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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 25-HYDROXYVITAMIN D_2 AND 25-HYDROXYVITAMIN D_3 IN HUMAN PLASMA

USE OF ISOTACHYSTEROLS AND A COMPARISON WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for estimating plasma 25-hydroxyvitamin D_2 (25-OHD_2) and 25-hydroxyvitamin D_3 (25-OHD_3) is described. The method involves plasma extraction, Lipidex 5000 chromatography and HPLC on straight-phase Zorbax-SIL, collecting the 25-OHD_2 + 25-OHD_3 fractions. These secosteroids are isomerised to their isotachysterol derivatives and re-run in the same HPLC system, monitoring at 290 nm. ^{3}H-Labelled 25-OHD_3 is used as an internal standard. The method was evaluated in terms of reproducibility, and recovery of added secosteroids was quantitative. Values obtained using this method were in close agreement with those values obtained on the same plasma sample using gas chromatography—mass spectrometry.

INTRODUCTION

Of the derivatives of vitamin D* circulating in human plasma, the 25-hydroxylated metabolite is in the highest concentration [1]. Since it is relatively easy to measure, estimates of plasma levels of 25-hydroxyvitamin D (25-OHD) have therefore been widely used for the assessment of vitamin D status in a variety of clinical situations [2]. Until recently, most methods for the estimation of

*Systematic and trivial names of vitamin D and its derivatives used in this paper are as follows: Vitamin D_2 (9,10-seco-5,7,10(19),22-ergostatetraen-3β-ol): D_2. Vitamin D_3 (9,10-seco-5,7,10(19)-cholestatrien-3β-ol): D_3. 25-Hydroxyvitamin D_2: 25-OHD_2. 25-Hydroxyvitamin D_3: 25-OHD_3. Isotachysterol isomer formed from 25-OHD_2: 25-$CHITS_2$. Isotachysterol isomer formed from 25-OHD_3: 25-$OHITS_3$.

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25-OHD in human plasma have relied upon competitive protein-binding assays with and without chromatography prior to assay (reviewed in ref. 3). The majority of these assays, being derived from the procedure of Haddad and Chyu [1], did not distinguish between 25-OHD_2 and 25-OHD_3. Over the last five years, the introduction of high-performance liquid chromatography (HPLC) has greatly simplified and improved the specificity of methods for the estimation of plasma levels of 25-OHD_2 and 25-OHD_3 [4]. The sensitivity of ultraviolet (UV) detection, used in these HPLC methods, can be enhanced by forming isotachysterol isomers* prior to HPLC [5]. This paper describes the application of this procedure to the estimation of 25-OHD_2 and 25-OHD_3 in human plasma.

Few of the previous HPLC methods have been evaluated in terms of specificity, except that the eluent HPLC fractions have been collected and subjected to mass spectrometry (MS) [6], gas chromatography (GC) and mass spectrometry [7, 8], and UV spectra have been obtained [6—8] in order to demonstrate the purity of the final HPLC fraction. This paper compares the results obtained by the HPLC method described with the results obtained by a mass fragmentographic procedure [9].

MATERIALS AND METHODS

Materials

Pure secosteroids were obtained from the following sources: vitamin D_2 (Koch-Light Labs., Colnbrook, Great Britain), 25-OHD_2 and 25-OHD_3 (Dr. J.A. Campbell, The Upjohn Company, Kalamazoo, MI, U.S.A.). These secosteroids were purified by HPLC before use. 25-Hydroxy[23,24-^{3}H]vitamin D_3 (specific activity around 110 Ci/mmole) supplied by the Radiochemical Centre (Amersham, Great Britain) was found to be radiochemically pure on receipt. Radioactive steroids were re-purified every three months by HPLC. Lipidex 5000 was obtained from Packard Instrument Co. (Reading, Great Britain). Other reagents were as specified by Seamark et al. [9, 10] and were analytical reagent grade wherever possible. HPLC was carried out with a Model 750/03 pump, a Rheodyne 7125 injection valve, and Model SF770 variable-wavelength (190—700 nm) detector (Schoeffel Instruments) all supplied by Applied Chromatography Systems (Luton, Great Britain). A Zorbax-SIL (5 μm, 250 × 0.46 mm) column from DuPont (U.K.), Hitchin, Great Britain was eluted with a solvent system of hexane—isopropanol (9:1) as described by Seamark et al. [5]. Mass fragmentography was carried out using an LKB 2091 gas chromatograph—mass spectrometer as described by Seamark et al. [9].

