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CHROMATOGRAPHIC SEPARATION OF 24(*R*),25-DIHYDROXYVITAMIN D_3 AND 25-HYDROXYVITAMIN D_3-26,23-LACTONE USING A CYANO-BONDED PHASE PACKING

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SUMMARY

A high-performance liquid chromatographic system is described for the baseline resolution of 25-hydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3-26,23-lactone, the three principal circulating metabolites of vitamin D_3 in the vitamin D-replete animal. The system is based upon a cyano-bonded phase packing and the solvent hexane—isopropanol—methanol (94:5:1). Of particular interest is the strong retention of carbonyl-containing vitamin D metabolites. The new system can be used for unequivocal analysis of vitamin D metabolites in plasma samples from clinical and animal studies and in the separation and identification of renal metabolites generated in vitro.

INTRODUCTION

During the early seventies, it was believed that the major metabolism of 25-hydroxyvitamin D_3 [25(OH)D_3] occurring in the kidney was into 1,25-$(OH)_2D_3$, the biologically active form of vitamin D_3, or into 24(*R*),25-$(OH)_2D_3$ [1]. Recently it has become clear that the kidney is capable of synthesising a number of other side-chain hydroxylated metabolites including: 25(*S*),26-$(OH)_2D_3$ [2, 3], 23(*S*),25-$(OH)_2D_3$ [4—6] and 25(OH)D_3-26,23-lactone [7]. The close structural similarity of the side-chain hydroxylated metabolites has placed an increased burden on the chromatographic techniques used in the routine separation of vitamin D compounds. Methods based upon silica microparticles and isopropanol—hexane mixtures, established for the separation of 25(OH)D_3, 24(*R*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ [8, 9] offer poor resolution of 24(*R*),25-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone. Alternatively, methods [10, 11] utilizing silica and methylene chloride in the mobile phase give good resolution of 24(*R*),25-$(OH)_2D_3$ and 25(OH)D_3-

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26,23-lactone but offer poor resolution of $25(OH)D_3$ and $25(OH)D_3$-26,23-lactone.

The recent surge of interest in the metabolism and biological activity of $24(R),25\text{-}(OH)_2D_3$ [12—14] has created a need for chromatographic systems capable of baseline resolution of $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone. Such a system would permit detailed study of renal metabolism of $25(OH)D_3$ and also allow for convenient assay of $24(R),25\text{-}(OH)_2D_3$ in clinical and animal plasma without the interference of $25(OH)D_3$-26,23-lactone. This paper describes a high-performance liquid chromatographic (HPLC) system based upon a cyano-bonded packing that takes advantage of the strong retention of $25(OH)D_3$-26,23-lactone and other carbonyl-containing vitamin D_3 molecules making possible excellent resolution of $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone.

EXPERIMENTAL

Vitamin D metabolites

Crystalline $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$, $25(S),26\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ were generous gifts of Dr. Milan Uskokovic of Hoffman-LaRoche, Nutley, NJ, U.S.A. 24-Keto-$25(OH)D_3$ [15, 16] and $25(OH)D_3$-26,23-lactone [17] were kindly supplied by Dr. Sachiko Yamada, Teikyo University and Dr. Tatsuo Suda, Showa University, Japan. Compound A, identified as 24-keto,$23,25\text{-}(OH)_2D_3$ [18] was generated from $25(OH)D_3$ using the perfused rat kidney [19].

Solvents

All solvents were distilled-in-glass spectroscopic grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Chromatography

HPLC was performed on a Model LC204 fitted with a Model 6000A pumping system, WISP automatic sample injector and Model 440 UV fixed-wavelength (254 nm) detector (all from Waters Assoc., Milford, MA, U.S.A.). Integration of peaks was performed using a Sigma 10 chromatography data station in the peak area mode (Perkin-Elmer, Norwalk, CT, U.S.A.). A stainless-steel Zorbax-CN column (25 cm × 4.6 mm I.D., 6 μm microspheres) was a gift of Dr. John Larmann (DuPont Instruments, Wilmington, DE, U.S.A.). Solvent systems used in the study were originally developed for silica column and based upon mixtures of hexane—isopropanol—methanol [9].

