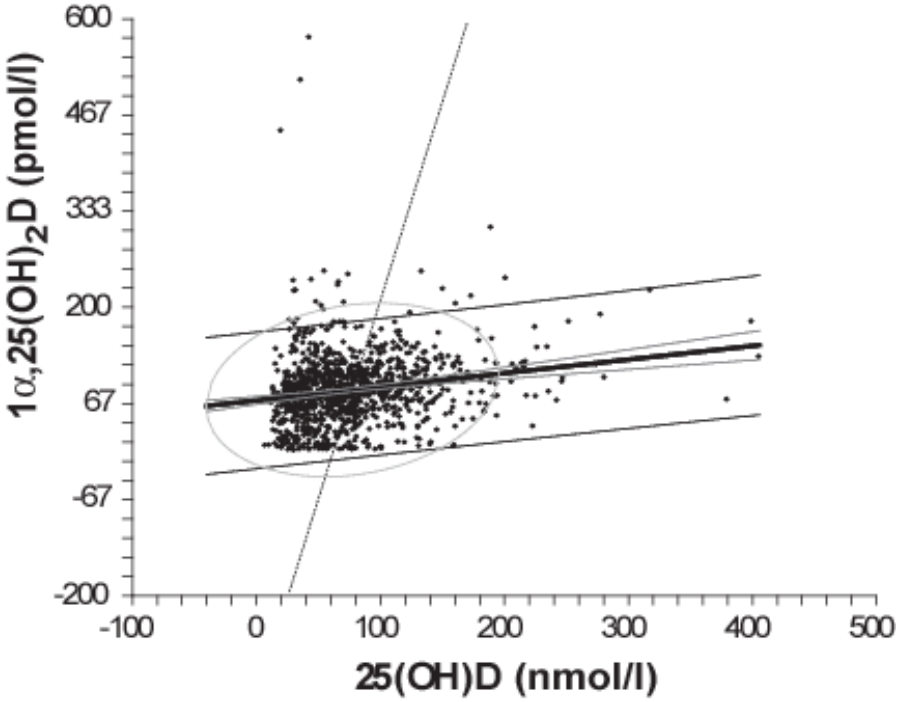

Fig 2 The relationship (Y=71.1+0.19*X) between parallel determination of serum 25(OH)D and 1α,25(OH)$_2$D (n = 1065, 928 women, 137 men) with showed Y on X line, X on Y line (dotted), probability ellipse, 95% confidence limits and prediction limits.

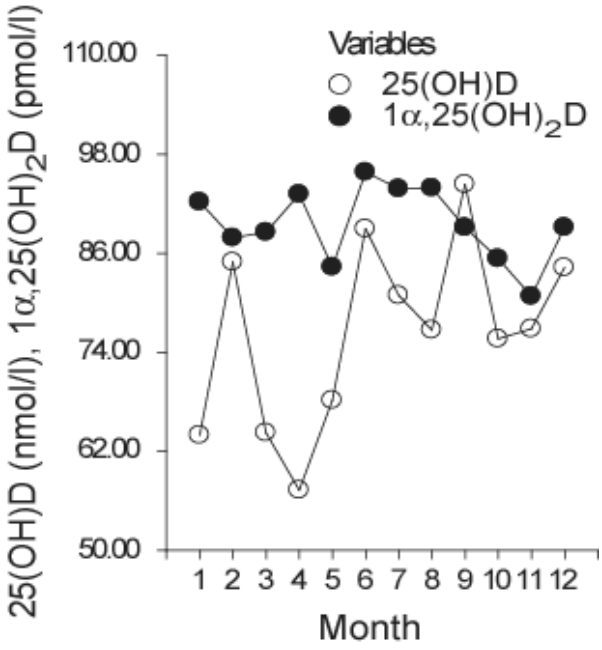

Fig 3 Seasonal variations of serum 25(OH)D and 1α,25(OH)$_2$D.

diagnosis, the lowest mean concentrations of 25(OH)D (n=188, 58.9 +/- 38.6 nmol/l) or 1α,25(OH)$_2$D (n=224, 53.8 +/- 53.5 pmol/l) were found in patients with hyperparathyroidism, and the highest mean concentrations of 25(OH)D (n=242, 89.2 +/- 55.2 nmol/l) or 1α,25(OH)$_2$D (n=234, 100.0 +/- 41.4 pmol/l) in patients treated with cholecalciferol (group of patients with osteoporosis and vitamin D deficiency).

