Simultaneous Determination of 25-Hydroxyvitamin D, 24,25-Dihydroxyvitamin D, and 1,25-Dihydroxyvitamin D in Plasma or Serum


We describe a simultaneous assay for the principal vitamin D metabolites: 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D, and 1,25-dihydroxyvitamin D. Special attention has been paid to simplification of the extensive extraction and purification procedures used in previously described simultaneous assays. All three metabolites were isolated with a single extraction step, followed by only one gradient liquid-chromatographic procedure. For final quantitation we used competitive protein binding assays, involving readily available binding proteins and commercially purchased tritiated vitamin D metabolites. Concentrations in the plasma of healthy subjects (mean age, 27 years), sampled during December were 51 (SD 17) nmol/L, 4.1 (SD 1.3) pmol/L, and 124 (SD 26) pmol/L for 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D, respectively. Intra- and interassay CVs for the three metabolites were 4.4 and 3.9%, 6.7 and 8.0%, and 7.0 and 4.8%, respectively.

Additional Keyphrases: gradient liquid chromatography • competitive protein binding • reference interval

In the past decade, knowledge of the metabolism and the mechanism of action of vitamin D has substantially progressed as a result of availability of radiolabeled vitamin D metabolites for metabolism studies and the development of sensitive assays for the biologically active vitamin D metabolites (1–9).

We have described the concurrent determination of these metabolites in blood, including 25(OH)D, 24,25(OH)2D, and 1,25(OH)2D (4–8a). However, these methods are all too laborious for routine use with large numbers of samples. Here we report a relatively fast and reliable procedure for concurrently determining 25(OH)D, 24,25(OH)2D, and 1,25(OH)2D.

25 (OH) D metabolites. Crystallized 25(OH)D3 and 25(OH)D2 were obtained from Philips-Duphar, Weesp, The Netherlands, and The Upjohn Co., Kalamazoo, MI 49001, respectively. All other vitamin D3 metabolites (24R,25-dihydroxyvitamin D3, 25RS,26-dihydroxyvitamin D4, and 1α,25-dihydroxyvitamin D4) were from Hoffmann-La Roche, Basle, Switzerland. 25-Hydroxy(27-methyl-3H)cholecalciferol (spec. act. 5–15 kCi/mol), 24R,25-dihydroxy(23,24(3H)cholecalciferol (spec. act. 68 kCi/mol), and 1α,25-dihydroxy(23,24(3H)cholecalciferol (spec. act. 70–110 kCi/mol) were purchased from the Amersham International Ltd., Amersham, U.K. All vitamin D metabolites were stored in absolute ethanol at −20 °C. 1,25(OH)D3 for recovery monitoring was purified by HPLC on a Nucleosil 10-N20 column developed in n-hexane/isopropanol/water (90/10/0.35 by vol) at a flow rate of 2 mL/min. 25(OH)D3 and 24,25(OH)2D were used for recovery determination without further purification.

Reagents. All solvents for extraction and HPLC were of analytical grade and were used without further purification. Dextran (Grade C) was obtained from BDH Chemicals, Poole, U.K. Activated charcoal (analytical grade) was from Merck Nederland, Amsterdam, The Netherlands. Nucleosil 10-N20 packing material for HPLC columns was obtained from Machery-Nagel, Duren, F.R.G. Conical 13 × 55 mm polyethylene tubes for competitive protein binding were from Baker Chemicals, Deventer, The Netherlands. Gelatin was from Brocades-ACF, Maarssen, The Netherlands. Radioactivity in aqueous solutions was measured in a Lumagel liquid scintillation fluid (Baker Chemicals), with 36% counting efficiency. The scintillation fluid used in liquid scintillation counting for recovery measurement was Permafluor III (Packard Instrument Belenex S.A., Brussels, Belgium) diluted 10-fold with toluene.

