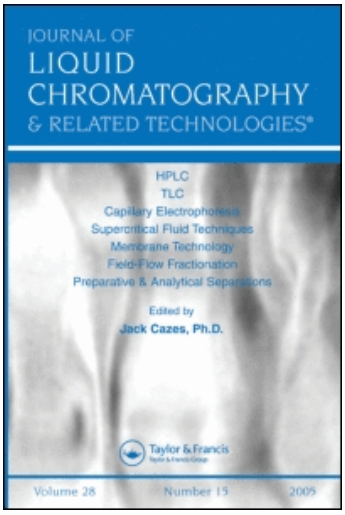

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IMPROVED HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF SERUM 25-HYDROXYCHOLECALCIFEROL AND 25-HYDROXYERGOCALCIFEROL AFTER REVERSE-PHASE SEP-PAK C_{18} CARTRIDGE PREPARATION OF SAMPLE

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ABSTRACT

A simplified method is described for extracting and purifying 25-hydroxycholecalciferol and 25-hydroxyergocalciferol from serum for quantitation by high-pressure liquid chromatography. The method involves extracting and purifying these metabolites from serum (1-10 ml) with a reverse-phase octadecylsilane bonded silica cartridge (Sep-Pak C_{18}). This method is faster than a previously described method involving extraction with dichloromethane and purification by Sephadex LH-20 chromatography. The correlation between the two methods was excellent ($r^2 = 0.96$, $p<0.0001$). The coefficient of variation for the new method is 4.3%. The new method allows measurement of 25-hydroxyergocalciferol from human serum since both 25-hydroxycholecalciferol and 25-hydroxyergocalciferol are extracted equally. This allows the use of [^{3}H]25-hydroxycholecalciferol to monitor the recovery of both the D_2 and the D_3 forms of the metabolite.

INTRODUCTION

High-pressure liquid chromatography (HPLC) (1) has been shown to resolve 25(OH)D_3 and 25(OH)D_2 from each other and from other

metabolites of the vitamin D_2 and D_3 family (2,3). It has been used to measure 25(OH)D_2 and 25(OH)D_3 in bovine (4) and human (5-10) sera. HPLC methods are more precise and expedient than previously reported competitive protein-binding (CPB) methods for assay of 25-hydroxyvitamin D (5-10). CPB methods do not distinguish the D_2 from the D_3 forms of 25-hydroxyvitamin D, whereas HPLC methods can. Quantitation by HPLC requires extraction and purification of 25(OH)D_3 from serum to a degree that analytical resolution of the sample becomes possible. The published HPLC methods are based upon lipid extraction and some purification of the extract via column chromatography with Sephadex LH-20 (5,6,8), Sephadex LH-20 and Lipidex 5000 (10), or silica gel (9) prior to analytical chromatography. These methods suffer in that the measurement of 25(OH)D_2 relies upon the assumption that the fractional recoveries of 25(OH)D_2 and 25(OH)D_3 through sample extraction and purification are the same. This assumption allows the use of [^{3}H]25(OH)D_3 to monitor overall recovery of both 25(OH)D_2 and 25(OH)D_3. The assumption is hazardous and can lead to error in measurement of 25(OH)D_2 as has been pointed out by Stryd and Gilbertson (11). One group (5) has synthesized and used [^{3}H]25(OH)D_2 to validate this assumption for their preparative procedure. However, [^{3}H]25(OH)D_2 is not commercially available and the routine use of [^{3}H]25(OH)D_3 to monitor 25(OH)D_2 recovery is not valid without demonstration of equal recovery of both congeners. An improved method for extracting and purifying 25(OH)D_2 and 25(OH)D_3 from human serum is presented in this report. It involves precipitation of serum

proteins with methanol and rapid extraction and purification of 25(OH)D_2 and 25(OH)D_3 with a reverse-phase octadecylsilane bonded silica cartridge (Sep-Pak C_{18}). This gives results for 25(OH)D_3 indistinguishable from those obtained by one of the published procedures (8), which employed Sephadex LH-20 chromatographic purification. The time and labor per assay are reduced substantially. Since the Sep-Pak C_{18} preparative method reported herein extracts 25(OH)D_2 and 25(OH)D_3 equally, recovery of both metabolites can be monitored by use of [^{3}H]25(OH)D_3 added initially to serum.

