Stable isotope-labeled vitamin D, metabolites and chemical analogs: synthesis and use in mass spectrometric studies

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Methods for the measurement of vitamin D and its metabolites using stable isotope-labeled internal standards and mass spectrometry are reviewed. The synthesis of both labeled and unlabeled standards is illustrated, and details of the synthesis of (26,26,27,27,27-$^2H_5$)-25,26-dihydroxyvitamin D_3 and (28,28,28-$^2H_3$)-24,25-dihydroxyvitamin D_2 are given. The use of in vitro biologic systems for the production of further metabolites of deuterated 25-hydroxyvitamin D_3 is discussed. Use of deuterated 25-hydroxydihydrotachysterol$_3$ as a substrate in the isolated perfused rat kidney has provided valuable data for the assignment of structure to a number of metabolites of 25-hydroxydihydrotachysterol$_3$ formed in this system. (*Steroids* **55**:418–432, 1990)

Keywords: steroids; vitamin D metabolites; deuterium labeling; gas chromatography; mass spectrometry

Introduction

Assays for vitamin D‡ and its metabolites are widely used by those interested in studies of the mechanism of calcium and phosphate homeostasis in humans and experimental animals. The methodology available for such assays has recently been reviewed.[1] It is, however, quite clear that results obtained using many of these assays in different laboratories are unreliable in that results from one laboratory cannot generally be compared with results on the same sample from different laboratories[2,3]; indeed, the intralaboratory precision for some assays can be so poor that some laboratories may be unable to detect important physiologic variations in analyte concentration.[3] This is a typical situation that arises when assays developed for specific purposes in research laboratories become widely disseminated and no interlaboratory quality control has been established. Good quality-control assurance schemes, which are essential for the maintenance of high standards in the multitude of different laboratories using such assays, are usually based on assigned target values for the samples circulated to participating laboratories. Target values are usually provided by prior assay by gas chromatography/mass spectrometry (GC/MS) (e.g., ref. 4), where such an analysis is appropriate. Vitamin D and its metabolites are low molecular weight hydrophobic molecules that can be separated by GC; therefore, we and others have been developing mass fragmentograpic assays for vitamins D_2 and D_3 and their metabolites in human body fluids (reviewed in refs. 5 and 6).

The best mass fragmentographic methods use stable isotope labeled analytes as internal standards, and these, if added in excess concentration to that of the analyte, also act as protective agents against destruction or loss during preassay purification. Unfortunately, neither labeled nor unlabeled standards for many vitamin D metabolites are available commercially and thus have to be synthesized before suitable GC/MS assays can be developed. This report therefore reviews early work in the development of GC/MS assays for vitamin D and its metabolites, and describes chemical and biochemical methods used by us and oth-

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‡ Abbreviations: vitamin D_2 (ergocalciferol, 9,10-ergostaseco-5,7,10(19),22-tetraen-3β-ol), D_2; vitamin D_3 (cholecalciferol, 9,10-seco-cholesta-5,7,10(19)-trien-3β-ol), D_3; 25-hydroxyvitamin D_2, 25-OH-D_2; 25-hydroxyvitamin D_3, 25-OH-D_3; 24,25-dihydroxyvitamin D_2, 24,25-$(OH)_2D_2$; 24,25-dihydroxyvitamin D_3, 24,25-$(OH)_2D_3$; 25,26-dihydroxyvitamin D_2, 25,26-$(OH)_2D_2$. Unless it is required to distinguish between vitamin D_2 and vitamin D_3, the term vitamin D is used.

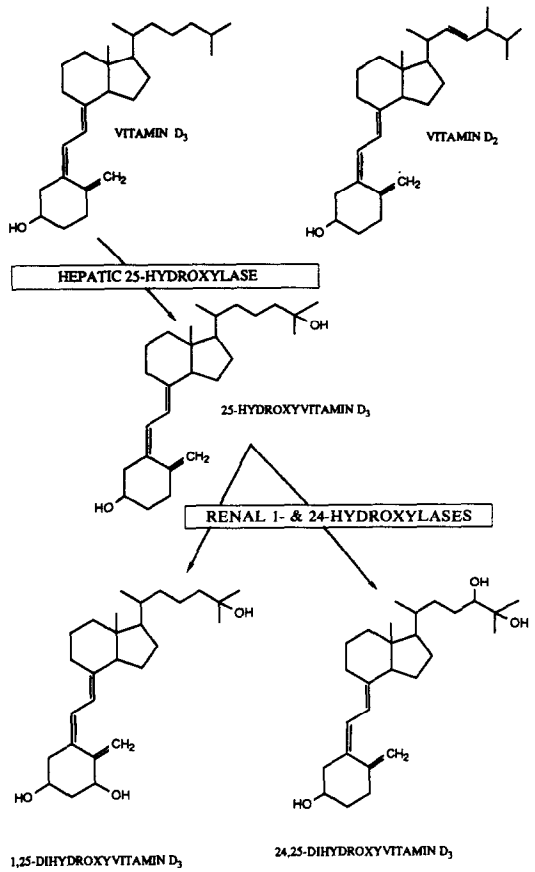


Figure 1 Structures of vitamins D_2 and D_3 and the major metabolites of vitamin D_3 (25-hydroxy-, 1,25-dihydroxy-, and 24,25-dihydroxyvitamin D_3).

ers for the synthesis of both labeled and unlabeled standards and their use in the development of mass fragmentographic assays.

Vitamin D_3, the natural compound synthesized in the skin in vivo from 7-dehydrocholesterol, is metabolized to a large number of different metabolites, mainly by modifications in the side chain. Initially, a hepatic 25-hydroxylation occurs producing 25-OH-D_3. The major calcium homeostatic hormone, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), is synthesized from 25-OH-D_3 by insertion of a hydroxyl at the 1α position under the influence of a parathyroid hormone-regulated renal 1α-hydroxylase system. 1,25-$(OH)_2D_3$ circulates in normal human plasma at a concentration of approximately 30 pg/ml; thus, any comprehensive assay system for vitamin D metabolites must be capable of measuring down to this concentration. Metabolites of vitamin D_2 also occur in human plasma, particularly in countries that fortify foodstuffs with calciferol (vitamin D_2). Vitamin D_2 differs from vitamin D_3 in the side chain and has an extra methyl group (C-28) at C-24 and a double bond at C-22. Because of these chemical modifications to the side chain of vitamin D_2, it undergoes less extensive side chain metabolism than does vitamin D_3. Vitamin D_2 and its metabolites appear to be less stable than their counterparts in the vitamin D_3 series and synthesis of labeled and unlabeled standards are thus more difficult. Figure 1 illustrates the structures of vitamins D_2 and D_3 and some of the more important metabolites of vitamin D_3. Table 1 lists the concentrations of some of these metabolites in human plasma as determined by GC/MS.