Competitive protein binding assay

$25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$, $25(S),26\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone were measured in some experiments by a competitive-protein binding assay utilizing vitamin D binding globulin (DBP) present in rachitic rat serum. Such an assay is based upon that of Belsey et al. [20] modified to include bovine serum albumin [21]. The metabolite under investigation was tested for its ability to displace $[^3H]25(OH)D_3$ from DBP and compared to a standard curve of non-radioactive $25(OH)D_3$ ranging from 25 to 1000 pg per

tube. Since 25(OH)D_3, 24(*R*),25-(OH)$_2D_3$ and 25(*S*),26-(OH)$_2D_3$ each displace [^{3}H]25(OH)D_3 equally well [22, 23] results obtained with these metabolites are directly comparable. However, 25(OH)D_3-26,23-lactone has been reported to have a 5-fold higher affinity for [^{3}H]25(OH)D_3 than 25-(OH)D_3 in this assay [10]. Though in our hands this was only a 2.5-fold difference a correction factor must be applied to the results for this compound when using a standard curve of 25(OH)D_3.

Animal experiments

Adult male Wistar rats (Camm Laboratories, Toronto, Canada) were fed a standard rodent diet (Masterfeed Laboratory, Toronto, Canada). Kidneys were cannulated, surgically removed and perfused with a Krebs—Henseleit buffer containing 6% bovine serum albumin [19]. 25(OH)D_3 (500 ng/ml) mixed with 1 μCi [26,27-^{3}H]25(OH)D_3 (20 Ci/mmol, Amersham, Arlington Heights, IL, U.S.A.) was added to the perfusate (100 ml) in 200 μl ethanol. Aliquots of perfusate (2 ml) were removed at hourly intervals and lipid extracted according to the method of Bligh and Dyer [24]. After nitrogen evaporation of the organic phase each extract was redissolved in hexane—isopropanol—methanol (94:5:1), centrifuged at 1000 *g* for 5 min and the supernatant fluid (200 μl) subjected directly to chromatography.

Female rabbits (Reimans Fur Ranch, St. Agatha, Canada) were orally dosed with 250 μg vitamin D_3/kg body weight/day (in ethanol). Daily blood samples were taken from the ear vein. Lipid extraction of 250-μl samples of plasma paralleled the analysis of kidney perfusates. Chromatography of plasma extracts again employed direct injection of the total lipid material.

RESULTS

The baseline separation of 25(OH)D_3, 24(*R*),25-(OH)$_2D_3$, 24-keto,23,25-(OH)$_2D_3$ and 25(OH)D_3-26,23-lactone on Zorbax-CN using hexane—isopropanol—methanol (94:5:1) is shown in Fig. 1. Retention times of these com-

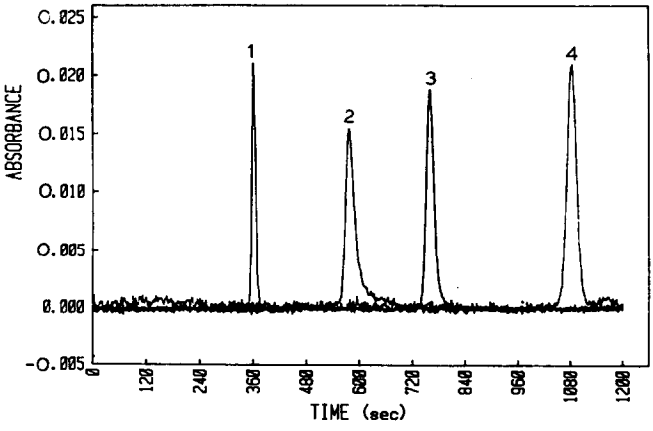

Fig. 1. Chromatographic separation of 25(OH)D_3 (1), 24(*R*),25-(OH)$_2D_3$ (2), 24-keto,23,25-(OH)$_2D_3$ (3) and 25(OH)D_3-26,23-lactone (4) on Zorbax-CN using the solvent system hexane—isopropanol—methanol (94:5:1) and a flow-rate of 1.4 ml/min.

TABLE I

RETENTION TIMES OF VITAMIN D METABOLITES ON ZORBAX-CN AND ZORBAX-SIL

Metabolites	Retention time (min)	
	Zorbax-CN*	Zorbax-SIL**
Vitamin D_3	3.87	3.60
25(OH)D_2	6.66	6.28
25(OH)D_3	7.11	6.78
24-Keto,25-OH-D_3	9.78	7.54
24(*R*),25-$(OH)_2D_2$	11.30	10.39
24(*R*),25-$(OH)_2D_3$	11.43	11.08
24-Keto,23,25-$(OH)_2D_3$	14.82	11.32
25(*S*),26-$(OH)_2D_3$	14.09	14.49
1α,25-$(OH)_2D_3$	18.66	21.90
25(OH)D_3-26,23-lactone	20.71	11.71