MATERIALS

Reagent grade methanol, isopropyl alcohol, chloroform, and dichloromethane were redistilled in glass. Hexanes (Mallinckrodt, Inc., St. Louis, Mo., certified), toluene, and tetrahydrofuran (reagent) were used as received. Chromatography columns of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were used as previously reported (8). Reverse-phase cartridges (1.2 x 0.9 cm) with an octadecylsilane bonded silica adsorbent (Sep-Pak C_{18}, Waters Associates, Inc., Milford, Mass.) were washed with 10 ml of tetrahydrofuran followed by 10 ml of glass-distilled water. High-pressure liquid chromatography was effected with two μPorasil columns (0.4 by 25 cm, Waters Associates) connected in series. [^{3}H]25(OH)D_3 (17 Ci/mmole, New England Nuclear, Boston, Mass.) was used after chromatographic purification through two

μPorasil columns. Concentrations of solutions of purified $25(OH)D_2$ and $25(OH)D_3$ were determined by uv absorbance at 265 nm (Beckman Model 24 Spectrophotometer); ε_{265} of 18,000 was used (8). [^{3}H]Vitamin D_3 (22 Ci/mmol, New England Nuclear, Boston, Mass.) and [^{3}H]$1,25(OH)_2D_3$ (110 Ci/mmol, New England Nuclear, Boston, Mass.) were used as received. Both $25(OH)D_2$ and $25(OH)D_3$ were gifts from the Upjohn Co., Kalamazoo, Mich. Serum obtained from subjects (informed consent obtained) was stored at -20°C until use.

METHODS

High-pressure liquid chromatography of purified serum extracts was performed as previously described (8) except that isopropyl alcohol:hexanes (4:96, v:v) was used as eluent. Chromatography was conducted at room temperature with a solvent flow rate of 1.0 ml/min (800 psi). Under these conditions $25(OH)D_2$ and $25(OH)D_3$ were eluted 16 and 18 minutes following injection, respectively. For each analysis the fraction corresponding to $25(OH)D_3$ (17.5 to 20.0 ml) was collected. The recovery of [^{3}H]$25(OH)D_3$ was then determined by liquid scintillation counting with correction for quenching. The areas of the $25(OH)D_2$ and $25(OH)D_3$ peaks were determined by triangulation (peak absorbance at 254 nm x peak width at half-height in ml); ε_{254} of 16,900 was used (8). Extraction and purification of $25(OH)D_3$ from serum on Sephadex LH-20 chromatography was performed as previously reported

(8). Extraction and purification of 25(OH)D_2 and 25(OH)D_3 from serum with the Sep-Pak C_{18} cartridges were performed as follows. All steps were carried out at room temperature. Each serum sample (1-10 ml) was labeled with 5000 dpm of [^{3}H]25(OH)D_3 (in 0.02 ml of isopropyl alcohol) to monitor overall recovery of 25(OH)D_3. The sample was added dropwise to a volume of methanol two times the volume of serum with vortexing to precipitate serum proteins. The concentration of methanol was adjusted to 50% by addition of glass-distilled water, and the precipitated proteins were removed by centrifugation at 1000 x g for 10 min. After separation of pellet and supernatant fractions, the pellet was washed with a volume of methanol:water (1:1, v:v) two times the volume of serum. The supernatant fraction from this washing was combined with the initial supernatant fraction and both were decanted into a glass syringe which was coupled to a Sep-Pak C_{18} cartridge. The supernatant fraction was passed through the cartridge with gentle pressure on the plunger. The cartridge was washed with 10 ml of methanol:water (7:3, v:v). Then, 25(OH)D_2 and 25(OH)D_3 were eluted with 10 ml of methanol:water (85:15, v:v) and concentrated to dryness in a stream of nitrogen. The residue was dissolved in 0.25 ml of isopropyl alcohol:hexanes (4:96, v:v), and this solution was analyzed by HPLC (8).