Apparatus. The HPLC system consisted of two Model 110A pumps controlled by a Model 420 microprocessor (Altex Scientific, Berkeley, CA 94710), a 1203 UV III detector operated at 254 nm (LDC, Riviera Beach, FL 33404), and a WISP 710A automatic sample processor (Waters Associates, Milford, MA 01757). HPLC columns were packed by use of a Model 70.00 packing apparatus (Knauer, Berlin, F.R.G.). In train with the HPLC column, a low-dump-volume three-way valve (Model 7030A, fitted with a Model 7163 solenoid valve; Rheodyne, Inc., Berkeley, CA 94710) was mounted to direct the flow to the fraction collector or to the ultraviolet detector. Fractions were collected with a Redirac 2112 fraction collector (LKB, Bromma, Sweden). Both the fraction collector and the three-way valve were controlled by the Model 420 micropro-

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Plasma and serum samples. Blood was sampled from 20 healthy subjects in December 1979. From a part of this blood a plasma and a serum pool were made for use in determination of intra- and interassay CVs. Reference intervals were derived from individual plasma and serum samples.

Extraction. Five milliliters of plasma or serum was fortified with approximately 15 000 dpm of 25(OH)\(^{[3H]}\)D\(_3\), 5000 dpm of 24,25(OH)\(^2\)[\(^{3H}\)]D\(_3\), and 6000 dpm of 1,25(OH)\(^{[3H]}\)D\(_3\), each in 50 \(\mu\)L of absolute ethanol. Extraction was done as described previously (9). After the extract was evaporated, the residue was dissolved in 210 \(\mu\)L of n-hexane/isopropanol (9/1 by vol) and transferred to minivials for the automatic sample processor.

Liquid chromatography. Nucleosil 10-NO\(_2\) was slurried in approximately 15 mL of carbon tetrachloride and poured into the empty column and the reservoir of the packing apparatus. The reservoir was then carefully filled with n-hexane and closed. Subsequently about 200 mL of n-hexane was forced through the column at maximum flow rate. After relief of pressure the column was closed and equilibrated overnight with n-hexane/isopropanol/water (90/10/0.35 by vol) at a flow rate of 0.2 mL/min. The number of theoretical plates was calculated by injecting 1,25(OH)\(^{2}\)D\(_3\) at a flow rate of 2 mL/min. Each new column was primed before use with 10 extracts of 5-mL aliquots of plasma.

We separated vitamin D metabolites in extracts of plasma on a 12 \(\times\) 0.46 cm Nucleosil 10-NO\(_2\) column fitted with a 4 \(\times\) 0.46 cm Nucleosil 10-NO\(_2\) precolumn by gradient elution with two solvents, A and B, described in the legend to Figure 1. Lipids were removed with 100% solvent B. 25(OH)D\(_3\), 24,25(OH)\(^2\)D\(_3\), and 25,26(OH)\(^2\)D\(_3\) were separated with 30% A, after which 1,25(OH)\(^2\)D\(_3\) was eluted with 50% A. The remaining interfering compounds were removed from the column with 100% solvent A (Figure 1A). The fractions containing 25(OH)D\(_3\), 24,25(OH)\(^2\)D\(_3\), and 1,25(OH)\(^2\)D\(_3\) were collected and evaporated under nitrogen. The residues were dissolved in, respectively, 4000, 530, and 215 \(\mu\)L of absolute ethanol. For each metabolite CBP was done in duplicate with 50-\(\mu\)L aliquots. Analytical recovery was measured in duplicate with 1800, 200, and 50 \(\mu\)L, respectively. That the metabolites were separated was verified by monitoring displacement activity of the fractionated eluent of an extract of a 5-mL aliquot of the plasma pool. Fractions were collected at 1-min (0–6 min), 0.5-min (6–29 min), and 2-min (29–57 min) intervals. After evaporation the fractions were dissolved in 175 \(\mu\)L of absolute ethanol. We assayed 50-\(\mu\)L aliquots in both the 24,25(OH)\(^2\)D\(_3\) and the 1,25(OH)\(^2\)D\(_3\) assay.

