High-Performance Liquid Chromatographic Method for the Determination of 25-Hydroxycholecalciferol in the Bovine Liver, Kidney, and Muscle

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A high-performance liquid chromatographic procedure (HPLC) was developed for the determination of 25-hydroxycholecalciferol (25-OH-D_3) in bovine liver, muscle, and kidney. The procedure involved extraction by blending of the tissues with ethanol, followed by shaking with diethyl ether, a set of solvent partitions, adsorption column chromatography on silica gel, a partition column chromatography on diatomaceous earth support, and finally determination by reversed phase HPLC on a C_{18} bonded microparticulate silica column. The identity of the compound in the extract was confirmed by correlation of retention times on an adsorption HPLC column (nonbonded microparticulate silica) and also by correlation of radioactivity in the extract and the effluent from the HPLC of the peak corresponding to 25-OH-D_3 from a cow that had received a dose of ^{14}C-labeled 25-OH-D_3. In the muscle, the identity was also confirmed by GLC/mass spectrometry of 25-OH-D_3 as the diheptafluorobutyrate. Recoveries from liver, muscle, and kidney fortified with 10–70 ppb of 25-OH-D_3 were 88, 86, and 89%, respectively. The endogenous level of 25-OH-D_3 in the liver, muscle, and kidney appeared to vary from cow to cow. In the samples analyzed from cows raised in Michigan, it was in the range of 5 to 10 ppb in the kidney, 3 to 5 ppb in the liver, and 1.5 to 3.4 ppb in the muscle.

Over the last decade, there have been major advances in our knowledge of the metabolism and mode of action of cholecalciferol (vitamin D_3). It is now well established that vitamin D_3 is initially converted to 25-hydroxycholecalciferol (25-OH-D_3) in the liver (DeLuca, 1971) and that this compound is the major circulating form of the vitamin. Ergocalciferol (vitamin D_2) from the diet similarly undergoes a conversion to 25-hydroxyergocalciferol (25-OH-D_2). A more polar metabolite of 25-OH-D_3 has been shown to be the most active form of vitamin D_3 and this was identified as 1,25-dihydroxycholecalciferol (Holick et al., 1971). This metabolite is formed in the kidney (Fraser and Kodicek, 1970).

25-OH-D_3 was found useful in reducing the incidence of parturient paresis in cows (Bringe et al., 1971; Olson et al., 1973a,b). We previously developed an HPLC method for 25-OH-D_3 in cow plasma (Koshy and VanDerSlik, 1976). The present report describes an HPLC method for the determination of 25-OH-D_3 in cow liver, kidney, and muscle. There have been no previous reports for a procedure for 25-OH-D_3 in these tissues.

EXPERIMENTAL SECTION

Sample. A 50-g sample was used for each analysis. If the sample is to be stored for any length of time, it should be weighed into individual containers and frozen.

Solvents. All solvents except 3A alcohol and anhydrous ethyl ether were distilled in glass (Burdick and Jackson, Muskegon, Mich.).

Extraction. Fifty grams of the thawed tissue was transferred into a 250-mL stainless steel blender cup (Waring Products Division, New Hartford, Conn.) to which about 0.25 g of sodium ascorbate, 0.5 g of sodium bicarbonate, and 100 mL of 3A alcohol (or 95% alcohol) were added. The contents were blended at high speed for 2–3 min. The slurry was transferred to a 500-mL mixing cylinder with 150 mL of anhydrous ethyl ether AR, mixed thoroughly, and poured into two 250-mL centrifuge cups. After centrifugation at about 2000 rpm for 5 min, the clear extract was poured into a 500-mL separatory funnel to which 60 mL of water was added. The residue in the centrifuge cups was reextracted with 75 mL of alcohol and 130 mL of ethyl ether in the 500-mL mixing cylinder (liver and kidney) or in the blender (muscle), and the extract was centrifuged as above. The supernatant was added to the 500-mL separatory funnel, mixed, and allowed to separate. The aqueous phase and any solids that separated at the bottom were discarded.