RESULTS

Application of [^{3}H]25(OH)D_3 in methanol:water (1:1, v:v) to the Sep-Pak C_{18} cartridge resulted in almost quantitative

retention of tritium. Washing the Sep-Pak C_{18} cartridge with 10 ml of methanol:water (7:3, v:v) resulted in recovery of 6% of total activity in the effluent. Washing of the cartridge with 10 ml of methanol:water (85:15, v:v) eluted 93% of the total activity. [^{3}H]Vitamin D_3 and [^{3}H]1,25(OH)$_2D_3$ were also retained quantitatively when applied with methanol:water (1:1, v:v). [^{3}H]1,25(OH)$_2D_3$ was eluted along with [^{3}H]25(OH)D_3. Only ten percent of the [^{3}H]vitamin D_3 was eluted by methanol:water (85:15, v:v). Virtually 100% was eluted by methanol. HPLC of serum extracts prepared with the Sep-Pak C_{18} method yielded chromatograms with clear resolution of 25(OH)D_2 and 25(OH)D_3 peaks upon a substantially flat baseline (Figure 1).

The extraction and purification procedure for serum 25(OH)D_3 by the Sep-Pak C_{18} cartridges was compared with that by Sephadex LH-20 (8). Both methods were used to assay sera (1 ml sample volume) obtained from 15 healthy adults. Recovery of tritium activity averaged 52.5% for the Sep-Pak C_{18} purification as compared with 31% for Sephadex LH-20 purification. The range of concentrations of 25(OH)D_3 in the 15 serum samples was 14-57 ng/ml. Linear regression analysis of the results (Figure 2) demonstrated no statistically significant difference in the assay of sera prepared by these two methods (intercept = -0.2, slope = 1.006, $p<0.0001$, $r^2 = 0.96$). The coefficient of variation (C.V.) for seven separate assays of a single serum (mean 25(OH)D_3 of 37 ng/ml) purified by the Sep-Pak C_{18} method was 4.3% compared to the C.V. of 5.1% for the Sephadex LH-20 method (8).

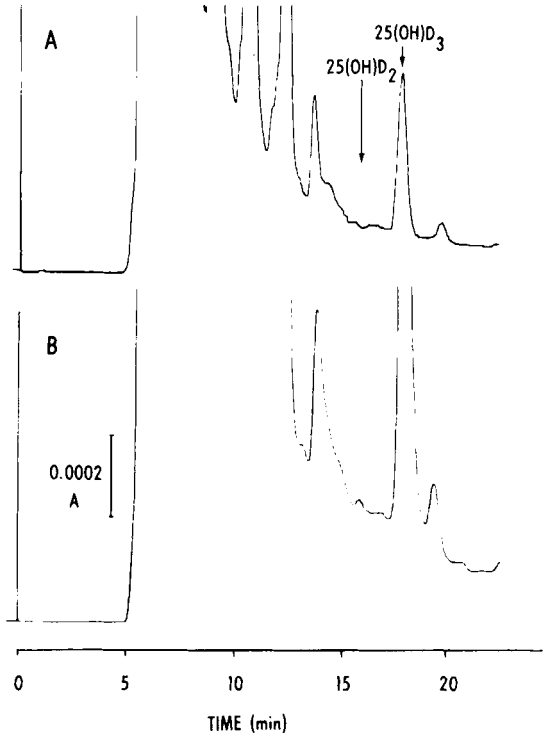

FIGURE 1. HPLC chromatograms of 2 aliquots of the same human serum prepared by the Sep-Pak C_{18} method. A, 1-ml aliquot (25(OH)D_2 not detected; 25(OH)D_3=14 ng; recovery=55%. B, 10-ml aliquot (25(OH)D_2 peak area far less than 3 ng; 25(OH)D_3=120 ng; recovery=48%). See Methods Section for conditions of chromatography.