Competitive protein binding. CBP procedures for 25(OH)D\(_3\) and 24,25(OH)\(^2\)D\(_3\) were modified from the method described by Weisman et al. (10). Nearly identical procedures were used for 25(OH)D\(_2\) and 24,25(OH)\(^2\)D\(_2\). The amounts in parentheses refer to 24,25(OH)\(^2\)D\(_3\). One hundred microliters (50 \(\mu\)L) of barbiturate buffer (0.1 mol of diethyl barbiturate and 1 g of gelatin per liter; pH 8.6) was pipetted into conical polyethylene tubes and 50 \(\mu\)L of standard or sample in ethanol was added. Then 1.5 ng (0.75 ng) of 25(OH)\(^{[3H]}\)D\(_3\) in 50 \(\mu\)L of ethanol/water (40/10 by vol) was added with a Hamilton PB 600-1 repeating dispenser. Finally, 700 \(\mu\)L (750 \(\mu\)L of normal rat serum, diluted approximately 2700 (6000-fold with barbiturate buffer was added. After 2 h at 0 °C, incubation was terminated by adding 200 \(\mu\)L of barbiturate buffer containing 20 g of charcoal and 2 g of dextran per liter. After another 30 min at 0 °C the tubes were centrifuged for 10 min at 4000 rpm and 1 mL of the supernatant fluid was taken for liquid scintillation counting. Non-specific binding was determined in the presence of 150 ng of 25(OH)D\(_3\) [25(OH)D\(_3\) assay] or 75 ng of 24,25(OH)\(^2\)D\(_3\) [24,25(OH)\(^2\)D\(_3\) assay]. Standard curves were constructed by plotting the reciprocal of specific binding vs the amount of 25(OH)D\(_3\) or 24,25(OH)\(^2\)D\(_3\) in the range of 0.1–4.5 ng per tube and 0.1–2.0 ng per tube, respectively.

The procedure for the 1,25(OH)\(^2\)D\(_3\) assay, including preparation of binding protein, has been detailed previously (9).

Results

Extraction. The vitamin D metabolites in question could be extracted in high yield with the described procedure. Recoveries of 25(OH)\(^{[3H]}\)D\(_3\), 24,25(OH)\(^2\)\(^{[3H]}\)D\(_3\), and 1,25(OH)\(^{2}\)[\(^{3H}\)]D\(_3\) added to 5 mL of plasma were, respectively, 93.7(SD 2.2)%, 89.4(SD 2.1)%, and 89.7(SD 1.3)% (n = 10).

Liquid chromatography. The number of theoretical plates for a 12-cm column was about 14 000 plates per meter. Typical column useful life was approximately 150 sample injections. The chromatographic profile obtained with the 1,25(OH)\(^2\)D\(_3\) CBP procedure has been already discussed (9). With the 24,25(OH)\(^2\)D\(_3\) CBP assay, five peaks were detected (Figure 1B), the first three peaks coeluting with 25(OH)\(^{[3H]}\)D\(_3\), 24,25(OH)\(^2\)[\(^{3H}\)]D\(_3\), and 25,26(OH)\(^2\)D\(_3\), respectively. In the polar region of the chromatogram two minor peaks were detected, the first one having the same retention time as the unknown compound detected with the 1,25(OH)\(^2\)D\(_3\) CBP assay and the second one emerging a few minutes later. There was baseline separation of all compounds that possessed displacement activity in the 24,25(OH)\(^2\)D\(_3\) CBP assay. The retention times were constant enough to allow unattended sample processing during at least 24 h.

All three metabolites were undetectable in 5-mL water
blanks carried through the whole procedure. Detection limits were 2.5 nmol/L, 0.5 nmol/L, and 5 pmol/L for 25(OH)D, 24,25(OH)2D, and 1,25(OH)2D, respectively. Overall analytical recoveries (n = 40) were 77.4(SD 3.1)%%, 64.7(SD 2.9)%%, and 77.3 (SD 5.2)%%, respectively.

Competitive protein binding. The reciprocal of specific binding (total binding minus nonspecific binding) was plotted vs amount of unlabeled metabolite. The straight lines obtained for each metabolite (Figure 2) were used to calculate sample concentration by linear interpolation. Detection limits (expressed as two standard deviations of binding without added unlabeled metabolite) for 25(OH)D, 24,25(OH)2D, and 1,25(OH)2D were 125, 120, and 2–5 fmol per tube (50, 50, and 1–2 pg per tube).

Precision and reference intervals. The 25(OH)D concentration in the plasma pool was measured in octuplicate on six different days. Interassay CVs, derived from the mean of these eight determinations on six days, and the mean intra-assay CVs were 3.9 and 4.4%, respectively. The 25(OH)D concentration in the plasma pool was 50.4(SD 3.0) nmol/L. Individual plasma concentrations averaged 51.3(SD 16.5) nmol/L (range 32–106 nmol/L; n = 20).