Solvent Partition. To the extract, 60 mL of saturated NaCl (whose pH was adjusted to about 8) was added, mixed, allowed to separate, and the aqueous phase discarded. The extract was evaporated to about 40–50 mL in a 1-L round-bottom flask under vacuum in a water bath at about 40 °C. To this, 100 mL of 5% $NaHCO_3$ was added and transferred to a 250-mL separatory funnel and extracted with 4 × 50 mL of methylene chloride using each methylene chloride portion to first rinse the flask. The combined methylene chloride extract was evaporated from a 500-mL round-bottom flask just to dryness under vacuum. The residue was immediately dissolved in 100 mL of *n*-hexane and extracted with 4 × 50 mL of acetonitrile using each acetonitrile portion to rinse the round-bottom flask (the hexane and acetonitrile were mutually saturated with each other prior to use). The acetonitrile extract was evaporated just to dryness under vacuum in a water bath at about 40 °C from a 500-mL round-bottom flask. The residue was quantitatively transferred to a 5-mL tapered test tube using 1:1 hexane–ether and concentrated to about 0.5 mL under a stream of N_2 with the tube immersed in water at about 40 °C.

Silica Gel Column Chromatography. Neutral Silic AR (1.8 g), 200–325 mesh (Mallinckrodt Chemical Works, St. Louis, Mo.), or equivalent, was slurry packed with *n*-hexane–ether 1:1 in a 5-mL disposable serological pipet (Kimble Products, Toledo, Ohio) with a wad of absorbent cotton at the bottom. The extract from the solvent partition step was transferred to the top of the column using a 9-in. disposable Pasteur pipet and allowed to go down to the top of the packing. The tube was rinsed with small portions of hexane–ether 1:1 and the rinsing transferred to the column allowing each rinsing to go down to the top of the column before the addition of the next. The column was connected with a glass tube of the same dimension using a Teflon adaptor so that the head of the column had a capacity of about 7–8 mL of the solvent. The tube was filled with about 3.5 mL of the solvent, eluted under N_2 pressure at a flow rate of about 0.75–1 mL/min, and the effluent discarded. The mobile phase was changed to ethyl ether–ethyl acetate 9:1. The first 2 mL of the

The Upjohn Company, Kalamazoo, Michigan 49001.

effluent was rejected and the next 4–5 mL collected in a tapered glass tube. The solvent was removed under N_2 while the tube was immersed in water at about 40 °C. The residue was reconstituted in a small volume of *n*-pentane.

Celite Partition Column Chromatography. The column was made from 6 mm i.d. Pyrex glass tubing. It was about 12 in. long with a 10/18 standard taper female joint at the top to fit a small reservoir to hold about 12–13 mL of solvent. The lower end of the column was drawn to a bore of about 1 mm diameter. The reservoir was made from a 15-mL volumetric pipet whose stem was cut off below the bulb and fitted with a 10/18 standard taper male joint. Alternately, we have used as the column, a 5-mL disposable serological pipet (Kimble Products, Toledo, Ohio). A glass tube of the same dimension was attached to it by a Teflon adapter to hold about 8–10 mL of the mobile phase.

Two hundred and fifty milligrams of sodium ascorbate was dissolved in 20 mL of water and mixed with 80 mL of methanol in a 500-mL separatory funnel and shaken with 400 mL of *n*-pentane to effect mutual saturation. The former was the stationary phase and the latter, the mobile phase. The methanol–water will get discolored with age, but is usable for at least a week if protected from light.

Two grams of Celite 545 (Johns Manville Product Corp., New York, N.Y.) was transferred to a 20-cm^3 test tube and mixed well with 1.6 mL of 80:20 methanol–water using a spatula. A small wad of glass wool or cotton was placed in the outlet of the column. The wide end was inserted into the test tube containing the wet Celite and a portion of the adsorbent collected in the column and tamped very gently with a glass rod. In this manner, all the Celite was packed in the column. The extract from the silica gel column was transferred to the top of the Celite column with a 9-in. Pasteur pipet using two or more 100-μL washings of the mobile phase. The reservoir was now attached to the column and filled with the mobile phase and the column eluted with a small N_2 pressure to get a flow rate of about 0.75 mL/min. The first 8.0 mL was discarded. This fraction will contain the less polar impurities and also any 25-OH-D_2 that may be present. The next 6 mL containing 25-OH-D_3 was collected in a tapered glass tube. It was evaporated to dryness under N_2 while the tube was immersed in water at 30–35 °C. The residue was reconstituted in an appropriate amount of ethyl acetate or acetonitrile (usually 100 μL) for the high-performance liquid chromatographic analysis.