Synthesis of labeled standards of vitamin D and metabolites

It has been the intention to synthesize standards that retain the chemical properties of the unlabeled material and simply differ in molecular weight. This enables the labeled material to be used as an internal standard to correct for losses that occur prior to the quantitation step by MS. However, while it is not essential, it is desirable, when developing stable isotope dilution assays, to ensure that the differences in molecular weight between the labeled and unlabeled standard is sufficient to avoid the effects of the natural abundance of many isotopes. In the case of steroids, this means that the difference in molecular weight should be at least 3 amu but probably not more than 6 to 10 amu, since the greater the difference in molecular weight, the greater the possibility that chromatographic differences may emerge during isolation and purification prior to assay. There are only two candidates for the stable isotope: carbon 13 and deuterium. The difficulty of introducing ^{13}C into the cyclopentanoperhydrophenanthrene structure of vitamin D precursors has meant that all such labeled standards have been synthesized by introducing deuterium. There are many chemical methods for the synthesis of such deuterated compounds, but we propose in this paper to deal only with those that have led to the synthesis of such standards which have been used in GC/MS methods for the measurement of vitamin D or its metabolites.

Björkhem and Holmberg,[9] in the first description of a mass fragmentographic assay for a vitamin D metabolite, 25-hydroxyvitamin D_3, used a trideuterated standard labeled at C-26. The route adopted for this synthesis is illustrated in Figure 2, since it outlines the route followed by other syntheses of deuterated standards used in mass fragmentographic procedures. These routes usually involve the synthesis of labeled 7-dehydrocholesterol or the appropriate hydroxylated derivative, which is then converted into the analogous vita-

Table 1 Concentrations of vitamin D and metabolites in human plasma determined by gas chromatography/mass spectrometry

Metabolite	Concentration (mean ± SD)	Reference
Vitamin D_3	8.1 ± 4.8 ng/ml	7
25-OH-D_2	0.9 ± 0.2 ng/ml	7
25-OH-D_3	27 ± 10 ng/ml	9
	18 ± 7 ng/ml	7
24,25-$(OH)_2D_3$	1.6 ± 0.6 ng/ml	12
	1.7 ± 0.8 ng/ml	7
25,26-$(OH)_2D_3$	0.4 ± 0.2 ng/ml	11
1α,25-$(OH)_2D_3$	55 ± 10 pg/ml	10
	26 ± 4 pg/ml	13

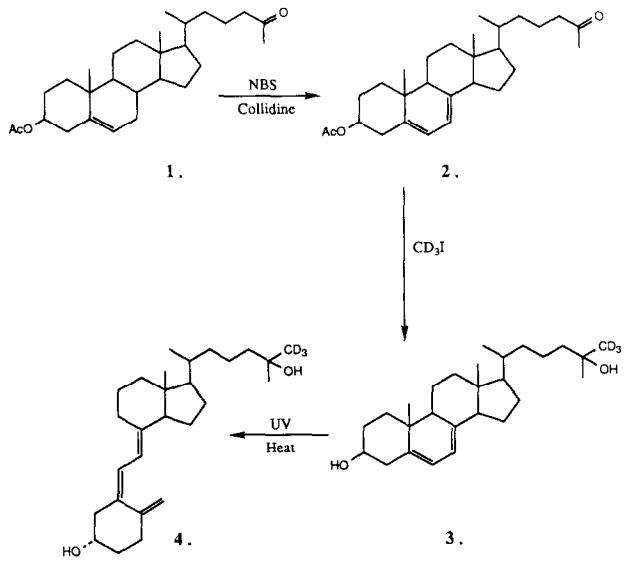


Figure 2 Schematic outline of the synthetic route to 25-hydroxy-[26-$^2H_3$]vitamin D_3 (ref. 4). (1) 3β-Acetoxy-27-norcholest-5-en-25-one, (2) 3β-acetoxy-27-norcholesta-5,7-dien-25-one, (3) [26-$^2H_3$]cholesta-5,7-diene-3β,25-diol. (Reprinted with permission from Elsevier Science Publishers, Amsterdam.)

min D compound by opening the B ring by standard methods under the influence of UV light at 310 nm and heating. In the case illustrated,[9] three deuterium atoms were introduced into the side chain at C-26 using a Grignard synthesis (CD_3I/Mg), which produced the labeled 25-hydroxylated-7-dehydrocholesterol. Irradiation opened the B ring and heating produced the required secosteroid. The final product of this synthesis, after purification by thin-layer chromatography, was 0.3 mg trideuterated 25-OH-D_3 of isotopic abundance 92% (an overall yield from the starting material of 0.026%). A similar approach was adopted by Kirk et al.[14] for the synthesis of hexadeuterated and pentadeuterated standards used in the development of mass fragmentographic methods for 24,25- and 25,26-dihydroxyvitamin D_3; this synthetic route is discussed more fully below. Other deuterated standards ([3,4-$^2H_2$]vitamin D_3, [2,2,3,4,4,6-$^2H_6$]vitamin D_3) have been synthesized,[15,16] but have not been used in a fully evaluated mass fragmentographic procedure, although they have been used in studies of the occurrence of vitamin D fatty acid esters.[17,18]

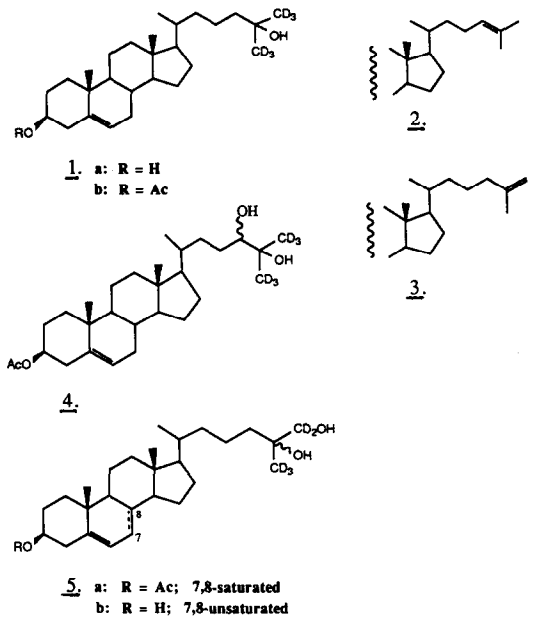
Detailed synthesis of the trideuterated (26,26,26-$^3H_2$) analogs of vitamin D_3, 25-OH-D_3, 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, was described by Whitney et al.[19] The starting material for these syntheses, 3β-hydroxy-26-nor-cholest-5-ene-25,-one, and some of the chemical procedures used for the synthesis of these compounds were similar to those described above.[9] Insertion of hydroxyls at C-24 and C-26 utilized osmium tetroxide.[20] Insertion of the 1α-hydroxyl was effected by production of the delta-1,4,6-triene using dichlorodicyanobenzoquinone,[21] formation of the 1α,2α-epoxide with alkaline hydrogen peroxide,[22] followed by treatment with liquid ammonia/tetrahydrofuran/ammonium chloride.[23] B ring fission was carried out by standard photolytic methodology. Detailed characterization of the labeled compounds was carried out by MS and nuclear magnetic resonance (NMR). Capillary GC and high-performance liquid chromatography (HPLC) methods were described for the separation of all of these metabolites and their use in the development of mass fragmentographic procedures was suggested. However, no detailed investigation of these suggestions was carried out in this or any subsequent publication.