All glassware was silanised by soaking overnight in 1% (v/v) dimethyldichlorosilane in toluene and washed with ethanol. Blood was taken from apparently healthy volunteers of both sexes into heparinised containers; the plasma was separated and analysed immediately. Liquid scintillation counting was carried out using an Intertechnique Model SL30 (Kontron Intertechnique, St. Albans, Great Britain) and 5 ml of NE250 liquid scintillation fluid. At least 10,000

*See p. 351.

counts were collected. Tritium counting efficiency in this system was 40% with a background count rate of around 30 cpm.

Method

The procedure is summarised in Fig. 1. Plasma (2 ml), to which approximate-

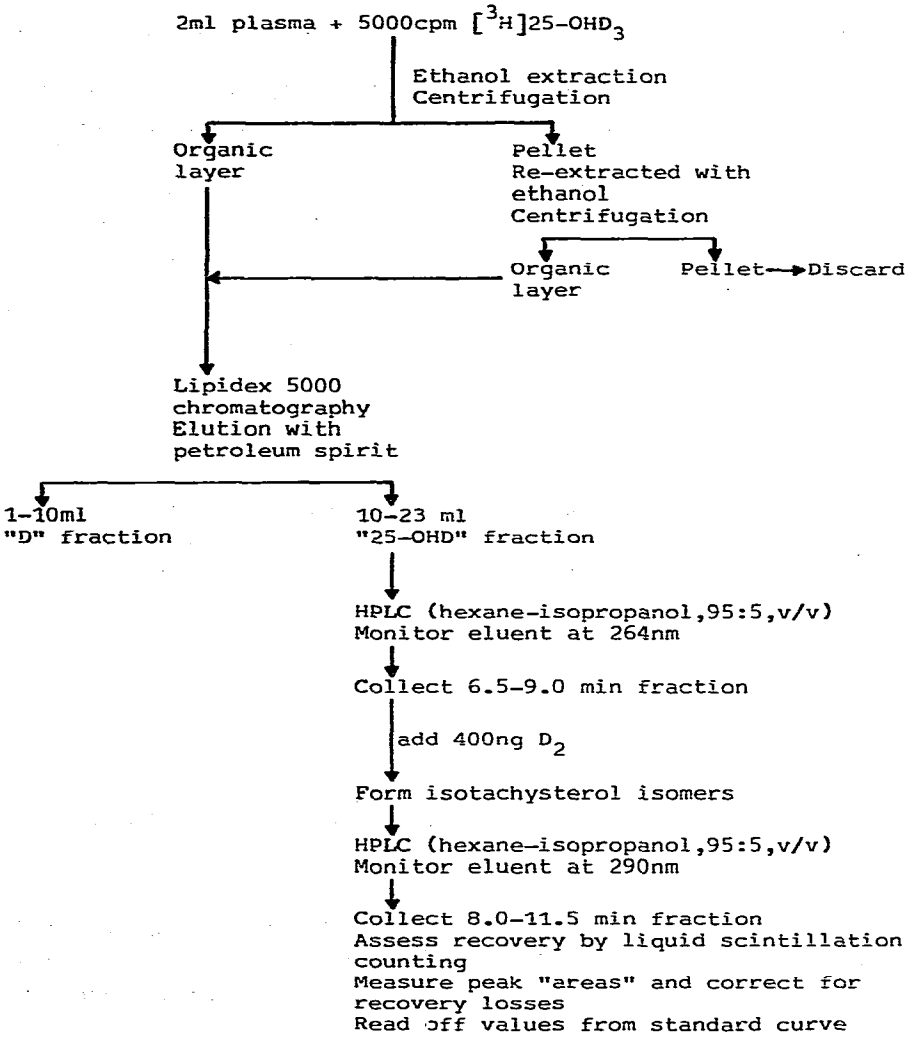

Fig. 1. Flow diagram for the two-stage HPLC assay for 25-OHD_2 and 25-OHD_3.

ly 5000 cpm of ^3H-labelled 25-OHD_3 had been added, was extracted with 18 ml of redistilled ethanol by mechanical shaking for 4 min. The precipitated protein was centrifuged (2000 *g* for 10 min), the supernatant removed and the protein pellet re-extracted with 10 ml of redistilled ethanol. The two ethanol extracts were combined and evaporated to dryness in a rotary evaporator at 40°C.