HPLC data. The extraction effectiveness of 0.5 ml of serum containing ^{3}H-25(OH)D$_3$ with 2 ml acetonitrile was 91 % and reduction of the acetonitrile volume to 1 ml resulted in a decrease in extraction effectiveness to 88 %.

RP HPLC separation of vitamin D metabolites and vitamins A, E, K$_1$ is shown in Fig. 4. In view of the subsequent radioimmunoanalytical detection of 25(OH)D, the chromatographic parameters of the system were adjusted, so that the retention times of 25(OH)D$_2$ and 25(OH)D$_3$ were the same. Fig. 4 shows that within the RP HPLC system used the analytical sensitivity of the UV detector working at 254 nm is insufficient for the measurement of real serum concentrations not only of 1α,25(OH)$_2$D, but also of 25(OH)D.

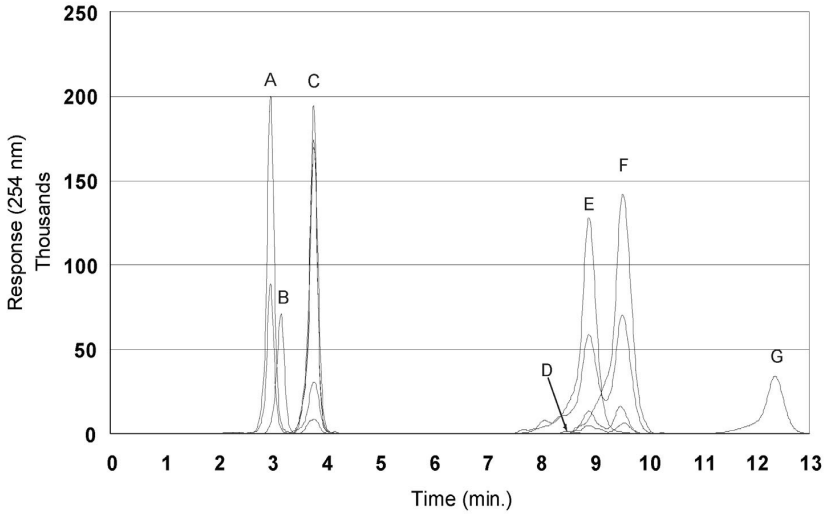

Fig 4 RP HPLC separation of standard solutions of 1α,25(OH)$_2$D$_3$ (10 a 3 µg/100 µl, t$_r$ = 3,0 min, peak A), vitamin A (5 µg/100 µl, t$_r$ = 3,2 min, peak B), 25(OH)D$_2$ and 25(OH)D$_3$ (10, 2, 0.4 µg/100 µl, t$_r$ = 3,8 min, t$_r$ = 3,9 min, peak C), vitamin E (5 µg/100 µl, t$_r$ = 8,7 min, peak D), ergocalciferol (10, 5, 1, 0.2 µg/100 µl, t$_r$ = 8,9 min, peak E), cholecalciferol (10, 5, 1, 0.2 µg/100 µl, t$_r$ = 9,6 min, peak F) and vitamin K$_1$ (5 µg/100 µl, t$_r$ = 12,4 min, peak G). The separation was performed isocratically using reverse stationary phase (C18, 250 x 4 mm i.d.) and mobile phase which consists of acetonitrile - methanol 95:5 (flow rate 1,5 ml/min). Standards were detected by UV detector working at 254 nm.