Chemical synthesis of deuterium labeled 25,26-$(OH)_2D_3$ and 24,25-$(OH)_2D_2$

The synthesis of hydroxylated vitamin D_2 standards is more difficult because of the lability of these compounds. As an example of the chemical methods we have used for the synthesis of deuterated standards, the synthesis of pentadeuterated 25,26-dihydroxyvitamin D_3 and trideuterated 24,25-dihydroxyvitamin D_2 is described.

A reported[24] synthesis of unlabeled cholesta-5,7-diene-3β,25ξ,26-triol (25ξ,26-dihydroxyprovitamin D_3), starting from a degradation product of ergosterol, has little in common with our chosen route for preparation of the 26,27-$^2H_5$-labeled form, which started from 25-hydroxy-[26,27-$^2H_6$]cholesterol (Figure 3, **1a**), prepared as described previously.[14] The 3-hydroxy group was protected by acetylation (Figure 3, **1b**), and the 25-hydroxy group was eliminated by treatment with phosphoryl chloride/pyridine to give a mixture of the corresponding 24-ene (Figure 3, **2**) and 25-ene (Figure 3, **3**), which was directly hydroxylated by use of osmium tetroxide to give a separable mixture of the [26,27-$^2H_6$]-24ξ,25-diol (Figure 3, **4**) and the [26,27-$^2H_5$]-25ξ,26-diol (Figure 3, **5a**). The latter was transformed into the corresponding vitamin D, derivative (Figure 3, **6**) by the usual route via photolysis of the derived 5,7-diene (Figure 3, **5b**). The product (Figure 3, **6**) was a mixture of the (25R) and (25S) isomers, suitable for use as an MS standard.

For the synthesis of unlabeled (Figure 4, **12b**) and deuterated samples of 24-hydroxyercalcidiol (Figure 4, **12a**) (24,25-dihydroxyvitamin D_2), our starting material was the 22-alcohol (Figure 4, **7**), derived[25] from the 3-tetrahydropyranyl ether derivative of 3β-hydroxy-23,24-dinorchol-5-en-22-oic acid. Oxidation of the alcohol (Figure 4, **7**) with pyridinium chlorochromate gave the 22-aldehyde (Figure 4, **8**), which was converted into the enone (Figure 4, **9**) (54% yield overall from the alcohol [Figure 4, **7**), by a modification of the method of Eyley and Williams[26] and Jones and co-workers,[27] via an aldol condensation with the enolate of 3-methyl-3-(tetrahydropyran-2-yloxy)butan-2-one. The (E) configuration of the enone (Figure 4, **9**) was apparent from the ^1H NMR spectrum ($J_{22,23}$ 15 Hz) and is consistent with previous work.[26] However, the enone proved to be unstable unless stored in the dark below −20 C; without these precautions, it rapidly afforded a mixture (HPLC) that appeared to comprise the enones isomeric



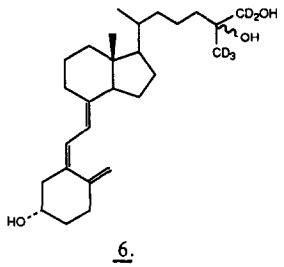


Figure 3 Intermediates in the synthesis of stable isotope-labeled 25,26-$(OH)_2D_3$.

about the C(22)-C(23) olefinic bond (IR, NMR; $J_{22,23}$ 11 Hz for the Z isomer). This instability was not investigated further, but may be significant (see below). Treatment of the enone (Figure 4, **9**) with [$^2H_3$]methylmagnesium iodide in diethyl ether-benzene at reflux gave, after acetylation, a mixture of the (24S)- and (24R)-diols (Figure 4, **10a** and **10b**, respectively, in a 1.24 : 1 ratio after separation by HPLC. Jones and co-workers[27] report a ratio of 7 : 3 for the corresponding nondeuterated diols (Figure 4, **10c** and **10d**), prepared using methyllithium in tetrahydrofuran at 0 C. Our samples had melting points substantially higher (by approximately 20 C) than those reported,[27] although the NMR spectra were virtually identical with those reported[27] for the (R,S) material. It is possible that the starting enone used by Jones and co-workers may have undergone some isomerization, as described above, leading to 24,25-diols contaminated by the corresponding (22Z)-isomers.

The deuterated (24R)-diol (Figure 4, **10b**) and the mixture of the nondeuterated diols (Figure 4, **10c** and **10d**), prepared in a similar manner using unlabeled methylmagnesium iodide, were converted into the corresponding 24,25-dihydroxyvitamin D_2 derivatives (Figure 4, **11a** and **11b**), respectively. Bromination of the diols at C-7 with an excess of 1,3-bromo-5,5-dimethylhydantoin followed by immediate dehydrobromination with collidine in boiling xylene gave, after alkaline hydrolysis of the 3-acetate, a crude mixture of the corresponding 5,7-diene and the 4,6-diene, with some unreacted 5-ene. The 5,7-ene (36%) was separated from this mixture by semipreparative HPLC. Ultraviolet irradiation and HPLC of the product gave the crude previtamin that was isomerized thermally to give the required vitamin D_2 derivative (Figure 4, **12a** or **12b**). The [$^2H_3$] species constituted more than 98% of the deuterated compound (Figure 4, **12a**) on the basis of MS of the 24,25-cyclic-*n*-butylboronate-3-trimethylsilyl ether derivative.