The residue was applied to a Lipidex 5000 column (7 cm × 0.5 cm I.D.) in 3 × 0.5 ml of petroleum spirit and the "25-OHD" fraction [9] was collected.

The solvent was evaporated to dryness, dissolved in 100 μl of hexane—isopropanol (95:5, v/v) and injected into the HPLC system [Zorbax-SIL, eluted with hexane—isopropanol (95:5, v/v) at a flow-rate of 1.5 ml/min], monitoring the effluent at 264 nm. The fraction containing 25-OHD_2 and 25-OHD_3 (usually between 6.5 and 9.0 min after injection) was collected and evaporated to dryness. Chloroform (75 μl) and vitamin D_2 (400 ng) were added and the isotachysterol isomers prepared using the HCl procedure described by Seamark and coworkers [5, 10]. Once formed, these isomers were dissolved in 100 μl of hexane—isopropanol (95:5, v/v) and injected into the HPLC system using the same column and eluting solvent as before, monitoring the effluent at 290 nm. The fraction containing the isotachysterol isomer of 25-OHD_3 (25-$OHITS_3$) (around 8.0—11.5 min) was collected and counted for radioactivity to assess overall recovery. Peak "areas" [5] for the isotachysterol isomers 25-$OHITS_2$ and 25-$OHITS_3$ were measured and corrected for recovery losses, assuming that the recovery of 25-OHD_3 was the same as that for 25-OHD_2.

A standard curve covering the range 0—100 ng of 25-OHD was obtained by injecting 5000 cpm of ^{3}H-labelled 25-OHD_3 plus increasing amounts of standard 25-OHD_2 and 25-OHD_3 which, together with 400 ng of vitamin D_2, had been isomerised to the isotachysterol derivatives. Peak "areas", corrected for recovery as described above, were measured and plotted against the amount of 25-OHD added. The response was linear over the range examined and equations of the lines were $y = 0.28x + 0.12$ (correlation coefficient 0.9999, for 25-OHD_2) and $y = 0.28x + 0.11$ (correlation coefficient 0.9996, for 25-OHD_3), where y represents the corrected peak area and x represents the mass in ng. 25-$OHITS_2$ and 25-$OHITS_3$ have the same UV absorbance per unit mass and thus, in the absence of standard 25-OHD_2, standard curves produced using 25-OHD_3 can be used to quantitate both 25-hydroxylated vitamins.

EVALUATION OF THE METHOD

The formation of isotachysterol derivatives of 25-OHD_2 and 25-OHD_3 increased their retention times on the straight-phase Zorbax-SIL column used.

TABLE I

RETENTION TIMES OF SOME SECOSTEROIDS AND THEIR CORRESPONDING ISOTACHYSTEROL DERIVATIVES ON A STRAIGHT-PHASE ZORBAX-SIL COLUMN

The solvent system used was hexane—isopropanol (95:5, v/v) at a flow-rate of 1.5 ml/min.

Secosteroid	Retention time (min)
D_3	2.00
D_2	2.00
25-OHD_2	6.80
25-OHD_3	8.36
ITS_3	1.78
ITS_2	1.78
25-$OHITS_2$	8.78
25-$OHITS_3$	11.20

Table I shows the retention times of vitamins D_2 and D_3, 25-OHD_2, 25-OHD_3, 25-$OHITS_2$ and 25-$OHITS_3$. Formation of these isotachysterol isomers did not significantly alter the separation of the calciferols in the HPLC system used here. Samples of the HPLC traces obtained when monitoring the effluent at 264 nm before isomerisation and when monitoring at 290 nm after isomerisation are shown in Fig. 2.