High-Performance Liquid Chromatography. High-performance liquid chromatography was carried out using a Varian Model 8500 instrument (Varian Instrument Division, Palo Alto, Calif.): column, Zorbax ODS, 5–8 μm, 2.1 mm i.d., 25 cm (DuPont De Neumours, Inc., Wilmington, Del.); mobile phase, CH_3CN, CH_3OH, H_2O, 90:5:5; solvent flow, 0.5 mL/min; pressure, ≈1400 psi; detection, 254 nm UV (Model 440, Waters Associates, Inc., Framingham, Mass.); sample size, 4–6 μL depending on instrument response; sensitivity, 0.02 AUFS.

Standard solution of 25-OH-D_3 was prepared from a 100 μg/mL stock solution in ethanol. Appropriate aliquots were evaporated to dryness under N_2 and then reconstituted in suitable volumes of ethyl acetate or acetonitrile to match the concentrations expected in the sample. The concentrations of these solutions were usually 5 to 10 ng/μL. The concentrations of 25-OH-D_3 in the samples were calculated from the peak height response of the samples and standard injected onto the column.

RESULTS AND DISCUSSION

It is well known that 25-OH-D_3 is not a very stable compound. In view of this and the fact that the concentrations of 25-OH-D_3 in the three tissues are in low ppb levels, the whole procedure should be carried out with due care. Glassware cleanliness is important as is the avoidance of exposure to excessive heat, light, and air.

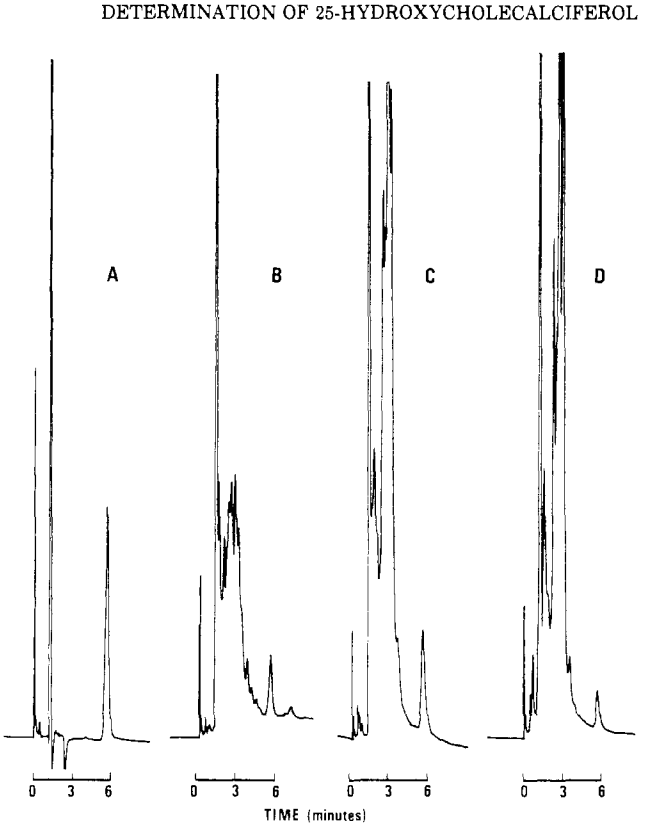

Figure 1. Liquid chromatograms showing 25-OH-D_3 peaks from: (A) standard (23.8 ng on column); (B, C, D) liver, kidney, and muscle, respectively, at 0.02 AUFS.

