Blood biomarkers of vitamin D status[1–4]

Joseph E Zerwekh

ABSTRACT

In the past quarter century, more than 50 metabolites of vitamin D have been described. To date, only a few of these have been quantified in blood, but this has widened our understanding of the pathologic role that altered vitamin D metabolism plays in the development of diseases of calcium homeostasis. Currently, awareness is growing of the prevalence of vitamin D insufficiency in the general population in association with an increased risk of several diseases. However, for many researchers, it is not clear which vitamin D metabolites should be quantified and what the information gained from such an analysis tells us. Only 2 metabolites, namely, 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], have received the greatest attention. Of these, the need for measuring serum $1,25(OH)_2D$ is limited, and this metabolite should therefore not be considered as part of the standard vitamin D testing regimen. On the other hand, serum 25(OH)D provides the single best assessment of vitamin D status and thus should be the only vitamin D assay typically performed. Currently, numerous formats exist for measuring serum 25(OH)D concentrations, each with its own advantages and disadvantages. This article reviews the currently available methods for serum 25(OH)D quantitation and considers important issues such as whether both the D_2 and the D_3 forms of the vitamin should be assayed, whether total or free concentrations are most important, and what measures should be taken to ensure the fidelity of the measurements. *Am J Clin Nutr* 2008;87(suppl): 1087S–91S.

INTRODUCTION

It has been >80 y since the discovery of vitamin D and its ability to cure rickets in children. The central role this secosteroid plays in calcium and phosphate homeostasis is well appreciated. However, recent epidemiologic and clinical studies have indicated that vitamin D may have far greater actions than to solely prevent rickets in children. Diseases such as osteoporosis, muscle weakness, several types of cancer, diabetes, hypertension, and cardiovascular disease may result from subtle and chronic vitamin D deficiency (1). Indeed, recent data from the third National Health and Nutrition Examination Survey concluded that nearly 90% of women aged >70 y did not meet the recommended daily intake of vitamin D and were at risk of developing vitamin D deficiency (2). With a new appreciation of such widespread vitamin D deficiency and its potential impact on health, awareness is growing of the need for accurate assessment of vitamin D status in the general population as well as in large populations typically found in epidemiologic studies. Although >50 different vitamin D metabolites have been reported to date, vitamin D assay methods have focused primarily on quantitating the parent sterol vitamin D and its metabolites 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [$1,25(OH)_2D$]. This article will address which vitamin D metabolites should be measured and what analytic methods are available for performing this analysis.

WHICH VITAMIN D METABOLITES SHOULD BE MEASURED?

The development of sensitive and specific assays for vitamin D, 25(OH)D, and $1,25(OH)_2D$ has proved to be an invaluable asset in the research laboratory in defining vitamin D action and several diseases that result from altered vitamin D metabolism. The development and application of these assays to large population-based studies or in the clinical biochemistry laboratory has proceeded much more slowly. This has been due, in large part, to the inherent problems associated with these assays and the detection of these vitamin D metabolites that circulate in the nanomolar to micromolar concentration range.

Another complicating factor is the presence of 2 forms of vitamin D. Vitamin D_3, or cholecalciferol, is produced from the action of ultraviolet radiation on the skin and represents the most important source of vitamin D in humans. Only a few foods naturally contain vitamin D_3. Intake from the diet or in the form of supplements can be either as vitamin D_3 or as a closely related molecule, vitamin D_2 (ergocalciferol). This form of vitamin D is produced from the irradiation of ergosterol. Although some of the currently available assays for vitamin D metabolites can provide a measure of both the D_3 and D_2 forms in the circulation, separate assessment of both forms of vitamin D metabolites in large epidemiologic studies is usually not performed, and only the total vitamin D metabolite concentration is measured.

There are exceptions to this, however. For example, in studies designed to assess the efficacy of vitamin D supplementation with the D_2 form or to examine the contribution of both the D_2 and

the D_3 forms to total vitamin D status, it would be necessary to measure both the D_2 and the D_3 metabolites of the vitamin in the circulation. This is especially important when assessing the serum 25(OH)D response to vitamin D_2 administration, because several studies have suggested that the D_2 form of the vitamin is much less effective than is vitamin D_3 in raising serum 25(OH)D over an extended dosing interval (3–5). This is believed to be due to a lesser affinity of 25(OH)D_2 to the vitamin D binding protein (DBP) than that of 25(OH)D_3, resulting in a more rapid clearance of 25(OH)D_2 (6). However, for most epidemiologic studies concerned with only an assessment of overall vitamin D status, it would be necessary only to measure total vitamin D. Thus, most of this discussion will focus on total vitamin D quantitation without consideration of separately assaying the 2 forms of vitamin D.