*Chromatographic conditions: column, 25 cm × 4.6 mm; mobile phase, hexane—isopropanol—methanol (94:5:1); flow-rate, 1.3 ml/min.
**Chromatographic conditions: column, 25 cm × 4.6 mm; mobile phase, hexane—isopropanol—methanol (91:7:2); flow-rate, 1.5 ml/min.

pounds, and a series of other D compounds, on Zorbax-CN are shown in Table I. For comparison are shown the retention times of the same compounds on Zorbax-SIL using a slightly more polar solvent system hexane–isopropanol—methanol (91:7:2). It is clear that in general a more polar solvent is required to elute vitamin D metabolites from Zorbax-SIL than from Zorbax-CN. However, it is also evident that within the vitamin D group, those metabolites containing a carbonyl residue [24-keto-25(OH)D_3, 24-keto-23,25-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone] are more strongly retained on Zorbax-CN than their dihydroxy and monohydroxy analogues. Thus, 24(*R*),25-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone, which are poorly resolved on Zorbax-SIL, become widely separated on Zorbax-CN with no appreciable change in the solvent selectivity.

We applied this chromatographic procedure to the analysis of rabbit plasma in an attempt to separate the principal circulating metabolites. Application of the method to the analysis of hypervitaminotic D plasma enabled us to observe the UV (254 nm) absorption of each separated metabolite and measure it using an integrator (Fig. 2). Alternatively, collection of 0.5-min fractions of chromatographic effluent, evaporation of mobile phase and dissolution in ethanol permitted the application of a competitive protein binding assay to quantitate the separated D metabolites (Fig. 3). Both assay techniques gave similar results with measurable peaks of 25(OH)D_3, 24(*R*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone. Note that peaks are displaced to the right in the binding assay trace because of delays involved during collection. Results for 25(OH)D_3-26,23-lactone in the binding assay are expressed in equivalents of 25(OH)D_3 and reflect its 2.5-fold higher affinity for DBP. Levels of other metabolites [e.g. 23,25-$(OH)_2D_3$] are below the

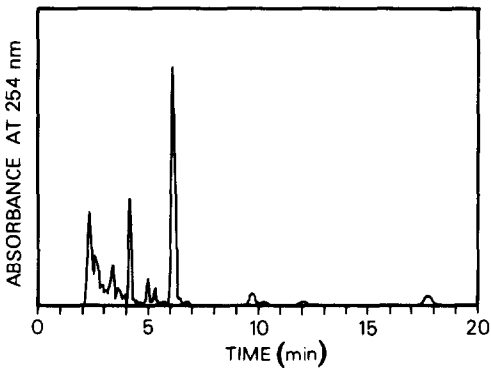

Fig. 2. UV_{254} chromatographic profile of hypervitaminotic rabbit plasma on Zorbax-CN. Conditions as in Fig. 1. Peaks were identified by comparison to standards. Absorbance scale = 0.1 absorbance units full scale. 250 μl plasma were extracted and the recovery of added tracer [^{3}H]25(OH)D_3 was 76%. Integrator gave 25(OH)D_3 = 6.15 min, 202 ng; 24,25-$(OH)_2D_3$ = 9.83 min, 21.5 ng; 25,26-$(OH)_2D_3$ = 12.20 min, 8.53 ng; 25(OH)D_3-26,23-lactone = 17.99 min, 32.2 ng.