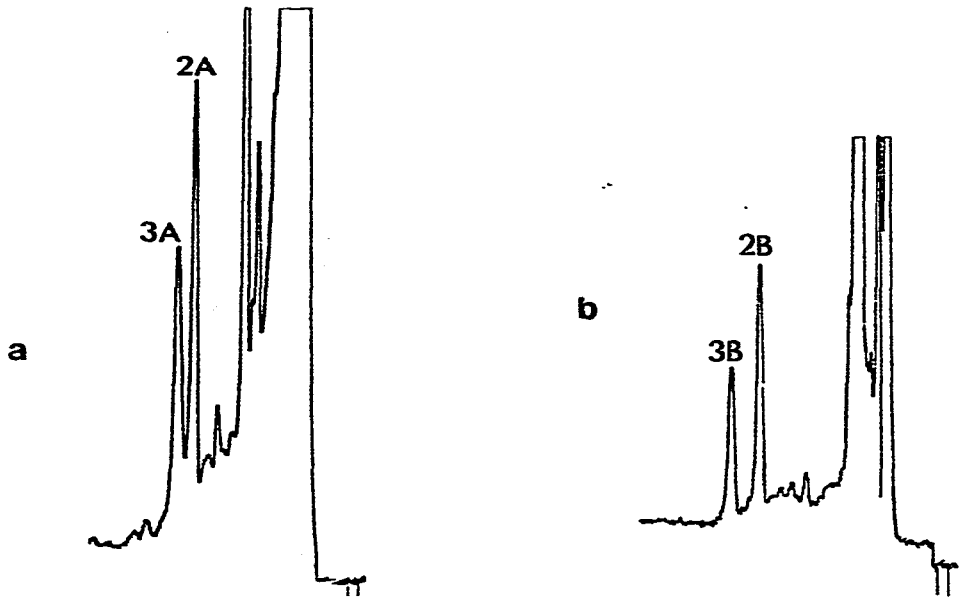

Fig. 2. HPLC traces obtained before and after isotachysterol formation. (a) First HPLC run, monitoring at 264 nm, 0.01 a.u.f.s. 2A and 3A indicate the positions of 25-OHD_2 and 25-OHD_3, respectively. (b) Second HPLC run (after isotachysterol formation), monitoring at 290 nm, 0.01 a.u.f.s. 2B and 3B indicate the positions of 25-$OHITS_2$ and 25-$OHITS_3$, respectively.

A single plasma sample was analysed in duplicate using four different volumes of plasma, ranging from 5 to 1 ml. Fig. 3 shows the amounts of 25-OHD_2 and 25-OHD_3 in each sample plotted against the volume of plasma used. Straight lines were obtained for both 25-OHD_2 and 25-OHD_3, indicating that values obtained were independent of the volume of plasma used for analysis. For a plasma sample containing around 20 ng/ml of 25-OHD_3 it should be possible to use around 0.5 ml of sample for analysis, but in practice, it was decided to use 2 ml to enable 25-OHD_2 to be measured as well.

Varying amounts of standard 25-OHD_2 and 25-OHD_3 were added in ethanol to 2-ml plasma samples in duplicate. After equilibration at 37°C for 15 min, the samples were analysed. The mean values for 25-OHD_2 and 25-OHD_3, together with the calculated recoveries, are given in Table II. In each case recoveries were quantitative and the amount of one secosteroid added did not affect the recovery of the other. The use of ^{3}H-labelled 25-OHD_3 to monitor the recovery of 25-OHD_2 appeared to give satisfactory results. Recoveries of added tritium at various stages in the method are given in Table III. Intra-assay and inter-

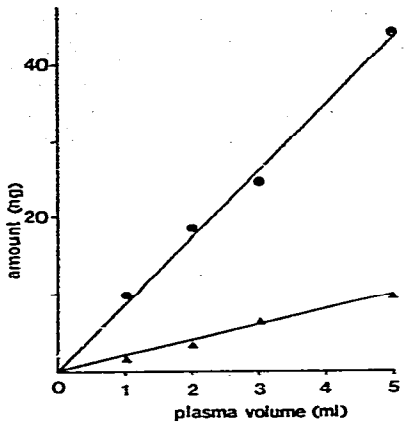

Fig. 3. Effect of analysing different volumes of plasma on the estimated amount (ng) of 25-OHD_2 (•) and 25-OHD_3 (▲). Correlation coefficients were 0.9950 (25-OHD_3) and 0.9910 (25-OHD_2); neither was significantly different from 1.000 ($p > 0.5$). Intercepts were +0.4 ng (25-OHD_3) and −0.8 ng (25-OHD_2); neither was significantly different from zero ($p > 0.5$).