To test the hypothesis that 25(OH)D_2 and 25(OH)D_3 are recovered equally from serum by the present method, assays were performed on a serum to which known amounts of these metabolites were added. These metabolites were initially undetectable in assay of 1 ml of the serum used in this experiment. The serum was obtained from a patient with primary biliary cirrhosis of greater

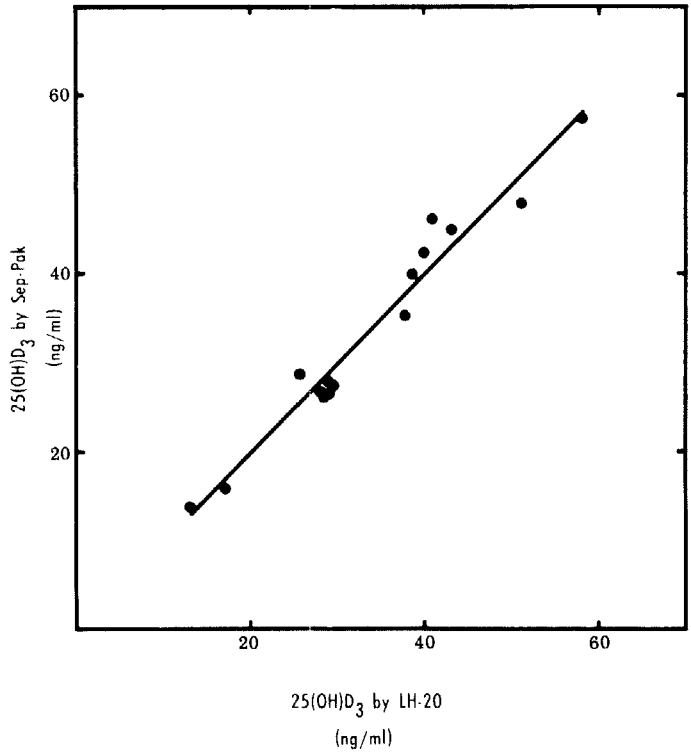

FIGURE 2. Concentration of $25(OH)D_3$ in serum determined by HPLC analysis. Data obtained by Sephadex LH-20 purification (X) are plotted along the abscissa. Data obtained by Sep-Pak C_{18} purification (Y) are plotted along the ordinate. ($Y = -0.2 + 1.006\ X$, $r^2 = 0.960$, $p<0.0001$).

than 3 years' duration. Marked depression of serum 25-hydroxy-vitamin D is a reported consequence of this illness (12). To 1-ml aliquots of this serum were added purified $25(OH)D_2$ and $25(OH)D_3$ in known but varying amounts (Table 1). These samples were assayed and the ratio of $25(OH)D_2$ to $25(OH)D_3$ recovered was determined for each sample. The ratios of $25(OH)D_2$ to $25(OH)D_3$ added to serum and recovered from serum were not statistically

TABLE 1

Recovery of $25(OH)D_2$ and $25(OH)D_3$ Added to Serum (see text for experimental design).

$25(OH)D_2$ (ng added)	$25(OH)D_3$ (ng added)	$\frac{25(OH)D_2}{25(OH)D_3}$ added*	$\frac{25(OH)D_2}{25(OH)D_3}$ recovered*
57.0	13.8	4.13	4.26
57.0	13.8	4.13	3.87
42.8	27.5	1.56	1.69
42.8	27.5	1.56	1.53
28.5	55.0	0.52	0.59
28.5	55.0	0.52	0.48
14.3	82.6	0.17	0.18
14.3	82.6	0.17	0.20
14.3	110	0.13	0.13
14.3	110	0.13	0.15

*Linear regression of ($\frac{25(OH)D_2}{25(OH)D_3}$ recovered upon $\frac{25(OH)D_3}{25(OH)D_3}$ added): slope = 0.981, intercept = 0.03, r^2 = 0.995.

different. Since the Sep-Pak C_{18} preparative method recovers $25(OH)D_2$ and $25(OH)D_3$ equally, $[^3H]25(OH)D_3$ can be used to determine the recovery of $25(OH)D_2$.