The procedure is relatively fast considering the levels of 25-OH-D_3 in the three tissues. One analyst can complete four samples in a day.

The method was the same for the three tissues except in the extraction step; the muscle was blended twice in the blender whereas the other tissues were blended only once. Under the HPLC conditions, 25-OH-D_3 had a retention time of about 5.5 min. Vitamins D_2 and D_3 had a retention time of about 14 min. The more polar metabolites, 1,25-dihydroxy-D_3, 24,25-dihydroxy-D_3, 25,26-dihydroxy-D_3, and *trans*-25-OH-D_3 all eluted soon after the solvent front and did not interfere. 25-Hydroxyvitamin D_2 (25-OH-D_2) had the same retention time as 25-OH-D_3, but this interference was removed by the Celite partition column chromatographic step. Even though it is known that 25-OH-D_2 and 25-OH-D_3 could be separated on a microparticulate silica column by HPLC (Jones and DeLuca, 1975), we chose to use the Celite partition column because this column removed not only 25-OH-D_2, but also served as an excellent clean-up step prior to HPLC regardless of which type of HPLC column was used (adsorption or reversed phase). It may be cautioned that the column profile for the separation of 25-OH-D_2 and 25-OH-D_3 may vary slightly depending on the batch to batch variation of Celite, the column packing technique, and the flow rate.

Figure 1 shows chromatograms of a 25-OH-D_3 standard (A) and extracts from the liver (B), kidney (C), and muscle (D) samples from cows on a regular diet in Michigan. The standard was a 4.8 ng/μL solution in ethyl acetate and 5 μL was injected on the column. As is readily seen the chromatograms are very clean and this was primarily due

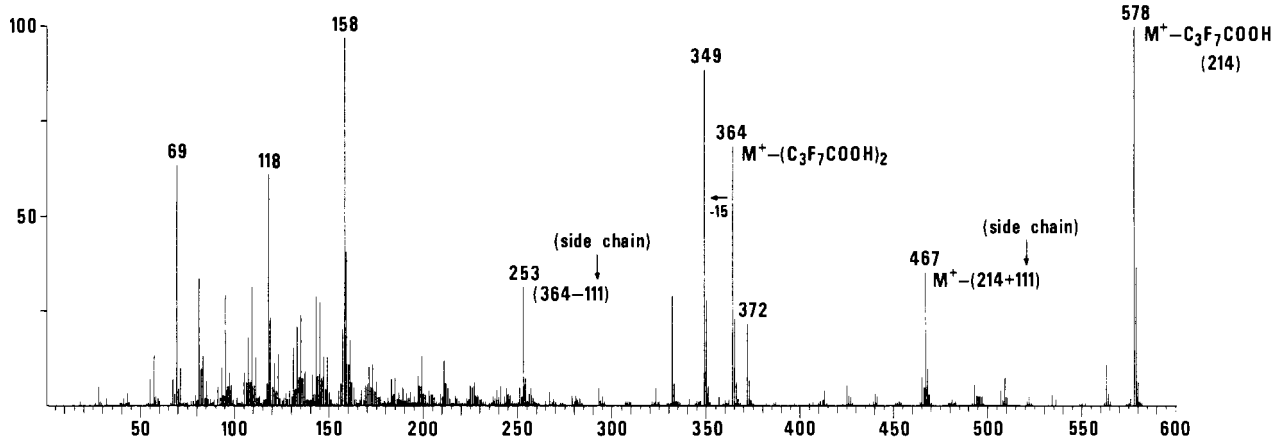

Figure 2. GLC/mass spectrum of 25-OH-D_3 isolated from cow muscle and derivatized to the diheptafluorobutyrate. Note that the parent ion is $M^+ - C_3F_7COOH$.