Jones and co-workers,[27] during the synthesis of (24R,S)-24,25-dihydroxyvitamin D_2, converted their mixture of 24,25-diols (Figure 4, **10c** and **10d**) into the 5,7-diene via the 5-en-7-one and decomposition of the derived toluene-*p*-sulfonylhydrazone in approximately

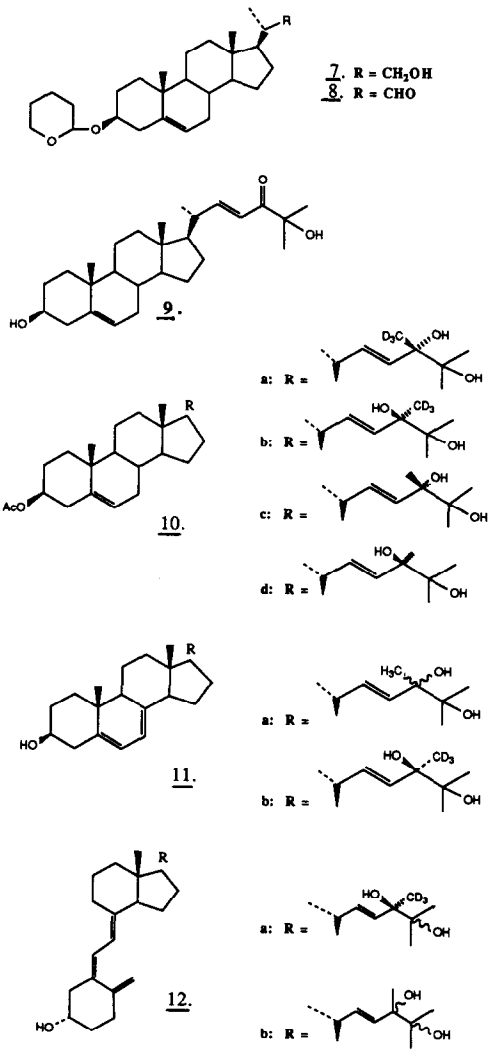


Figure 4 Intermediates in the synthesis of stable isotope-labeled 24,25-$(OH)_2D_2$.

13% overall yield. Our method appears to be more direct and is slightly higher yielding (approximately 16% overall).

Katsumi and co-workers[28] have recently reported the application of a similar series of side-chain reactions to the cholen-22-al derivative obtained by side-chain degradation of the Diels-Alder adduct of ergosterol and 4-phenyltriazoline-3,5-dione as a synthesis of unlabeled 24,25-dihydroxyvitamin D_2.

Experimental

Chemical synthesis

Melting points were determined on a Reichert hot-stage apparatus. Infrared spectra refer to KBr discs. Nuclear magnetic resonance spectra were recorded at 100 MHz for solutions in deuterochloroform with tetramethylsilane as internal standard. Ultraviolet spectra were recorded for ethanol solutions. Deuterium incorporation was measured by MS using a Hewlett Packard mass-selective detector (HP 5970) attached to a Hewlett-Packard gas chromatograph (HP 5890). Preparative HPLC was carried out with a Waters Associates Prep LC/System 500 equipped with Prep-pak-500 silica cartridges. Small-scale preparative HPLC was carried out on columns (25 cm, 100 mm internal diameter) packed with Nucleosil-50 (5 mμ). All solvents were distilled before use. Unless otherwise indicated, "light petroleum" refers to the fraction of bp 60 to 80 C. Tetrahydrofuran and dioxan were dried by distillation from lithium aluminum hydride. Pyridine, benzene, xylene, and cyclohexane were distilled from calcium hydride. Extracted solutions of organic products were washed as necessary with dilute hydrochloric acid and/or sodium hydrogen carbonate solution, dried over anhydrous sodium sulfate, and evaporated under reduced pressure below 40 C.

*Synthesis of [25R,S]-[26,27-$^2H_5$cholesta-5,7-diene-3β,25,26-triol (Figure 3, **5b**)*

(24R,S)-[26,27-$^2H_6$]Cholest-5-ene-3β,24,25-triol 3-acetate (Figure 3, 4) and (25R,S)-[26,27-$^2H_5$]cholest-5-ene-3β,25,26-triol 3-acetate (Figure 3, 5a) [26,27-$^2H_6$]Cholest-5-ene-3β,25-diol (Figure 3, **1a**) (408 mg) was treated with acetic anhydride (3 ml) and pyridine (10 ml) for 19 hours at room temperature. The mixture was then poured into water and the product was extracted by use of ether. The crude 3-acetate (Figure 3, **1b**), in pyridine (13 ml), was treated with phosphoryl chloride (450 μl) under reflux for 30 minutes. The dark brown solution was cooled and diluted with ether (100 ml) and water (30 ml). The ethereal solution was washed with dilute hydrochloric acid and water, dried, and the solvent was removed to give a crude mixture of the 24-ene (Figure 3, **2**) and 25-ene (Figure 3, **3**).

This crude residue in benzene (6 ml) and pyridine (73 μl) was treated with 1% osmium tetroxide in benzene (25.6 ml) at room temperature for 16 hours, then with sodium metabisulfite (1.33 g) in water (20 ml) and ethanol (60 ml). After 5 hours, the suspension was filtered, the residue was washed with ethanol, and the solvents were removed under reduced pressure. The organic products were extracted from the dry residue with chloroform and separated by HPLC on Nucleosil-50 (ethyl acetate/light petroleum 1 : 1) giving, in order of increasing polarity, (24R,S)-[26,27-$^2H_6$]cholest-5-ene-3β,24,25-triol 3-acetate (Figure 3, **4**; 209 mg, 45%) and (25R,S)-[26,27-$^2H_5$]cholest-5-ene-3β,25,26-triol 3-acetate (Figure 3, **5a**; 85 mg, 18%).