Another issue to be addressed is whether there is clinical utility in measuring the free vitamin D metabolite concentration. Most circulating 25(OH)D and 1,25(OH)$_2$D is transported in the circulation bound to DBP (80–90%) and to albumin (10–20%). A very small fraction [0.02– 0.05% of 25(OH)D and 0.2–0.6% of total 1,25(OH)$_2$D] remains free or unbound (7). Thus, under physiologic conditions, nearly all circulating vitamin D compounds are protein bound. However, the vitamin D–DBP complex can be taken up by target cells via an endocytic process involving megalin and cubilin (8). Once in the cell, the DBP is proteolytically degraded, leaving the intracellular vitamin D metabolite available for further action or metabolism. Furthermore, free vitamin D metabolite concentrations appear to be well maintained, even in subjects with liver disease and reduced DBP concentrations, which suggests that there is little utility in assessing free vitamin D metabolite concentrations as indicators of vitamin D status.

Free serum 25(OH)D and 1,25(OH)$_2$D can be directly measured by centrifugal ultrafiltration or equilibrium dialysis (7). Free plasma 25(OH)D and 1,25(OH)$_2$D concentrations can also be determined by calculation provided that both the total vitamin D metabolite and DBP concentrations are known as well as the affinity constants of albumin and DBP for the respective vitamin D metabolites (9). Because of the lack of data showing that free 25(OH)D or 1,25(OH)$_2$D concentrations are a better predictor of vitamin D deficiency, assessment of total vitamin D metabolite concentration is sufficient in most clinical settings.

Another concern in selecting which vitamin D metabolite should be measured is the half-life of these metabolites in the circulation. The half-life of vitamin D is ≈24 h (10), thus making the concentration in serum dependent on the most recent exposure to sunlight and vitamin D ingestion. Similarly, the half-life of 1,25(OH)$_2$D is ≈4 h (11), and its production is tightly regulated by the calcium needs of humans. Because of these reasons, there is little usefulness in measuring vitamin D or 1,25(OH)$_2$D in the circulation. Assessment of these metabolites will not be further considered. On the other hand, quantitation of serum 25(OH)D provides a clinically useful assessment of an individual's vitamin D status for several reasons. First, the serum half-life of 25(OH)D is ≈3 wk (12, 13). This rather long serum half-life serves as an accurate indication of vitamin D stores obtained from both ultraviolet irradiation and dietary intake over long periods. Second, liver production of 25(OH)D is not significantly regulated and is primarily dependent on substrate concentration. For these reasons, measurement of serum 25(OH)D provides the best estimate of a patient's vitamin D status.

METHODS CURRENTLY AVAILABLE FOR MEASURING 25-HYDROXYVITAMIN D_2 AND D_3

As mentioned above, assessment of serum 25(OH)D provides the best measure of vitamin D repletion in humans. It is therefore not surprising that there is a much greater selection of methodologies for measuring this vitamin D metabolite in serum. Two assays for the measurement of 25(OH)D were introduced in 1971 (14, 15). Both assays were competitive protein binding assays that used the serum DBP from vitamin D– deficient rat serum as the binding agent. Although there were differences between assays in the solvent system used for extraction and in incubation times, both assays yielded serum 25(OH)D concentrations that were remarkably close to the values seen with state-of-the-art assay systems. There have been several variations on this basic competitive protein binding assay that incorporate different extraction procedures or alternative purification schemes but with no real improvement in assay performance. A commercially available competitive protein binding assay for 25(OH)D uses a competition between 25(OH)D in the sample and 25(OH)D bound to a microplate for binding to the DBP (Immunodiagnostik AG, Bensheim, Germany). Quantitation is performed via the addition of a DBP antibody that is conjugated to peroxidase. Tetramethylbenzidine is added as an enzyme substrate, and color development is inversely proportional to the original concentration of 25(OH)D present in the samples and calibrators. The assay has 100% cross-reactivity with 25(OH)D_2 according to the manufacturer's product information sheet.