Table I. Percent Recovery of 25-OH-D_3 Added to Cow Liver Kidney, and Muscle

Level of fortification, ng/g	Percent recovery		
	Liver	Muscle	Kidney
9.9	86	80	86
9.9	86	95	101
9.9	84	86	108
9.9	80	91	71
9.9	89	80	
19.9	93	91	85
19.9	89	89	96
19.9	82	89	88
19.9	87	84	84
19.9	89		
39.8	88		89
39.9	87		85
39.9	96		84
39.9	94		94
46.7		72	
46.7		80	
46.7		85	
46.7		84	
69.6	93		84
69.6	90		85
69.6	85		92
69.6	85		84
74.6		91	
74.6		83	
74.6		80	
74.6		89	
99.4		88	
99.4		89	
99.4		86	
99.4		76	
99.4		89	
Mean	88.0 ± 4.2	86.0 ± 5.4	89.0 ± 8.4

to the clean up on the 2 g Celite partition column. The concentrations in these samples represented 2.9 ng/g in the liver, 5.0 ng/g in the kidney, and 1.7 ng/g in the muscle.

Confirmation of the Identity of the Peak. Preliminary confirmation of the identity of the peaks from the three tissues was by correlation of retention times of peaks from the standard and samples on a totally different HPLC system—a microparticulate silica column (Zorbax Sil, 2.1 mm × 25 cm, DuPont Instruments, Wilmington, Del.) using 4% ethanol in *n*-hexane as the mobile phase at a flow rate of 0.5 mL/min. It was also confirmed by tracer analysis of the extracts of three tissues from a cow that had received intramuscular injection of ^{14}C-labeled 25-OH-D_3. There was excellent correlation between the radioactivity in the effluent from the HPLC of the peak corresponding to 25-OH-D_3 and an equal amount of the extract not subjected to HPLC.

The identity of the peak was confirmed more conclusively in the muscle extract by GLC/mass spectrometry of the diheptafluorobutyrate. Muscle extracts from twenty 50-g samples were combined and the volume adjusted to about 400 μL of ethyl acetate. Ten–twelve microliters of this extract was repeatedly injected on the HPLC column, and the effluent corresponding to the 25-OH-D_3 peak was collected, evaporated under vacuum, and transferred to a 2.5-mL glass-stoppered tapered tube using *n*-pentane, and the solvent removed. To the residue, 25 μL of pyridine (freshly distilled over KOH) and 20 μL of heptafluorobutyrylimidazole (Pierce Chemical Co., Rockford, Ill.) were added and heated in an oil bath at 65 °C for 0.5 h. The reaction mixture was blown down under N_2 to an oily residue and extracted with a small quantity of *n*-hexane. The extract was transferred to another small tapered tube and reconstituted in about 25 μL of *n*-hexane. Four micrograms of pure 25-OH-D_3 was derivatized in a similar manner. The derivatives were subjected to GLC/mass spectrometry (LKB 9000, LKB-Produkter AB, Stockholm, Bromma, Sweden) on a 1% OV-17 on Gas-Chrom Q

Table II. Endogenous Levels of 25-OH-D_3 in the Liver, Muscle, and Kidney from Cows in Michigan (ng/g)

Liver		Muscle			Kidney		
Cow no. 1	Cow no. 2	Cow no. 1	Cow no. 4	Cow no. 5	Cow no. 1	Cow no. 2	Cow no. 3
5.0	2.8	1.8	1.3	3.1	10.5	5.5	8.4
5.3	2.9	2.5	1.7	3.0	9.5	5.0	10.0
7.7	2.3	3.0	1.4	3.1		4.9	8.2
6.8	2.6	3.4	1.9	3.1			8.7
4.6	2.8	2.7	1.7	3.9			9.6
4.8			1.3	4.0			8.6
4.4			1.3				11.2
4.0							
Mean 5.3 ± 1.3	2.7 ± 0.2	2.7 ± 0.6	1.5 ± 0.3	3.4 ± 0.5	9.8	5.1 ± 0.3	9.2 ± 1.2

100/120 3 mm i.d., 2 ft column at 210 °C. The ionizing current was 60 μA and the ionizing voltage 70 eV. There were two GLC peaks, most likely due to the pyro and the isopyro forms of the derivative. The first peak was very much larger than the second. Several spectra were obtained from the front, apex, and rear of the two peaks from the muscle extract and all were identical with that from the authentic 25-OH-D_3. A typical spectrum is shown in Figure 2 which shows molecular ion with m/e at 578 representing the 25-OH-D_3 diheptafluorobutyrate less a molecule of heptafluorobutyric acid. This loss takes place during GLC/mass spectrometry as the parent ion 792 was obtained when the derivative from the pure compound was introduced by the direct probe. Even by direct probe mass spectrometry, the first loss was a molecule of heptafluorobutyric acid, and thereafter the fragments were identical with that by GLC/mass spectrometry. The characteristic ions of 25-OH-D_3 diheptafluorobutyrate are indicated in the spectrum.