A similar preparation using nondeuterated materials gave first (24R,S)-cholest-5-ene-3β,24,25-triol 3-acetate (174 mg, 38%), mp 154 to 166 C (methanol) (literature,[29] mp 151 to 152.5 C); ν_{max} 3,470, 1,735, and 1,715 cm^{-1}; δ 0.70 (3H, s, 18-H_3), 0.94 (3H, d, J 7 Hz, 21-H_3), 1.02 (3H, s, 19-H_3), 1.18 and 1.23 (3H, s, and 3H, s, 26- and 27-H_3), 2.04 (3H, s, 3-OAc), ca 3.3 (1H, m, 24-H), ca 4.55 (1H, m, 3-H), and ca 5.35 (1H, m, $W_{1/2}$ 6-H). The deuterated sample (Figure 3, **4**) had no detectable signals at δ 1.18 and 1.23 due to 26- and 27-$^1H_3$. The second fraction was (25R,S)-cholest-5-ene-3β,25,26-triol 3-acetate (136 mg, 30%), mp 164 to 166 C (acetone-hexane) (literature, ref. 29, mp 169 to 171 C; ref. 20, mp 160 to 161 C); ν_{max} 3,420 and 1,730 cm^{-1}; δ 0.68 (3H, s, 18-H_3), 0.92 (3H, d, J 7 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.16 (3H, s, 27-H_3), 2.02 (3H, s, 3-OAc), 3.26 to 3.58 (2H, m, 26-H_2), ca 4.55 (1H, m, $W_{1/2}$ 3-H), and ca 5.35 (1H, m, $W_{1/2}$ 6-H). The deuterated sample (Figure 3, **5a**) had no detectable signals at δ 1.16 and 3.42, indicating essentially complete deuteration at C-26 and C-27.

(25R,S)-Cholesta-5,7-diene-3β,25,26-triol (Figure 3, 5b). The 5-ene-3β,25,26-triol 3-monoacetate (Figure 3, **5a**) (230 mg) was treated in pyridine (6 ml) with acetic anhydride (2 ml) for 18 hours at room temperature. The solution was then diluted with water and the product was extracted with ethyl acetate to give crude (25R,S)-cholest-5-ene-3β,25,26-triol 3,26-diacetate (256 mg). Without further purification, the diacetate (201 mg) in light petroleum (bp 40 to 60 C) (12 ml) and cyclohexane (6 ml) was stirred and heated under reflux with 1,3-dibromo-5,5-dimethylhydantoin (114 mg) for 30 minutes, then cooled. Collidine (0.8 ml) was then added, low-boiling solvents were removed under reduced pressure at less than 25 C, and the residue was diluted with xylene (8 ml), then stirred and heated under reflux under nitrogen for 1 hour. The cooled mixture was diluted with ethyl acetate (40 ml), which was washed with dilute sulfuric acid, aqueous sodium hydrogen carbonate, and water, dried (Na_2SO_4) and evaporated. The residual oil was dissolved in acetone (8 ml) containing toluene-*p*-sulfonic acid monohydrate (20 mg). After 21 hours at room temperature, the product was extracted using ethyl acetate. Semipreparative HPLC of the residue (ethyl acetate/light petroleum, 1 : 2) gave, in order of increasing polarity, recovered (25R,S)-cholest-5-ene-3β,25,26-triol 3,26-diacetate (46 mg) and (25R,S)-cholesta-5,7-diene-3β,25,26-triol 3,26-diacetate (52 mg, 26%) as a pale yellow crystalline solid that appeared to be homogeneous. Without further purification, this 5,7-diene diacetate in dioxan (1 ml) and methanol (3 ml)

was stirred at room temperature under nitrogen with methanolic 5% potassium hydroxide (0.33 ml) for 4 hours. Extraction by use of ethyl acetate gave (25R,S)-cholest-5,7-diene-3β,25,26-triol (Figure 3, **5b**) (44 mg), which was stored in the dark at less than 20 C under N_2 until required for photolysis, leading to the corresponding vitamin D_3 derivative.

*Synthesis of (24R,S)-ergosta-5,7,22-triene-3β,24,25-triol (Figure 4, **11a**) and its (24R)-28-$^2H_3$-labeled analog (Figure 4, **11b**)*

3β-(Tetrahydropyran-2-yloxy)-23,24-dinorchol-5-en-22-al (Figure 4, 8). Pyridinium chlorochromate (1.3 g, 24 mmol) was added to a stirred solution of 23,24-dinorchol-5-ene-3β,22-diol 3(tetrahydropyran-2-yl) ether[25] (Figure 4, **7**) (1.25 g, 12 mmol) in dichloromethane (30 ml, distilled from anhydrous K_2CO_3) containing suspended anhydrous sodium acetate (100 mg, 1.2 mmol). The suspension was stirred at room temperature for 1.5 hours, then diluted with diethyl ether (150 ml; peroxide-free) and filtered through a column of florisil (50 g). Elution with additional ether (approximately 200 ml) gave crude 3β-(tetrahydropyran-2-yloxy)-23,24-dinorchol-5-en-22-al (Figure 4, **8**) (1.13 g), which was used directly and with minimum delay in the next stage; ν_{max} 2,710 and 1,725 cm_{-1}; δ 0.76 (3H, s, 18-H_3), 1.05 (3H, s, 19-H_3), 1.40 (d, J 7 Hz, 21-H_3), 3.3 to 4.1 (4H, br, complex), 4.70 (1H, br s) (THP), 5.34 (1H, m, $W_{1/2}$ ≈8 Hz, 6-H), and 9.58 (1H, d, J 4 Hz, 22-H).

(22E)-3β,25-Dihydroxycholesta-5,22-dien-24-one (Figure 4, 9). A solution of *n*-butyllithium in hexane (1.6 M; 5.6 ml, 9 mmol) was added to a stirred solution of diisopropylamine (1.27 ml, 9 mmol, distilled from CaH_2) in dry tetrahydrofuran (19 ml) at 0 C under dry nitrogen. This solution was then treated at 0 C for 15 minutes with a solution of 3-methyl-3-(tetrahydropyran-2-yloxy)-butan-2-one[26] (1.675 g, 9 mmol) in tetrahydrofuran (19 ml). The mixture was stirred at 0 C for 1 hour, cooled to −70 C, and a solution of the above crude aldehyde (Figure 4, **8**) (1.13 g) in tetrahydrofuran (15 ml) was added over 10 minutes. After a further 10 minutes, the temperature was raised to 0 C and maintained there for 3 hours. Acetic acid (1 ml) was then added and the solution was allowed to warm to room temperature. The product was extracted by use of ether, then dissolved in tetrahydrofuran (25 ml) and methanol (19 ml) and treated with dilute hydrochloric acid (3 ml; concentrated HCl-H_2O, 1:4). After 18 hours at room temperature, the product was isolated by use of ethyl acetate. Preparative HPLC (ethyl acetate/light petroleum, 1:2, as mobile phase) gave (22E)-3β,25-dihydroxycholesta-5,22-dien-24-one (Figure 4, **9**) (676 mg, 54% from alcohol **7**), mp 184 to 187 C (acetone/hexane); ν_{max} 3,400, 1,690, and 1,620 cm^{-1}; δ 0.72 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 1.12 (3H, d, J 6 Hz, 21-H_3), 1.4 (6H, s, 26- and 27-H_3), ca 3.45 (1H, m $W_{1/2}$ ~25 Hz, 3-H), ca 5.3 (1Hm, m, $W_{1/2}$ ~8 Hz, 6-H), 6.3 (1H, d, J 15 Hz, 23-H), and 6.97 (1H, dd, J 15 and 8 Hz, 22-H). Found: C, 78.1; H, 10.2. $C_{27}H_{42}O_3$ requires C, 78.2; H, 10.2%.