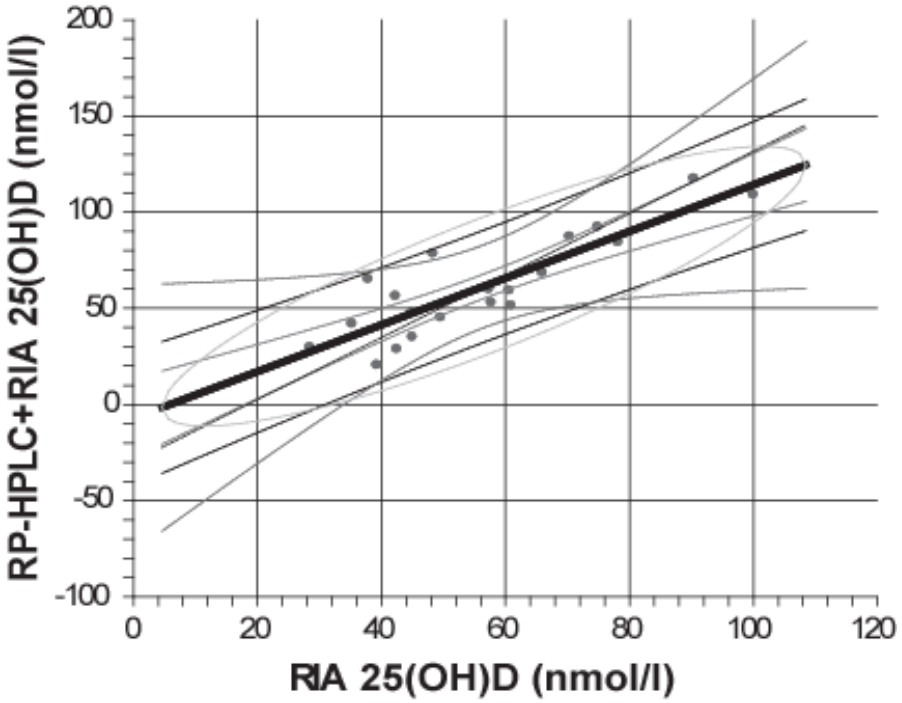

Fig 5 Scatter plot representing parallel determination (n=20) of serum 25(OH)D by commercially available RIA kit from IDS (X axis) and by RP HPLC isolation of 25(OH)D from serum with subsequent RIA qantitation of separated 25(OH)D using the same RIA kit (Y axis). There is showed probability ellipse, 95 % confidence limits and prediction limits. The results are described by the equation Y=1.21*X-7.1.

Detection of vitamin D metabolites at 290 nm wavelength was approximately 1/3 less effective as compared to that using 254 nm.

In 20 healthy volunteers, parallel determination of serum 25(OH)D was conducted by IDS RIA kit according company instruction, or in acetonitrile extract of serum, where 25(OH)D was isolated by RP HPLC and determined using the same IDS RIA kit. The results are shown in Fig. 5, and are described by the equation (HPLC+RIA)=1.2144*RIA-7.1000 with Pearson's correlation coefficient of 0.867, Spearman's correlation coefficient of 0.7820 and the coefficient of determination R^2 = 0.7529. The difference between both types of assays was highly significant ($p<0.0000$). The results obtained following HPLC separation (61.6 ± 26.6 nmol/l) were by 8 % higher than those obtained using the RIA kit only (56.6 ± 19.0 nmol/l).

Discussion

Quantification of vitamin D and its metabolites in body fluids belongs to very difficult analytical procedures of clinical chemistry. The reasons for this include the low concentrations of circulating metabolites, their chemical similarities, their physico-chemical instability (ultraviolet light, heat) and the presence of a large number of interfering substances within the biological matrix (especially of neutral lipids). This means that before the analysis itself it is necessary to use separation techniques, such as extraction with liquid solvents, extraction on solid phases, various chromatographic isolation procedures based on normal or reverse phase or on affinity chromatography etc. (Luque-Garcia and Luque de Castro 2001).