In 1977, the first useful direct ultraviolet detection assay for 25(OH)D was reported (16). 25(OH)D circulates at nanomolar concentrations, thus permitting direct quantitation via ultraviolet detection at 265 nm, the wavelength of maximal absorbance for vitamin D metabolites with the classic triene structure. This method has the advantage of being able to separate 25(OH)D_2 from 25(OH)D_3 and thus permits their individual quantitation. It requires equipment and expertise that is usually only available in research laboratories. However, for laboratories equipped with HPLC instrumentation, an HPLC application for determination of 25(OH)D_3 is commercially available from Immunodiagnostik. This product provides reagents and Sep-Pack C_{18} cartridges for sample extraction and prepurification before injection on the HPLC apparatus. It also provides authentic 25(OH)D_3 as a calibrator for the HPLC system, thus alleviating the need to prepare and determine the concentration of an in-house 25(OH)D standard. On the basis of the manufacturer's product insert, it is not clear whether the chromatographic system can fully resolve 25(OH)D_2 from 25(OH)D_3.

In order for the 25(OH)D assay to receive more widespread application in the clinical biochemistry laboratory, it became necessary to simplify the extraction procedure and to simplify or eliminate the chromatographic purification steps. This was accomplished through the development of the first valid radioimmunoassay (RIA) for 25(OH)D in 1985 (17). This assay does not require sample prepurification before the assay. In addition, the need for spiking of samples with ^{3}H-25(OH)D for subsequent quantitation of yield after purification was eliminated, because all standards and controls were in a serum matrix and were extracted exactly like the serum samples. Last, its sensitivity was increased, because the tracer was ^{125}I-25(OH)D. Because of the overall simplicity of this assay and its strong correlation with

values obtained from HPLC analysis, this assay has been approved by the Food and Drug Administration for clinical use in the United States (DiaSorin, Stillwater, MN). The antibody used in this equilibrium RIA recognizes both $25(OH)D_2$ and $25(OH)D_3$ as well as other hydroxylated metabolites, such as 24,25-dihydroxyvitamin D; 25,26-dihydroxyvitamin D; and $25(OH)D_3$-26,23-lactone. However, the overall contribution of these latter metabolites to the circulation is rather small ($\leq 6\%$) and thus their contribution can be ignored. A second $25(OH)D$ RIA is also available from IDS Inc (Immunodiagnostic Systems Inc, Fountain Hills, AZ). It is similar to the DiaSorin assay but uses a 2-step extraction procedure. Portions of the extracted samples, calibrators, and controls are incubated with ^{125}I-$25(OH)D$ tracer and a highly specific sheep anti-$25(OH)D$ polyclonal antibody. Separation of antibody-bound tracer from free is achieved by a short incubation with Sac-Cel. The antibody is reported to have 75% cross-reactivity with $25(OH)D_2$. This assay has been shown to correlate well with values obtained by HPLC ($r^2 = 0.89$) and by the DiaSorin RIA ($r^2 = 0.92$).

There are also 2 enzyme-linked immunosorbent assay (ELISA) format assays for $25(OH)D$ that are commercially available. The IDS Inc assay used biotin-labeled $25(OH)D$, which is added to the calibrators, controls, and 25 μL of nonextracted serum. The diluted samples are then incubated in microtiter wells that are coated with a sheep antibody to $25(OH)D$. After the wells are washed, color development is accomplished via incubation with horseradish peroxidase–labeled avidin and tetramethylbenzidine as the chromogenic substrate. This antibody (probably the same as in their RIA) has 75% cross-reactivity with $25(OH)D_2$. The second ELISA assay (Immunodiagnostik) is similar to the Immunodiagnostik competitive protein binding assay that uses the DBP described above. However, in this direct assay, standards, controls, and patient samples are incubated with a releasing agent to free the $25(OH)D$ from the DBP. An aliquot from each of these extractions is transferred to a microplate that has been coated with $25(OH)D$, and an anti-$25(OH)D$ antibody is added. After an overnight incubation step, $25(OH)D$ in the sample and a fixed amount of $25(OH)D$ bound to the microtiter well compete for the binding of the antibody. A peroxidase-conjugated secondary antibody is added, and the complex is detected after the addition of tetramethylbenzidine. Interestingly, the antibody used in this assay has only 23% cross-reactivity with $25(OH)D_2$.