A sample of the enone (Figure 4, **9**) that had been exposed to daylight at room temperature over several days was chromatographed by semipreparative HPLC (ethyl acetate/light petroleum, 1:2) to give, in order of decreasing polarity, the enone (Figure 4, **9**) and a second compound (probably the (22Z)-isomer; ratio approximately 3–1); ν_{max} 3,380, 1,690, and 1,620 cm^{-1}; δ 0.74 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), δ 1.05 (3H, d, J 6 Hz, 21-H_3), 1.38 (6H, s, 26- and 27-H_3), ca 3.5 (1H, m, $W_{1/2}$ ~32 Hz, 3-H), ca 5.3 (1H (1H, m, $W_{1/2}$ ~8 Hz, 6-H), and 6.00 and 6.20 (2H, d,d, J 11 Hz, 22- and 23-H).

(24S)- And (24R)-[28-$^2H_3$]ergosta-5,22-diene-3β,24,25-triol 3-acetates (Figure 4, 10a and 10b). A solution of the enone (Figure 4, **9**) (297 mg, 0.5 mmol) in benzene (39 ml, distilled from CaH_2) was added over 10 minutes to a stirred solution of [$^2H_3$]methyl magnesium iodide (prepared from [$^2H_3$]methyl iodide [1.92 ml, 16 mmol; 99% $^2H_3$] and magnesium [369 mg, 15 mmol]) in ether (20 ml, distilled from CaH_2) at room temperature under dry nitrogen. The mixture was stirred and heated under reflux for 3 hours, then cooled to 0 C and treated with aqueous 50% acetic acid (19 ml) and water (5 ml) over 5 minutes. Extraction by use of ethyl acetate gave a crude mixture of triols that was acetylated (acetic anhydride-pyridine at room temperature overnight), then subjected to semipreparative HPLC (ethyl acetate/light petroleum, 1:2, as mobile phase) to give, in order of increasing polarity,[29] (24S)-[28-$_2H_3$]ergosta-5,22-diene-3β,24,25-triol 3-acetate (Figure 4, **10a**; 93 mg, 39%), mp 175 to 178 C (acetone-hexane) (literature,[27] 157 to 158 C for nondeuterated material, see text) and (24R)-[28-$^2H_3$]ergosta-5,22-diene-3β,24,25-triol 3-acetate (Figure 4, **10b**; 75 mg, 32%), mp 184 to 187 C (acetone/hexane) (literature,[27] 164 to 165 C for nondeuterated material, see text); ν_{max} 2,230 cm^{-1} (C-D); no detectable NMR signal at δ 1.28 for 28-[$^1H_3$] for either isomer; more than 98% [$^2H_3$] (measured as the 24,25-cyclic *n*-butyl boronate). The corresponding nondeuterated materials (Figure 4, **10c** and **10d**) had identical IR and ^1H NMR spectra except for absence of the 2,230 cm^{-1} IR band and signals for the protons at C-28 in the NMR spectra; ν_{max} 2,430 and 1,730 cm^{-1}; δ (for both isomers) 0.71 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 1.055 (3H, d, J 5 Hz, 21-H_3), 1.20 and 1.22 (3H, s, and 3H, s, 26- and 27-H_3), 1.28 (3H, s, 28-H_3), 2.04 (3H, s, 3-OAc), ca 4.6 (1H, m, $W_{1/2}$ ~25 Hz, 3-dH), and ca 5.35 (1H, m, $W_{1/2}$ ~8 Hz, 6-H); (24R)-isomer: δ 5.51 (1H, s) and δ 5.55 (1H, s); (24S)-isomer: δ 5.52 (1H, d, J 1 Hz) and 5.56 (1H, s), (22-H and 23-H). Found (Figure 4, **10c**): C, 76.1; H, 10.4. Found (Figure 4, **10d**): C, 76.4; H, 10.3. $C_{30}H_{48}O_4$ requires C, 76.2; H, 10.2%.

(24R,S)-Ergosta-5,7,22-triene-3β,24,25-triol (Figure 4, **11a**) was prepared from an unseparated mixture of the 5,22-dienes (Figure 4, **10c** and **10d**) by essentially the procedure described above for (25R,S)-cholesta-

5,7-diene-3β,25,26-triol (Figure 3, **5b**). The yield was 36%, δ 0.64 (3H, s, 18-H_3), 0.94 (3H, s, 19-H_3), 1.06 (3H, d, J 6 Hz, 21-H_3), 1.20 and 1.22 (3H, s, and 3H, s, 26- and 27-H_3), 1.28 (3H, s, 28-H_3), ca 3.6 (1H, m, $W_{1/2}$ ~2, 3-H), and 5.3 to 5.7 (4H, overlapping complex multiplets, 6-, 7-, 22-, and 23-H).

The deuterated compound (24R)-[28-$^2H_3$]ergosta-5,7,22-triene-3β,24,25-triol (Figure 4, **11b**), obtained in the same way from the 5,22-diene (Figure 4, **10b**), had identical spectral characteristics except for absence of the 28-H_3 signal at δ 1.28.

The 5,7-dienes (Figure 3, **5b** and Figure 4, **11a** and **11b**), were converted into the corresponding vitamin D derivatives in the normal way by photolysis followed by thermal isomerization. The following products were purified by semipreparative HPLC (ethyl acetate light petroleum, 1 : 1):

[26,27-$^2H_5$]-25,26-Dihydroxy vitamin D_3 (Figure 3, **6**): ν_{max} 267 nm (ε 17,400); δ 0.57 (3H, s, 18-H_3), 0.95 (3H, d, J 6 Hz, 21-H_3), 1.20 (H, s, 27-H_3), 3.94 (1H, m, $W_{1/2}$ 20 Hz, 3.H), 4.83 (1H, d, J ~2 Hz) and 5.05 (1H, d, J 2 Hz) (19-H_2), 6.04 (1H, d), and 6.25 (1H, d) (J 11 Hz, 6- and 7-H): m/z (as 25,26-*n*-butyl boronate 3-trimethylsilyl ether) 559 (M^+, 20%), 454 ([M −90 −15)$^+$, 100) and 428 ([M − 131]$^+$, 28%). The corresponding $^2H_4$ species (M^+ 558) was undetectable; the $^2H_3$ (M^+ 557) and $^2H_2$ (M^+ 556) species were possibly present at ≤2% each. A nondeuterated sample prepared in the same way from nondeuterated 25-hydroxycholesterol showed an additional signal at δ 3.45 (2H, br s, $W_{1/2}$ 4 Hz, 26-Hz): m/z (as 25,26-*n*-butyl boronate 3-trimethylsilyl ether) 554 (M^+, 20%), 449 ([M −90 −15]$^+$, 100), and 423 ([M −131]$^+$, 20).