In our laboratory we use the extraction of (25(OH)D) and immuno-extraction (Fraser et al. 1997) of (1α,25$(OH)_2$D) by commercial RIA kits from IDS (England) for determining 25(OH)D and 1α,25$(OH)_2$D. In these kits, however, company data on the specific antibody manufactured by IDS does not show the same response to 25$(OH)D_2$ (75 %) as that to 25$(OH)D_3$ (100 %). Recovery of 25$(OH)D_2$ from samples of serum is only 21-29 % (Hollis 2000), and there, according to company data, also exists more than 100 % cross-

reactivity to 24,25$(OH)_2D_3$ in the case of the antibody specific against 25(OH)D. However, the plasma concentration of 24,25$(OH)_2D_3$ measured by RP HPLC with UV detection can be higher than 40 nmol/l (Boyer et al. 1999) and thus the circulating 24,25$(OH)_2D_3$ can substantially contribute to the imunoanalytically measured serum concentration of 25(OH)D. Similarly, immuno-extraction RIA determination of circulating 1α,25$(OH)_2D$ from IDS does not show any equivalent response to 1α,25$(OH)_2D_2$ (91 %) compared to 1α,25$(OH)_2D_3$ (100 %) (company data). Results of RIA assays of 25(OH)D (n = 2175) shown in Table 1 show that, in accordance with the normal reference range, only 4 % of individuals were inadequately supplied with 25(OH)D, 79 % were supplied adequately and almost 17 % were supplied more than adequately. Results of RIA assays of 1α,25$(OH)_2D$ (n = 1580) show inadequate concentrations of this main metabolite of vitamin D in 15 % of the individuals tested, 59 % of these individuals were within the reference range and 26 % had values exceeding the upper limit of the reference range. If we were to take into consideration the published comparison of parallel serum determination of serum 25$(OH)D_3$ (n=140) using the HPLC-ESI-MS (HPLC-electro-spray ionisation mass spectrometry) system or the RIA kit from IDS, there is shown that the RIA kit yielded in average by 25 % lower concentration of 25$(OH)D_3$ (47.8 nmol/l) than the determination by HPLC-ESI-MS (56.8 nmol/l) (Quesada et al. 2004). From this view the proportion of individuals with more than adequate level of 25(OH)D would be even higher.

This is one of the reasons why we attempted to compare, in a small group of healthy volunteers (n=20), the results obtained by the RIA kit from IDS with those obtained following the extraction, subsequent RP HPLC separation of 25(OH)D and detection of 25(OH)D using the same RIA kit from IDS. The correlation is shown in Fig. 5. Similarly as shown by Quesada et al. (2004), though not to the same extent, the results obtained following HPLC separation were by 8 % higher than those obtained only by the RIA kit. As shown in Fig, 4, by adjusting appropriately the chromatographic conditions, it is possible to separate not only the various hydroxylated metabolites of vitamin D_2 and D_3, but also other lipophilic vitamins. Their quantitative determination requires the detection with a higher degree of sensitivity than available by the system of UV detectors that we used. The HPLC/MS combination can be optimal for this type of analysis.

The concentration of both vitamin D metabolites was higher in women than in men (see Table 1), but the statistically significant differences were found only between the mean and median levels of 1α,25$(OH)_2D$ ($p<0.0000$). Unfortunately, the non-equivalence exists in both groups, whereby the number of men was approximately 8 times lower than that of women. The correlation between parallel determination of 25(OH)D and 1α,25$(OH)_2D$ was highly significant ($p<0.0000$), but Figure 2 shows that the dispersion of values was relatively high. Fig. 3, which depicts the changes in 25(OH)D and 1α,25$(OH)_2D$ concentrations depending on the individual months of blood sampling, shows that minimum values for both vitamin D metabolites were reached in the late winter season. This apparently corresponds to the extent of sunlight exposure. However, the variance of 25(OH)D is much higher than the variance of 1α,25$(OH)_2D$, and there is a notable peak of 25(OH)D in the February. This fact cannot be explained by the uneven distribution of proband numbers. The lowest mean concentrations of 25(OH)D or 1α,25$(OH)_2D$ were found in patients with hyperparathyroidism.