Two other $25(OH)D$ assay formats deserve mention. DiaSorin has developed a chemiluminescent $25(OH)D$ assay that is fully automated on the LIAISON analyzer (Stratec Biomedical Systems, Birkenfeld, Germany). Under this format, a $25(OH)D$-chemiluminescent tracer competes with $25(OH)D$ in the sample for binding to antiserum coated on paramagnetic beads. The polyclonal antiserum is the same as that used in the DiaSorin $25(OH)D$ RIA (described above). This antiserum has equal affinity for both the D_2 and the D_3 forms of the vitamin D metabolite, and the assay provides total $25(OH)D$ values. The method does not require any pretreatment of samples. Assay time is 40 min, and ≈ 90 samples per hour can be analyzed. When compared against the DiaSorin RIA, a correlation coefficient of 0.94 was obtained; when compared against liquid chromatography–tandem mass spectrometry (LC-MS/MS), the correlation coefficient was 0.95 (18).

The last method, LC-MS/MS, is considered to be the most accurate of all methods to date and is currently regarded as the gold standard. LC-MS/MS can separate and accurately quantitate both $25(OH)D_2$ and $25(OH)D_3$. In addition, this method has been shown to correlate well with the DiaSorin RIA ($r = 0.74$ to 0.96) (19, 20). However, all of the LC-MS/MS techniques require derivatization or deuterated internal standards. In addition, LC-MS/MS methods frequently encounter issues with ion suppression. Ion suppression represents difficulties with reproducibility and accuracy when analyzing small quantities of analytes in complex samples such as biological fluids. It may result from co-eluted matrix components, affecting the detection capability, precision, or accuracy of the analytes of interest. This change often is observed as a loss in response, thus the term ionization suppression. It frequently occurs when internal standards are used that do not share chemical and structural properties with vitamin D. Thus, it is critical to minimize or compensate for ion suppression if found to be present. A recently described method used a novel internal standard, deuterated Δ^9-tetrahydrocannabinol-D_3, to minimize ion suppression with shorter analysis times (19). Deuterated vitamin D compounds are also now available as suitable internal standards (20). Generally speaking, this assay method is best left to high-volume reference laboratories. A summary of the serum $25(OH)D$ assay platforms is provided in **Table 1**.

WHICH ASSAY PLATFORM SHOULD BE USED?

Deciding which vitamin D assay format to use can be a daunting task. As discussed above, assays for $25(OH)D$ are available in several platforms. Although establishing an in-house assay might be the most cost-effective means of performing $25(OH)D$ assays, without the appropriate equipment and expertise, such an undertaking can be formidable. Rather, one of the commercially available RIA or ELISA procedures may be suitable for use in the investigator's laboratory. Alternatively, if the research budget allows, investigators could obtain a quote from one of the many commercial laboratories performing this assay. Under this setting, the price per test typically diminishes as the number of samples increases.

Quality control concerns for each of the assays must also be considered, particularly in light of a recent report that highlighted interlaboratory variability in serum $25(OH)D$ results (21). Those authors reported unacceptable variation in circulating $25(OH)D$ measurements encountered from laboratory-to-laboratory as well as method-to-method and the confounding of the 2. For example, 42 specimens from postmenopausal women were sent to a commercial laboratory that used acetonitrile extraction and their own in-house RIA. Of the 42 patients, 17% were classified as being vitamin D insufficient [serum $25(OH)D < 80$ nmol/L]. Twenty different specimens from a nearly identical group of women went to laboratory B, which used a commercially available $25(OH)D$ RIA. In this case, 90% of the women were classified as being vitamin D insufficient on the basis of the serum $25(OH)D$ value. It is not surprising that discordant results were obtained, because these laboratories used substantially different methods to measure serum $25(OH)D$. Certainly, the variability among the methods to detect $25(OH)D_2$ may explain some of the differences. However, another likely source of variability may have been inaccurate preparation of the standards used in the assays. Although most (but not all) of the commercially available assay kits (RIA and ELISA) will contain the manufacturer's own quality control material, this will only indicate whether there has

TABLE 1
Commercially available assays for measuring 25-hydroxyvitamin D[1]