24,25-Dihydroxyvitamin D_2, (24R,S)-hydroxyercalcidiol (Figure 4, **12b**), was obtained from the 5,7,22-triene (Figure 4, **11a**); λ_{max} 263 nm (ε 18,300); δ 0.57 (3H, s, 18-H_3), 1.05 (3H, d, J 7Hz, 21-H_3), 1.20 and 1.22 (3H, s, and 3H, s, 26- and 27-H_3), 1.28 (3H, s, 28-H_3), ca 3.95 (1H, m, $W_{1/2}$ 18 Hz, 3-H), 4.82 (1H, m) and 5.04 (1H, m) (19-H_2), 5.55 to 5.59 (each 1H, apparent s, s, 22- and 23-H), 6.04 (1H, d), and 6.24 (1H, d) (J 11 Hz, 6- and 7-H); m/z (as 24,25-*n*-butyl boronate 3-trimethylsilyl ether) 566 (M^+, 29%), 461 (100), and 435 (20).

[28-$^2H_3$]-24,25-Dihydroxyvitamin D_2, (24R)-24-hydroxy-[28-$^2H_3$]ercalcidiol (Figure 4, **12a**): This compound, prepared from the (24R)-[28-$^2H_3$]-diol (Figure 4, **11b**), showed no detectable NMR signal at δ 1.18 for 23-$^1H_3$, but the UV absorption and all other parts of the NMR spectrum were identical to those of the previous sample: m/z (as 24,25-*n*-butyl boronate-3-trimethylsilyl ether) 569 (M^+, 29%), 464 (100), and 438 (23).

Biosynthesis of labeled metabolites

A less common but valuable approach to the production of deuterated vitamin D standards is to utilize in vitro enzyme systems to synthesize the required compound using a labeled precursor as substrate. Correct choice of the experimental system can lead to efficient production of the required compound with good yield, in many cases, better than the corresponding synthetic route. Efficient biologic systems are not available for all required conversions, but insertion of a 1α-hydroxyl group by kidney extracts is an excellent way of producing 1-hydroxylated vitamin D standards. Björkhem et al.[10] used this approach for the biosynthesis of (26-$^2H_3$)-1,25-dihydroxyvitamin D_3 from previously synthesized (26-$^2H_3$)-25-hydroxyvitamin D_3 (ref. 9) using a kidney homogenate from vitamin D-deficient chicks. Vitamin D deprivation enhances 1α-hydroxylase activity; even so, in this case, a yield of only 1.7% was reported. In our laboratory, we have recently produced labeled 1,25-OH-D_3 by a similar process, but with much higher yield than previously reported.[10] This process is detailed below.

Chicks were raised on a vitamin D-deficient diet from birth for 3 weeks and then killed. Their kidneys were removed and homogenized in 100 ml incubation buffer (0.2 mM sucrose containing 15 mM TRIS buffer, pH 7.4, 2 mM magnesium chloride, and 5 mM sodium succinate[30]). The homogenate was incubated under 95% O_2 : 5% CO_2 at 37 C for 1 hour with 100 μg (26,27-$^2H_6$)-25-hydroxyvitamin D_3, chemically synthesized by us.[14] The reaction was stopped with 100 ml acetonitrile, the precipitated protein removed by centrifugation, and the supernatant added to 50 ml 0.2 M sodium acetate buffer, pH 5.6, and extracted on several BondElut C_{18} cartridges as described previously.[31] The methanol extracts produced were further fractionated on Sep-Pak SIL cartridges[11] to yield a "polyhydroxylated vitamin D metabolite" fraction. Further purification was achieved by HPLC on Zorbax SIL (5 μm, 25 cm × 6.2 mm) using the solvent system isopropanol/methanol/hexane (6 : 3 : 91, v/v/v at 2 ml/min). The chromatogram obtained is shown in Figure 5, from which it can be seen that a substantial peak, labeled D, has been obtained with the same retention time as that for 1,25-OH-D_3. This peak was collected and repurified on reverse-phase HPLC and quantitated by UV absorption of 264 nm assuming a molar extinction coefficient of 18,300,[32] indicating a recovery of 15 μg (15%), nearly 10 times that reported previously.[10] An aliquot of this preparation was converted into the pertrimethylsilyl ether derivative by treatment with trimethylsilylimidazole, and GC/MS was carried out. Figure 6 shows the mass spectrum obtained in comparison to that obtained from pertrimethylsilyl 1,25-$(OH)_2D_3$. Pilot studies had previously been carried out using both D-deficient chick and rat kidney preparations, but the D-deficient chick preparation was finally utilized. D-deficient rat kidney preparations also produced putative 1,25-$(OH)_2D_3$, but in lower yield; in addition, further, more polar, metabolites were produced. Production of further deuterated standards can of course also be carried out in vivo, which is valuable if many metabolic steps, which may occur in different tissues, are required. In vivo systems are, however, not efficient, and very low yields are usually obtained.