It is known that a negative relationship exists between serum 25(OH)D and serum PTH (Lips 2006), but the situation concerning 1α,25$(OH)_2D$ is more complicated, because the production of 1α,25$(OH)_2D$ itself is stimulated by PTH. However, primary hyperparathyroidism and vitamin D insufficiency are common conditions that can occur in combination (Moosgaard et al. 2005). Also a secondary hyperparathyroidism frequently observed in patients with chronic kidney disease can result in impaired 1α-hydroxylation of 25(OH)D to 1α,25$(OH)_2D$ with concomitantly decreased circulating 1α,25$(OH)_2D$ levels (Cozzolino et al. 2006). In accord with such assumption is that the highest mean concentrations of 25(OH)D or 1α,25$(OH)_2D$ were found in patients treated with cholecalciferol.

The interpretation of existing and future studies dealing with vitamin D stores must be based on precise and correct analytical procedures. For this reason, clinical assays for determining 25(OH)D and 1α,25$(OH)_2D$ should be validated by HPLC-MS, or eventually MS/MS procedures (Bouillon et al. 2005a). It may be also necessary to reevaluate the recommended intake of vitamin D. Heaney (2005) stated that the complex realization of vitamin D biological functions is ensured only at concentrations of circulating 25(OH)D that are approximately equal to 80 nmol/l. This corresponds to an intake of more than 2200 IU of vitamin D daily.

Though this value exceeds the upper limit of the tolerated vitamin D intake (2000 IU), it is associated with optimal absorption of calcium from the intestines and a minimization of increased parathyroid gland activity which is characteristic of the aging population. Osteoporosis induced fractures are reduced and sufficient local production of 1a,25$(OH)_2$D is ensured (Heaney 2005). Table 1 shows that this optimal state as expressed by the average level (n = 2175) of circulating 25(OH)D equal to 76.4 nmol/l was achieved in the group studied. The question is, what is the influence of other vitamin D metabolites and conjugates on the concentration of 25(OH)D measured using commercially available kits. A few is known about this phenomena.

Acknowledgements

This work was supported by the grant 1A 8634-3 of the Internal Grant Agency, Ministry of Health, Czech Republic.

References

Axelson M: 25-Hydroxyvitamin D_3 3-sulphate is a major circulating form of vitamin D in man. FEBS Lett **191**, 171-175, 1985

Bouillon R, Moody T, Sporn M, Barrett JC, Norman AW: NIH deltanoids meeting on Vitamin D and cancer. Conclusion and strategic options. J Steroid Biochem Mol Biol **97**, 3-5, 2005a.

Bouillon R, Verlinden L, Eelen G, de Clerc QP, Vandewalle M, Mathieu C, Verstuyf A: Mechanisms for the selective action of Vitamin D analogs. J Steroid Biochem Mol Biol **97**, 21-30, 2005b

Boyer FO, Romero JMF, de Castro MDL, Quesadab JM: Determination of vitamins D2, D3, K1 and K3 and some hydroxy metabolites of vitamin D3 in plasma using a continuous clean-up preconcentration procedure coupled on-line with liquid chromatography – UV detection. Analyst **127**, 401-406, 1999

Cozzolino M, Gallieni M, Brancaccio D, Arcidiacono T, Bianchi G, Vezzoli G: Vitamin D retains an important role in the pathogenesis and management of secondary hyperparathyroidism in chronic v. J Nephrol **19**, 566-577, 2006

Fraser WD, Durham BH, Berry JL, Mawer EB: Measurement of plasma 1,25-dihydroxyvitamin D using a novel immunoextraction technique and immunoassay with iodine labelled vitamin D tracer. Ann Clin Biochem **34**, 632-637, 1997

Glendenning P: Issues of standardization and assay-specific clinical decision limits for the measurement of 25-hydroxyvitamin D. Am J Clin Nutr 77, 522-523, 2003

Heaney RP: The vitamin D requirement in health and disease. J Steroid Biochem Mol Biol **97**, 13-19, 2005

Hintze J: NCSS and PASS, Number Cruncher Statistical System. www.ncss.com, 2006

Hollis BW**,** Kamerud JQ**,** Selvaag SR**,** Lorenz JD**,** Napoli JL**:** Determination of vitamin D status by radioimmunoassay with an ^{125}I-labeled tracer. Clin Chem **39**, 529-533, 1993