Assay type and manufacturer	Sample type and volume	Extraction	Range of detection	Sensitivity	Intraassay CV	Interassay CV	Assay time	Comments
			nmol/L	*nmol/L*	%	%		
RIA								
DiaSorin	Serum or plasma, 50 μL	Acetonitrile	0–100	≤6	<8	<12	2.2 h	Calibrators and controls in serum matrix; no yield determination required
IDS Inc	Serum or plasma, 50 μL	Two-step reagent extraction	4–400	≤3	6.8	8.9	3 h	Calibrators and controls in serum matrix; no yield determination required; 75% cross-reactivity for $25(OH)D_2$
ELISA								
IDS Inc	Serum or plasma, 25 μL	None	6–360	≤5	<6	<9	3 h	75% cross-reactivity for $25(OH)D_2$
Immunodiagnostic	Serum or plasma, 30 μL	Proprietary extraction reagent	6.3–250	2	10	8	ON	Monoclonal antibody; 23% cross-reactivity for $25(OH)D_2$
CPB								
Immunodiagnostic	Serum or plasma, 50 μL	Acetonitrile	6.4–250	5.6	11	13	4.5 h	Uses DBP in EIA format; 100% cross-reactivity for $25(OH)D_2$
HPLC								
Immunodiagnostic	Serum, 500 μL	Acetonitrile and C_{18} cartridge extraction	Up to 1250	4	5.2	8.4	20 min	Laboratory must have HPLC unit with silica column; $25(OH)D_2$ and $25(OH)D_3$ separated with different column
Chemiluminescence								
DiaSorin Liaison	Serum or plasma, 25 μl	Automated	7.5–375	≤10	4	6	40 min	Fully automated on Liaison instrument; 100% cross-reactivity for $25(OH)D_2$

[1] RIA, radioimmunoassay; $25(OH)D_2$, 25-hydroxyvitamin D_2; ELISA, enzyme-linked immunosorbent assay; ON, overnight; CPB, competitive protein binding; DBP, vitamin D binding protein.

been a general problem with the assay performance and does not guarantee that the result is accurate simply because the control value came within the specified range. For commercial assays not providing quality control specimens, a suitable serum control for 25(OH)D is available from Bio-Rad Laboratories (Hercules, CA), but there is no commercially available control serum for 1,25(OH)₂D at present.

To help with this issue, 2 external quality assessment schemes (EQAS) are available for these vitamin D metabolites. They are located in the United Kingdom (22) and in Finland (Internet: www.labquality.fi). For DEQAS (Vitamin D External Quality Assessment Scheme) (22), 5 samples of normal human sera are sent out at 3-mo intervals. Participants perform the assays, send the results to the DEQAS central office, and, after statistical analysis of the results, are provided a report giving an all-laboratory trimmed mean (ALTM) and SD for each sample. The ALTM has been shown to be a good surrogate for the "true" (target) value obtained by gas chromatography–mass spectrometry. The accuracy of each result is defined by its percentage bias from the ALTM. Results for each sample are also grouped by method, and a method mean is also provided. The overall accuracy of each method can be assessed from the percentage bias of the method mean from the ALTM.

Another issue is whether researchers should collect multiple blood samples across seasons for 25(OH)D assessment to account for the well-known seasonal variation in serum 25(OH)D (23). Population-based longitudinal studies designed to assess

the adequacy of vitamin D supplementation must control for such variation in circulating 25(OH)D and should try to obtain, at a minimum, 2 samples during each year of study corresponding to the nadir and zenith of serum 25(OH)D concentration. Typically, this would correspond to January-February and July-August for most of the United States.

Last, most of the assays in use today for vitamin D metabolites can use either serum or plasma, which should be stored at −20 °C until assayed. One study (24) has suggested that both 25(OH)D and 1,25(OH)₂D are stable in uncentrifuged blood at 24 °C for as long as 72 h. In addition, exposure to ultraviolet light and repeated (up to 11 times) freezing and thawing of a serum pool was without apparent effect on analyte stability. Unlike the situation with purified vitamin D metabolites, vitamin compounds present in human serum and plasma were protected from degradation by heat or light, possibly by the presence of DBP.

SUMMARY

In the past few years, there has been an increasing awareness of the multisystem effects of vitamin D and its metabolites in humans. No longer is vitamin D regarded simply as a steroid hormone that prevents rickets in children. Rather, its roles in cancer prevention, diabetes, motor function, and immunologic processes, to name but a few, have opened the door for large population-based studies. Such studies have aimed to specifically define the role of vitamin D in these disease states and how

alterations in vitamin D sufficiency affect the risk of development of such diseases. The success of these and other epidemiologic studies of vitamin D require the ability to accurately and reproducibly measure serum vitamin D metabolites. There has been considerable progress in vitamin D assay methods, and today researchers can measure 25(OH)D by an array of different assay techniques. The choice of which assays to use will be dictated by the availability of required equipment, technical expertise, and whether a need exists to quantitate both the D_3 and D_2 forms of the vitamin D metabolites. Most importantly, there exists an external quality control mechanism for ensuring the accuracy and precision of such vitamin D measurements in each researcher's laboratory.