TABLE II

RECOVERIES OF STANDARD SECOSTEROIDS ADDED TO PLASMA

25-OHD_3			25-OHD_2		
Added (ng/ml)	Observed (ng/ml)*	Recovery (%)	Added (ng/ml)	Observed (ng/ml)*	Recovery (%)
0	19.9	—	0	0	—
10	29.1	92.0	10	10.2	102.5
20	40.0	100.5	15	14.3	95.3
30	51.3	104.5	25	23.8	95.3
50	65.5	91.2	30	31.0	103.3
0	19.0	—	35	38.3	109.3

*Mean of duplicate assays is recorded.

TABLE III

RECOVERIES, AT VARIOUS STAGES IN THE METHOD, OF ^{3}H-LABELLED 25-OHD_3 ADDED TO PLASMA

Results are expressed as mean ± standard deviation. n = number of samples.

Stage in method	Recovery (%)
After extraction	78.4 ± 5.9 ($n = 10$)
After Lipidex column	69.5 ± 2.9 ($n = 12$)
After first HPLC	60.5 ± 3.5 ($n = 8$)
After second HPLC	48.0 ± 3.6 ($n = 8$)

assay variability studies were also carried out and the results are given in Table IV.

TABLE IV

INTRA-ASSAY AND INTER-ASSAY VARIABILITY

	Secosteroid value (ng/ml)	C.V. (%)
Intra-assay		
25-OHD$_3$	19.2 ± 0.95	4.9 (n = 10)
25-OHD$_2$	14.3 ± 0.6	4.1 (n = 10)
25-OHD$_2$	3.9 ± 0.3	8.3 (n = 10)
*Inter-assay**		
25-OHD$_3$	18.7 ± 1.6	8.8 (n = 6)
25-OHD$_2$	4.2 ± 0.5	11.6 (n = 6)

*Plasma sample stored at −20°C, analysed over a period of four weeks.

The specificity of the HPLC assay described here was evaluated by comparing the values for 25-OHD$_2$ and 25-OHD$_3$ in plasma obtained from normals and from volunteers taking oral vitamin D$_2$ supplements, with values obtained on the same plasma using a GC—MS method previously described [9]. In addition, concentrations of 25-OHD$_2$ and 25-OHD$_3$ were also estimated after the first HPLC separation using the effluent trace monitored at 264 nm. Concentrations of 25-OHD$_2$ and 25-OHD$_3$ after only one HPLC separation, without isomerisation, were higher than those obtained by GC—MS. On occasion it proved impossible to discern a single peak at the appropriate retention times. However, the second HPLC separation, after isomerisation to the isotachysterol

TABLE V

PLASMA CONCENTRATIONS OF 25-OHD$_3$ AND 25-OHD$_2$ IN TWELVE PLASMA SAMPLES ASSAYED BY THREE DIFFERENT PROCEDURES

Sample	25-OHD$_3$ (ng/ml)			25-OHD$_2$ (ng/ml)		
	HPLC$_{264}$	→ HPLC$_{290}$	GC—MS	HPLC$_{264}$	→ HPLC$_{290}$	GC—MS
1	35.0	16.8	15.8	50.5	22.5	21.3*
2	44.3	8.8	11.5	27.5	6.3	5.4*
3	28.0	12.8	13.0	3.0	1.5	1.6
4	54.8	27.1	21.3	27.2	3.4	3.0
5	24.0	10.3	8.7	22.8	5.9	4.5
6	18.5	12.8	13.0	3.0	3.1	2.8
7	25.0	12.5	14.9	19.0	6.4	6.1*
8	24.5	12.2	16.9	28.5	14.2	12.7*
9	26.0	13.8	15.8	24.1	15.7	13.7*
10	I**	40.0	46.2	45.6	4.9	2.6
11	I	18.4	18.2	30.4	3.1	3.2
12	41.5	29.4	26.9	I	ND***	ND

*Plasma samples from volunteers taking vitamin D$_2$ (3000 IU/day, orally).
**I = interference, making the estimation impossible.
***ND = Not detectable.

isomers, gave clearer traces and produced lower values which were in close agreement with those obtained by GC—MS. The values obtained are given in Table V. Regression analysis performed on these results gave correlation coefficients of 0.9941 (25-OHD_2) and 0.9639 (25-OHD_3) and the equation of the lines were y (GC—MS) = $0.93x$ ($HPLC_{290}$) − 0.32 (for 25-OHD_2) and y (GC—MS) = $1.06x$ ($HPLC_{290}$) + 0.34 (for 25-OHD_3). Normal values obtained from healthy volunteers of both sexes, using plasma taken in Great Britain in August are given in Table VI.