Hollis BW: Comparison of commercially available (125)I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. Clin Chem **46**, 1657-1661, 2000

Hsieh JC, Sisk JM, Jurutka PW, Haussler CA, Slater SA, Haussler MR, Thompson CC: Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. J Biol Chem **278**, 38665-38674, 2003

Lind C**,** Chen J**,** Byrjalsen I**:** Enzyme immunoassay for measuring 25-hydroxyvitamin D_3 in serum. Clin Chem **43**, 943-949, 1997

Lips P: Vitamin D physiology. Prog Biophys Mol Biol **92**, 4-8, 2006

Luque-Garcia JL, Luque de Castro MD: Extraction of fat-soluble vitamins. J Chromatogr A **935**, 3-11, 2001

Mata-Granados JM**,** Luque de Castro MD**,** Quesada JM**:** Fully automated method for the determination of 24,25$(OH)_2$ and 25$(OH)D_3$ hydroxyvitamins, and vitamins A and E in human serum by HPLC. J Pharm Biomed Anal **35**, 575-582, 2004

Moosgaard B, Vestergaard P, Heickendorff L, Melsen F, Christiansen P, Mosekilde L: Vitamin D status, seasonal variations, parathyroid adenoma weight and bone mineral density in primary hyperparathyroidism. Clin Endocrinol (Oxf) **63**, 506-513, 2005

Quesada JM, Mata-Granados JM, Luque de Castro D: Automated method for the determination of fat-soluble vitamins in serum. J Steroid Biochem Mol Biol **89-90**, 473-477, 2004

SHIMADA K, HIGASHI T, MITAMURA K: Development of Analyses of Biological Steroids Using Chromatography – Special Reference to Vitamin D Compounds and Neurosteroids. Chromatography **24**, 1-6, 2003

SHIMADA K**,** MITAMURA K**,** KITAMA N**,** KAWASAKI M**:** Determination of 25-hydroxyvitamin D_3 in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection. J Chromatogr B Biomed Sci Appl **689**, 409-414, 1997

SHIMADA K, MITAMURA K, KITAMA N: Quantitative determination of 25-hydroxyvitamin D_3 3-sulphate in human plasma using high performance liquid chromatography. Biomed Chromatogr **9**, 229-233, 1995

SIU-CALDERA ML**,** SEKIMOTO H**,** PELEG S**,** NGUYEN C**,** KISSMEYER AM**,** BINDERUP L**,** WEISKOPF A**,** VOUROS P**,** USKOKOVIC MR**,** REDDY GS**:** Enhanced biological activity of 1alpha,25-dihydroxy-20-epi-vitamin D_3, the C-20 epimer of 1alpha,25-dihydroxyvitamin D_3, is in part due to its metabolism into stable intermediary metabolites with significant biological activity. J Steroid Biochem Mol Biol **71**, 111-121, 1999

STEWART LV, LYLES B, LIN MF, WEIGEL NL: Vitamin D receptor agonists induce prostatic acid phosphatase to reduce cell growth and HER-2 signaling in LNCaP-derived human prostate cancer cells. J Steroid Biochem Mol Biol **97**, 37-46, 2005

TRANG HM**,** COLE DEC**,** RUBIN LA**:** Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. Am J Clin Nutr **68**, 854-858, 1998

TURPEINEN U, HOHENTHAL U, STENMAN UH: Determination of 25-hydroxyvitamin D in serum by HPLC and immunoassay. Clin Chem **49**, 1521-1524, 2003

VAN ETTEN E**,** MATHIEU C**:** Immunoregulation by 1,25-dihydroxyvitamin D_3: basic concepts. J Steroid Biochem Mol Biol **97**, 93-101, 2005

WHITING SJ, CALVO MS: Dietary recommendations to meet both endocrine and autocrine needs of Vitamin D. J Steroid Biochem Mol Biol **97**, 7-12, 2005

Corresponding author: Doc. Ing. Radovan Bílek, CSc.
Institute of Endocrinology,
Národní 8,
116 94 Praha 1
Czech Republic
Phone: +420 224 905 251
Fax: +420 224 905 325
email: rbilek@endo.cz