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REFERENCES

1. Holick MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest 2006;116:2062–72.
2. Moore C, Murphy MM, Keast DR, Holick MF. Vitamin D intake in the United States. J Am Diet Assoc 2004;104:980–3.
3. Trang HM, Cole DE, Rubin LA, Pierratos A, Siu S, Vieth R. Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. Am J Clin Nutr 1998;68:854–8.
4. Tjellesen L, Hummer L, Christiansen C, Rodbro P. Serum concentration of vitamin D metabolites during treatment with vitamin D_2 and D_3 in normal premenopausal women. Bone Miner 1986;1:407–13.
5. Armas LAG, Hollis BW, Heaney RP. Vitamin D_2 is much less effective than vitamin D_3 in humans. J Clin Endocrinol Metab 2004;89:5387–91.
6. Hollis BW. Comparison of equilibrium and disequilibrium assay conditions for ergocalciferol, cholecalciferol, and their major metabolites. J Steroid Biochem 1984;21:81–6.
7. Bikle DD, Gee E, Halloran B, Haddad JG. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. J Clin Invest 1984;74:1966–71.
8. Nykjaer A, Dragun D, Walther D, Vorum H, et al. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D_3. Cell 1999;96:685–95.
9. Al-oanzi ZH, Tuck SP, Raj N et al. Assessment of vitamin D status in male osteoporosis. Clin Chem 2006;52:248–54.
10. Clemens TL, Adams JS, Holick MF. Measurement of circulating vitamin D in man. Clin Chim Acta 1982;121:301–8.
11. Gray AW, Caldas AE, Wilz DR, Lemann J, Smith GA. Excretion of ^{3}H-1,25-dihydroxyvitamin D_3 in healthy adults. J Clin Endocrinol Metab 1978;46:756–65.
12. Barraguy JM, France MW, Corless D, et al. Intestinal cholecalciferol absorption in the elderly and in younger adults. Clin Sci Mol Med 1978;55:213–20.
13. Clemens TL, Zhou X, Myles M, Endres D, Lindsay R. Serum vitamin D_2 and vitamin D_3 metabolite concentrations and absorption of vitamin D_2 in elderly subjects. J Clin Endocrinol Metab 1986;63:656–60.
14. Belsey R, DeLuca HF, Potts JT Jr. Competitive binding assay for vitamin D and 25-OH vitamin D. J Clin Endocrinol Metab 1971;33:554–7.
15. Haddad JG, Chyu KJ. Competitive protein binding radioassay for 25-hydroxycholecalciferol. J Clin Endocrinol Metab 1971;33:992–5.
16. Eisman JA, Shepard RM, DeLuca HF. Determination of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in human plasma using high-pressure liquid chromatography. Anal Biochem 1977;80:298–305.
17. Hollis BW, Napoli JL. Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. Clin Chem 1985;31:1815–9.
18. Frenzel EM, Friedberg MA, Olien PJ, Olson GT, Schmidt JA. Development of the Liaison® 25-OH vitamin D assay with improved sensitivity and precision. J Bone Miner Res 2006;21(suppl):S450 (abstract).
19. Saenger AK, Laha TJ, Bremner DE, Sadrzadeh SMH. Quantification of serum 25-hydroxyvitamin D_2 and D_3 using HPLC-tandem mass spectrometry and examination of reference intervals for diagnosis of vitamin D deficiency. Am J Clin Pathol 2006;125:914–20.
20. Tsugawa N, Suhara Y, Kamao M, Okano T. Determination of 25-hydroxyvitamin D in human plasma using high performance liquid chromatography-tandem mass spectrometry. Anal Chem 2005;77:3001–7.
21. Binkley N, Krueger D, Cowgill CS, et al. Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. J Clin Endocrinol Metab 2004;89:3152–7.
22. Bates CJ. Vitamin analysis. Ann Clin Biochem 1997;34:599–626.
23. Stryd RP, Gilbertson TJ, Brunden MN. A seasonal variation study of 25-hydroxyvitamin D_3 serum levels in normal humans. J Clin Endocrinol Metab 1979;48:771–5.
24. Lissner D, Mason RS, Posen S. Stability of vitamin D metabolites in human blood, serum, and plasma. Clin Chem 1981;27:773–4.