Intra- and interassay CVs for the 24,25(OH)2D assay were determined separately. The serum pool was analyzed eight times on one day, and one time on six other days. Intra- and interassay CVs were 6.7 and 8.0%, respectively. The 24,25(OH)2D concentration of the plasma pool was 5.5(SD 0.1) nmol/L. Individual concentrations in serum averaged 4.1(SD 1.3) nmol/L (range 1.7–6.2 nmol/L; n = 11).

For the 1,25(OH)2D assay, intra- and interassay CVs were determined as described for 25(OH)D and were, respectively, 7.0 and 4.8%. The 1,25(OH)2D concentration in the plasma pool was 129(SD 12) pmol/L. Individual plasma concentrations averaged 124(SD 26) pmol/L (range 77–189 pmol/L; n = 20).

Discussion

To meet the demands for an assay of the main metabolites of vitamin D for clinical investigation, our aim was to design as simple a procedure as possible. Hitherto, five reports have been published on the simultaneous determination of 25(OH)D, 24,25(OH)2D, and 1,25(OH)2D in human blood (4–8). All of them involve multiple extraction steps, open column chromatography on Sephadex LH-20 or silicic acid, and final purification of the metabolites in one or more HPLC systems. The use of multiple chromatographic systems, involving several evaporation steps, makes these methods especially laborious. Because of the lack of receptor proteins specific for one particular vitamin D metabolite, a chromatographic separation is required for accurate measurement of these metabolites. As indicated from the chromatographic profile (Figure 1B), a nonchromatographic assay for 25(OH)D would result in overestimates of 25(OH)D concentration by about 5 to 15% because of the presence of 24,25(OH)2D and 25,26(OH)2D. Reports on the comparison of chromatographic and non-chromatographic procedures reveal considerable overestimation of 25(OH)D concentrations without at least one open-column chromatographic purification (10–15). 24,25(OH)2D can only be satisfactorily purified by HPLC, because it must be completely separated from 25,26(OH)2D and the large excess of 25(OH)D. With regard to the 1,25(OH)2D assay, for samples with vitamin D concentrations within the normal physiological range, this metabolite requires isolation from 25(OH)D and the unknown polar interfering compound. This separation might be achieved with open column chromatography, but for simultaneous isolation of several vitamin D metabolites HPLC remains necessary.

The chromatographic profile of the plasma pool showed peaks that comigrated with authentic vitamin D3 metabolites. Although 25(OH)D3 and 25(OH)2D3 had almost identical retention times, we could not confirm this for dihydroxyated vitamin D2 metabolites, because these compounds are not available in pure form.

Horst et al. (16) reported the presence of 25(OH)D-26,23-lactone in the blood of pigs and cows, a compound that could interfere in the CPB assay for 25(OH)D and 24,25(OH)2D with rat-serum binding protein. This metabolite appeared to be present in humans only when vitamin D concentrations were greater than physiologically (17). The results of Aksnes (8a) seem to confirm this, because he could not detect additional displacement peaks after rechromatography of comparable metabolite fractions on straight-phase and reversed-phase HPLC. Although we find a very small peak between the 25(OH)D and the 24,25(OH)2D fraction (Figure 1B), the level of displacement is so small that this does not interfere in our assays.

Vitamin D metabolites are quantitated with CPB proce-
durea because such assays are sensitive enough to detect the very low physiological concentrations of all three vitamin D metabolites and because they are suited for the analysis of many samples. Moreover, the use of receptor proteins increases specificity with regard to plasma components not derived from vitamin D (18, 19). The concentrations in plasma that we found for 25(OH)D and 24,25(OH)2D agreed well with values for 25(OH)D (4, 8b, 20, 21) and 24,25(OH)2D (4, 8b, 22) reported in Western Europe in the recent literature. As discussed previously (9), our 1,25(OH)2D concentrations were somewhat higher than earlier values.

We conclude that our extraction and HPLC procedures offer a relatively fast and more compact way to isolate the main metabolites of vitamin D from plasma or serum. With the mechanized HPLC apparatus 20 samples can be fractionated unattended within 24 h. The compact isolation procedure, together with the modified methods, result in a low detection limit and high precision for the concurrent determination of 25(OH)D, 24,25(OH)2D, and 1,25(OH)2D in plasma or serum samples.

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References