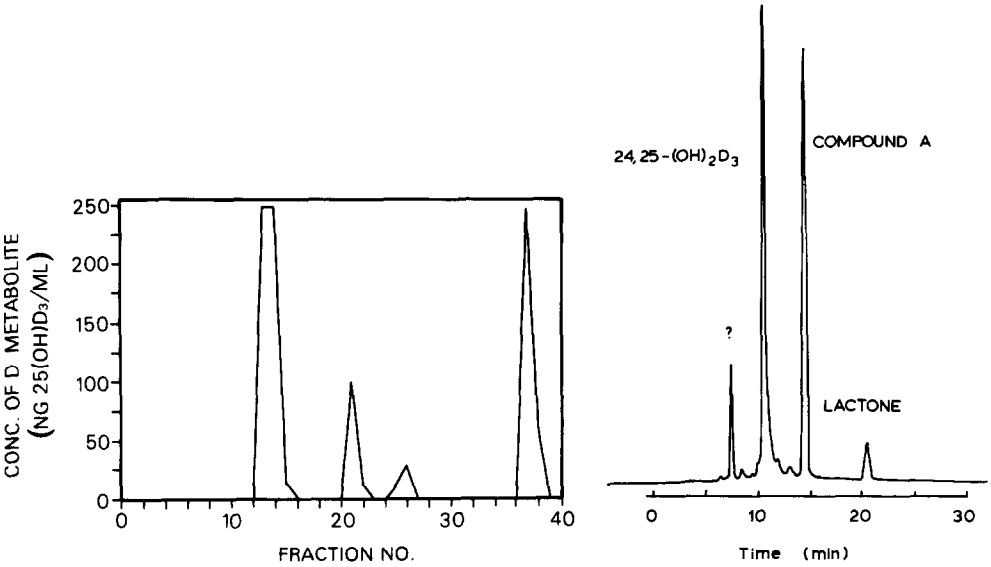

Fig. 3. Competitive protein binding assay of fractions from the chromatographic run shown in Fig. 2. Each fraction was dissolved in 1 ml of ethanol and 50 μl were assayed for ability to displace [^{3}H]25(OH)D_3 from DBP. Each data point represents the mean of triplicate determinations and results are expressed in nanogram equivalent of 25(OH)D_3 per ml plasma.

Fig. 4. Separation of the 24,25-$(OH)_2D_3$ fraction from Zorbax-SIL by Zorbax-CN chromatography. Conditions: Solvent, hexane—isopropanol—methanol (94:5:1); flow-rate, 1.4 ml/min. Compound A was subsequently identified as 24-keto,23,25-$(OH)_2D_3$.

detection limit of each assay technique or else it is possible that these minor components co-migrate with one of the major components.

Kidney perfusate extracts were also well resolved by Zorbax-CN chromatography (Fig. 4). The 24(*R*),25-$(OH)_2D_3$ region from Zorbax-SIL chromatography containing a number of closely migrating, partially resolved peaks was

well separated on Zorbax-CN. Again, the presence of a >C=0 group in 24-keto-23,25-$(OH)_2D_3$ increased its retention on Zorbax-CN permitting it to be obtained in pure form for identification and enabling us to measure its production rate independently of 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone.

DISCUSSION

We report in this paper the novel use of a cyano-bonded phase packing in the separation of vitamin D metabolites. The chromatographic system is particularly useful for the separation of >C=0 containing vitamin D compounds from their hydroxylated analogues and in particular for the resolution of 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone. This is a difficult separation using isopropanol—hexane solvent mixtures [8] and although feasible using methylene chloride [10] it is only possible at the expense of losing the separation of 25$(OH)D_3$ and 25$(OH)D_3$-26,23-lactone. The new system utilizing Zorbax-CN and hexane—isopropanol—methanol solvent mixtures permits resolution of all three principal circulating metabolites.

The new chromatographic system described here allows for the simultaneous analysis of 25$(OH)D_3$, 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone by a procedure involving extraction, one chromatographic step and measurement by either UV_{254} peak integration or competitive protein binding assay. This is of great potential in the clinical setting where unequivocal assay of these metabolites is required in order to determine the clinical usefulness of each.

The chromatographic system using UV_{254} peak integration promises to be of great potential to the clinician monitoring vitamin D-intoxication in patients given large doses of vitamin D_3. Small plasma volumes (e.g. 250 μl used here) are sufficient for this purpose. Where greater sensitivity is required the chromatographic system must be used in conjunction with a competitive protein binding assay of the type described here (B_{50} ca. 200 pg per tube or less). Such a combination permits analysis of 25$(OH)D_3$, 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone in as little as 250 μl if the plasma concentration of each metabolite is greater than 1 ng/ml. Plasma levels below 1 ng/ml are best measured by scaling up the conditions employed here (e.g. 500 μl or 1 ml plasma extracted and directly chromatographed on a 25 cm × 6.2 mm Zorbax-CN column) along the lines suggested by our previous work [25].

The other area where Zorbax-CN chromatography has proved to be of value is in the purification of vitamin D metabolites prior to identification. The technique has already allowed us to recognise the existence of and identify a potentially important renal product, 24-keto,23,25-$(OH)_2D_3$. In addition, it will provide a useful procedure to aid the researcher who wishes to confirm identity of a vitamin D peak during metabolic studies.

In previous work [9] we have demonstrated the importance of the chemical composition (or selectivity, α) of the solvent in the separation of vitamin D metabolites. Here we have demonstrated the importance of the chemical properties of the column packing on this separation. We assume that the increased retention of >C=0 containing vitamin D metabolites is due to their

chemical interaction with —C≡N on the surface of the chromatographic packing. This example serves to illustrate the potential value of this —C≡N packing to the more general field of steroids containing >C=0 groups.

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