To test the hypothesis that as much as 10 ml of serum could be extracted for assay with the present method, 1-ml and 10-ml aliquots were prepared from 3 different sera and assayed for $25(OH)D_3$ concentration. Essentially the same results were obtained for the 3 sera (25.4 and 24.9 ng/ml, 28.6 and 29.1 ng/ml, 38.7 and 36.6 ng/ml for the 1-ml and 10-ml aliquots, respectively). This demonstrates that the performance of the Sep-Pak C_{18} cartridge is independent of sample volume to at least 10 ml.

If 10 ml of serum are prepared by this technique, as little as 0.6 ng/ml of either $25(OH)D_2$ or $25(OH)D_3$ should be reliably

measured by HPLC and uv detection (on the assumption of 50% recovery and of a lower limit of detector sensitivity of 3 ng) (8). To test this lower limit of sensitivity, serum with a very low concentration of 25(OH)D_2 was assayed. A 10-ml aliquot of this serum had no measurable 25(OH)D_2 (Figure 1). To another 10-ml aliquot of this serum was added 7.3 ng of purified 25(OH)D_2. Results from the assay of this sample were 3.6 ng with 48% recovery. Total amount assayed was 7.5 ng, which is within 3% of the expected result.

The Sep Pak C_{18} preparative method and HPLC assay for 25(OH)D_2 and 25(OH)D_3 were applied to 1-ml samples of 49 sera from healthy individuals (22-66 years of age) residing in San Antonio and the surrounding area. Blood was sampled in April, August, and November. Samples were obtained from many of the individuals during all three periods (Table 2). Serum 25(OH)D concentration (the sum of 25(OH)D_2 and 25(OH)D_3) was lower in April than in either August or November ($p<0.001$). There was no significant difference between the August and November values. The majority of individuals had no measurable 25(OH)D_2, regardless of season. When 25(OH)D_2 was found to be present, it contributed little to the total 25(OH)D concentration. The highest concentration of 25(OH)D_2 was 11.3 ng/ml, which was 36% of that subject's total 25(OH)D concentration.

All of the sera sampled in August and November were analyzed for differences in 25(OH)D concentration as a function of sex. The mean 25(OH)D concentration was 27.1 ng/ml $\pm$ 1.6 (SEM) in men (n=27) and 31.9 ng/ml $\pm$ 1.6 in women (n=18). These are

TABLE 2

Total 25(OH)D and 25(OH)D_3 Concentrations(ng/ml) in Normal Adults*

Month	Total 25(OH)D	25(OH)D_3	Sera with measurable 25(OH)D_2**
April			
mean ± sem (n=20)	21.4 ± 1.5	19.6	5
range	12.8 - 36.4		
August			
mean ± sem (n=18)	28.0 ± 2.0	26.7	3
range	14.8 - 42.2		
November			
mean ± sem (n=30)	29.4 ± 1.5	29.4	1
range	12.1 - 47.7		

* Assay of 1 ml serum from 49 healthy adults living in or near San Antonio, Texas.

** Measurable if at least 3 ng eluted. Another 7 sera had 25(OH)D_2 peaks smaller than 3 ng.

statistically different with $p<0.05$. Linear regression analysis of 25(OH)D concentration versus age showed no correlation.

DISCUSSION

Extraction and purification of serum 25(OH)D_3 by the Sep-Pak C_{18} method in preparation for HPLC is an improvement over the previously-reported Sephadex LH-20 method (8). Sephadex LH-20 chromatography imposes a limitation upon the rate at which sera can be assayed. Sephadex LH-20 purification involves reuse of the columns and requires stripping with one solvent and re-equili-

brating with yet another solvent. This is the major inconvenience in time and labor encountered in the routine use of these columns. Although an assay can proceed from serum to HPLC within half a workday (with 6 prepared columns, 6 sera require 4 to 5 hours), preparation for reuse of the Sephadex LH-20 columns requires an additional 11 to 12 hours.