Recovery Data. Table I shows the recovery of 25-OH-D_3 from the liver, muscle, and kidney samples, respectively. In these experiments known amounts of 25-OH-D_3 in ethanol were added to the tissue in the blender cup at the extraction step and carried through the entire procedure. An unfortified sample was run along with each set of fortified samples. The concentrations of 25-OH-D_3 in the fortified and unfortified samples were calculated from peak height responses by comparison with the responses from appropriate standards. The fortification levels were in the range of 10–70 ng/g for the liver and kidney and 10–100 ng/g for the muscle. The average values for the unfortified samples were subtracted from the fortified ones in calculating the percent recovery which were 88 ± 4.2, 86 ± 5.4, and 89 ± 8.4% for the liver, muscle, and kidney, respectively.

Endogenous Level of 25-OH-D_3. Since the endogenous level of 25-OH-D_3 in the blood will vary according to the diet and exposure to sunlight, it was expected that the endogenous level in the liver, muscle, and kidney will vary from animal to animal. Replicate analyses were made for each tissue obtained from the same cow raised in Michigan, and these are shown in Table II. No definite conclusions can be made because of the limited number of tissues analyzed. It appears that among the three tissues, the kidney level was the highest and the muscle the lowest. The levels observed were 5 to 10 ppb in the kidney, 3 to 5 ppb in the liver, and 1.5 to 3.4 ppb in the muscle. The data also indicate good reproducibility for replicate analyses for each tissue from the same cow.

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Received for review February 22, 1977. Accepted July 5, 1977. Presented at the Division of Agricultural and Food Chemistry, 174th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1977.

Effect of High Temperature on CPTA-Induced Carotenoid Biosynthesis in Ripening Tomato Fruits

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Detached fruits of the normal red and high-beta tomato genotypes (*Lycopersicon esculentum* cv.) were dipped in an aqueous solution containing CPTA. The treated fruits with corresponding untreated control fruits were ripened at either 21 or 32 °C for 6 and 12 days in order to determine the effect of the higher temperature on CPTA-induced carotenoid biosynthesis in the ripening tomato. The lycopene content of both the normal red and high-beta fruits treated with CPTA increased during ripening at 21 °C and decreased when ripened at 32 °C but the inhibitory effect of high temperature was more pronounced in the high-beta genotype. CPTA treatment did not overcome the temperature-inhibited step in carotenoid biosynthesis in the tomato.

The alteration of the carotenoid biosynthetic pathway induced by CPTA, 2-(4-chlorophenylthio)triethylamine hydrochloride, has been reported in numerous carotenogenic systems (Batra et al., 1973; Coggins et al., 1970; Elahi et al., 1973; Hsu et al., 1972; Poling et al., 1975; Yokoyama et al., 1971, 1972). The inhibition of the biosynthetic pathway at the cyclization step(s) with consequent accumulation of the acyclic intermediates is the most commonly observed effect of this bioregulator. The changes in the biosynthetic pattern are complex and extend beyond simple cyclase inhibition to include increased synthesis of the more saturated polyene precursors (Elahi et al., 1973) as well as other lipids (Hayman et al., 1974).

Lycopene synthesis in the fruit of the normal red tomato genotype (*Lycopersicon esculentum* cv.) is inhibited at a temperature of 32 °C or higher while β-carotene synthesis is unaffected (Goodwin and Jamikorn, 1952; Tomes, 1963). Rabinowitch and Rudich (1972) reported that treatment

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