TABLE VI

NORMAL VALUES FOR 25-OHD_2 AND 25-OHD_3 IN PLASMA

Plasma was taken from six females and five males (aged between 18 and 40 years) in August. Results are expressed as mean ± S.D.

	Plasma concentration (ng/ml)
25-OHD_3	23.9 ± 11.0
25-OHD_2	4.4 ± 1.3

DISCUSSION

Although the estimates of the concentration of 25-OHD in plasma, obtained after Lipidex chromatography and a single HPLC run monitoring the effluent at 264 nm (see Table V), were in many cases of the right order of magnitude, it is clear that these values do not reflect the lower, presumably more accurate, concentrations measured by mass fragmentography. In our hands, therefore, a single straight-phase HPLC run after one column chromatographic separation is inadequate. The interpolation of a specific chemical reaction — formation of isotachysterol isomers — between the first and second HPLC separation, increases the specificity and enhances the sensitivity of detection [5]. This type of alteration in chromatographic mobility after chemical transformation has been used in classical chromatography systems for steroids [11] and for organic acids [12] and is recommended as one procedure for use in establishing the identity of steroids [13]. Improvement in specificity can also be achieved by the use of reversed-phase systems for the second HPLC separation, without chemical transformation [6], or by the use of different solvent systems on an LH-20 column prior to HPLC [14]. Dual-column methods require the use of two separate HPLC systems. The method described here has the advantage that only a single straight-phase HPLC system is required. It is thus simpler than many of the previously published HPLC assays for 25-OHD.

Plasma concentrations of 25-OHD_2 and 25-OHD_3 using this method are in reasonable agreement with those obtained by a mass fragmentographic method [9] and agree with the results reported by Shepard et al. [15], although our mean value for 25-OHD_2 is perhaps high for Great Britain. Total 25-OHD values measured here were in agreement with values obtained on samples collected during the summer months in Great Britain using competitive protein-binding assays [16, 17] and HPLC in the U.S.A. [15].

A number of HPLC assays for 25-OHD_3 [7, 8, 18—20] or total 25-OHD [7, 8, 21] have been described, none of which have measured 25-OHD_2 directly. Shepard et al. [15] and Jones [6] describe HPLC methods which measure 25-OHD_3 and 25-OHD_2. Jones [6] used a straight-phase HPLC separation followed by a reversed-phase HPLC separation, and validated the method against a competitive protein-binding assay and obtained mass spectra from trapped fractions from the second HPLC column.

The majority of HPLC assays described above have utilised UV absorbance at 254 nm for quantitation. This wavelength is not the absorption maximum of vitamin D and its metabolites. The method described by Jones [6], however, monitored the effluent at 264 nm, which is the absorption maximum. The formation of isotachysterol isomers increased the absorbance at 290 nm approximately two-fold over that at 254 nm [5] and thus increased the sensitivity of detection.

The use of HPLC with UV detection has become increasingly widespread in the assay of 25-OHD_2 and 25-OHD_3. The cost of HPLC equipment is less than a mass spectrometer and requires less expertise to operate and maintain. The HPLC assays described here give comparable specificity to that obtained by mass fragmentography and, on occasions where specificity is in doubt, effluent fractions can be collected and checked on the mass spectrometer. HPLC assays are more expensive than competitive protein-binding assays which in general are relatively non-specific and measure only total 25-OHD. The HPLC assay described here is able to distinguish between 25-OHD_2 and 25-OHD_3, and the accuracy and degree of precision are higher than most published competitive protein-binding assays for 25-OHD. This simplified HPLC assay is estimated to have a minimum detection limit of approximately 0.5 ng/ml using a 2-ml plasma sample.

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