The Sep-Pak C_{18} purification method requires minimal preparation of the cartridges. Sera in groups of 6 to 10 can be processed through purification in about 3 hours, a rate faster than the rate at which HPLC analysis can be performed upon the purified samples. Therefore, the rate at which sera can be assayed is not limited by extraction and purification when the Sep-Pak C_{18} method is used.

Another advantage offered by the Sep-Pak C_{18} preparative method is that both $25(OH)D_2$ and $25(OH)D_3$ recoveries can be monitored with $[^3H]25(OH)D_3$, which is commercially available.

It has been demonstrated in this laboratory that at least 3 ng of $25(OH)D_3$ must be eluted to insure reproducibility and linearity of the uv detector response (8). With the Sep-Pak C_{18} preparative method the average recovery of $25(OH)D_2$ and $25(OH)D_3$ from serum is 52%. The lower limit of concentration for each metabolite in serum that can be measured is dependent upon the volume of serum extracted. For a 1-ml serum sample this lower limit of sensitivity is 6 ng/ml. For a 10-ml serum sample this lower limit is decreased to 0.6 ng/ml. The concentration of 25(OH)D in serum of normal individuals has been reported by various groups in North America, Europe and Japan (5,6,8-10,12-

22). The concentration of 25(OH)D has been found to be lower in Europe than in North America, and lower in winter and spring than in summer and autumn (Table 3). The lower limits of 25(OH)D concentration were less than 6 ng/ml in only 3 of the 17 cited studies. The lowest reported concentration for a normal subject was 3 ng/ml (in England during the winter) (17). No lower limit of "normal" in North American populations studied was less than 6 ng/ml. For practical purposes a 1-ml sample of serum prepared and assayed by the procedure reported herein would be sufficient to determine whether 25(OH)D concentration is depressed. With a

TABLE 3

Series Reporting 25(OH)D Concentrations in Various Populations.

Location	Season (if given)	25(OH)D range* (ng/ml)	reference**
Madison, Wis.	Summer	16 - 42	5
	Winter	19.6 - 41.9	10
Rochester, Minn.	Summer	21.2 - 29.6	6
San Antonio, Tx.	---	11 - 52	8
Toronto	Winter	8.2 - 23.8	9
Chicago	---	17.6 - 40	12
St. Louis	Spring	11 - 55	13
	---	8.2 - 29	16
Boston	---	18 - 36	14
Tuscon, Ariz.	---	25 - 40	20
France	---	6.6 - 23.4	15
Belgium	Spring	5.2 - 21.6	21
United Kingdom	Winter	3 - 22	17
	Spring	6.6 - 25.2	18
	Autumn	10.8 - 41.8	18
	---	4 - 26.4	19
Japan	---	13 - 33	22

* Range observed, if reported, otherwise ± 2 SD from mean.

** First 5 series employed HPLC methods, the others employed CPB methods.

10-ml serum sample, concentrations of $25(OH)D_2$ or $25(OH)D_3$ as low as 0.6 ng/ml can be reliably determined, a sensitivity approaching that reported for many of the CPB assays (13-15,17,20).

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REFERENCES AND NOTES

1. Abbreviations used:
HPLC, high-pressure liquid chromatography;
CPB, competitive protein-binding;
$25(OH)D_3$, 25-hydroxycholecalciferol;
[^{3}H]$25(OH)D_3$, 25-hydroxy[26(27)-methyl-^{3}H]cholecalciferol;
$25(OH)D_2$, 25-hydroxyergocalciferol;
[^{3}H]vitamin D_3, [1,2-^{3}H]cholecalciferol;
[^{3}H]$1,25(OH)_2D_3$, 1α,25-[26,27-^{3}H]-dihydroxycholecalciferol.

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