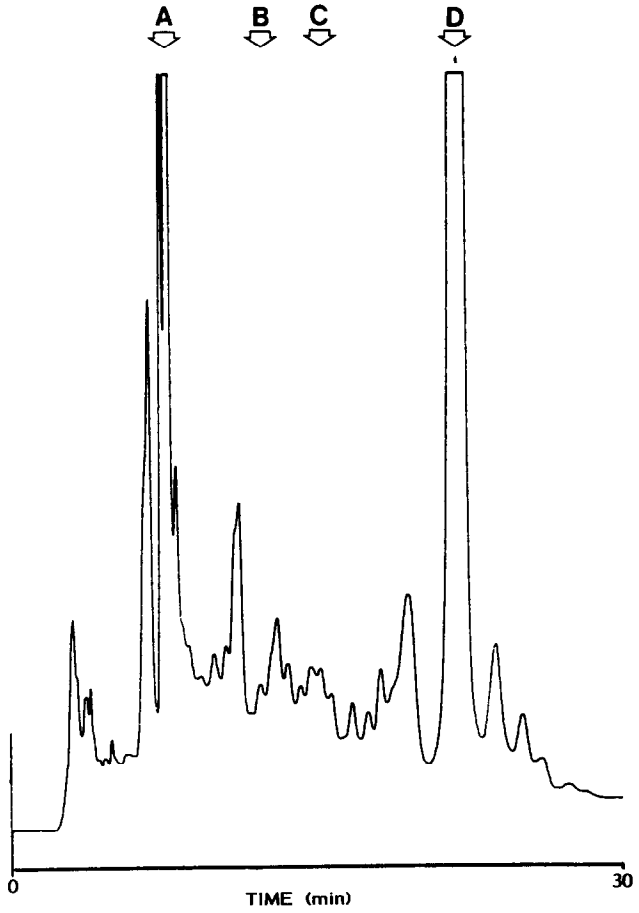


Figure 5 High-performance liquid chromatography (ZORBAX-SIL) of an extract of rachitic chick kidney homogenate incubated with [25,26-$^2H_6$]-25-OH-D_3. A, B, C, and D indicate the retention times of pure standards of 25-OH-D_3, 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 1α,25-$(OH)_2D_3$, respectively, run in the same system. The eluate was monitored by UV absorption at 264 nm.

Mass fragmentographic assays for vitamin D and metabolites using stable isotope dilution

Using standard material produced by the methods described above, several mass fragmentographic procedures have been described for the measurement of vitamin D and its metabolites in human plasma or serum since 1976. These are summarized in Table 2, which also includes early methods developed without the use of stable isotope-labeled internal standards.

Two groups in Europe have produced the majority of the fully evaluated methods described (Björkhem and colleagues in Sweden and Makin and colleagues in the United Kingdom). Both groups have used conventional packed glass columns for GC, with a diameter of 2 mm or an internal diameter 4 mm (Table 3). A few workers[15,35] describe the use of capillary GC columns, but only one evaluated method has been described using capillary columns.[13] The use of capillary columns theoretically should produce an improvement in both resolution and sensitivity of mass fragmentographic assays, but, for various practical reasons, there may be no improvement in sensitivity. There are considerable injection difficulties when applying vitamin D and its metabolites to capillary columns. Injection systems that "split" the injection volume obviously reduce sensitivity considerably and are therefore not suitable for assays designed to measure the very low circulating concentrations of some of the vitamin D metabolites. Using the injector in the "splitless" mode, the sample is introduced in a large solvent volume and peak shape may be severely distorted; we have experienced considerable difficulties using these systems. In addition, vitamin D and its metabolites are particularly susceptible to destruction and/or absorption during the injection system. Sample injection problems with capillary columns can be overcome by using an all-glass dropping needle injection assembly. This involves applying the sample, in stages, to a silanized glass needle and allowing the solvent to evaporate between each application. The dried concentrated sample is then released into the heated zone of the injection port, and the sample is efficiently transferred onto the capillary column. Unfortunately, all-glass dropping needle injection assemblies require adaptation of many injection ports and

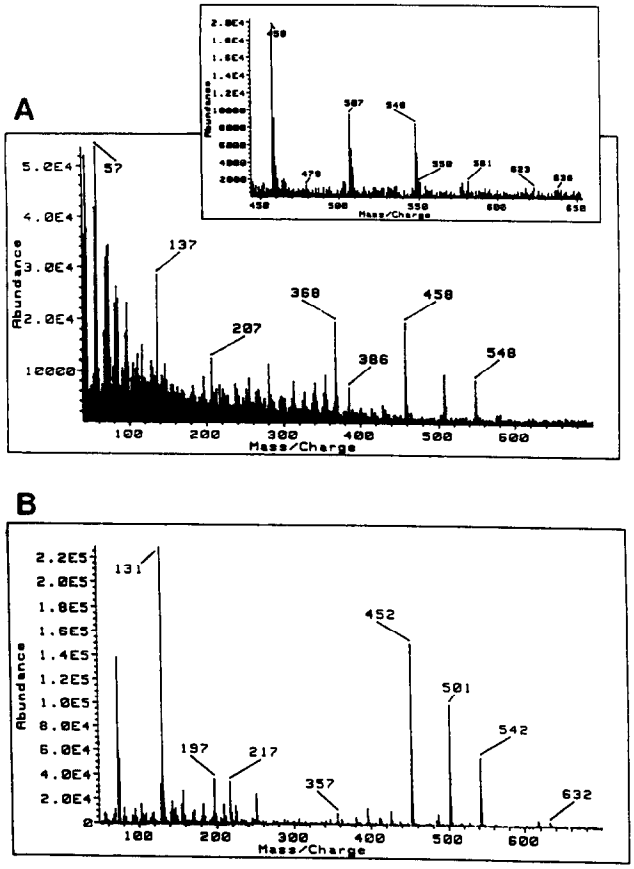


Figure 6 Mass spectra (EI(+) at 75 eV) of pertrimethylsilyl ethers of (A) putative [25,26-$^2H_6$]-1,25-$(OH)_2D_3$ (Peak D, Figure 5) isolated from rachitic chick kidney homogenate incubate as described in text and legend to Figure 5, and (B) standard 1,25-$(OH)_2D_3$. Mass spectra were obtained using a Hewlett-Packard mass selective detector (Model 5970) after separation by GC.

Table 2 Methods for the measurement of vitamin D and its metabolites in human plasma/serum using mass fragmentography

Metabolite	Derivative used	Ion(s) monitored	Internal standard(s) used	Reference
D_3	ITS-HFB	M^+ 586	DHT	33
	TMSi	$(M - 131)^+$ 325	D_2	34
D_3 fatty acid esters	TMSi	M^+ 456 (D_3) 468 (D_2)	D_3-d_8[a] D_2-d_3[a]	15–18
25-OH-D_3	TMSi	131 (side-chain fragment)$^+$	[26-$^2H_3$]-25-OH-D_3	9
	TMSi	$(M - 90 - 15)^+$ 439	None	35
	t-BDMSi/TMSi	M^+ 586	[26-$^2H_3$]-25-OH-D_3	8,36,39,42
	TMSi	131 (side-chain fragment)$^+$ $(M - 131)^+$ 413, $(M - 90 - 15)^+$ 439 M^+ 544	None	37
24,25-$(OH)_2D_3$	nBBA-TMSi	$(M - 90 - 15)^+$ 449	[26,27-$^2H_6$]-24,25-$(OH)_2D_3$	12
25,26-$(OH)_2D_3$	nBBA-TMSi	$(M - 90 - 15)^+$ 449	[26,27-$^2H_5$]-25,26-$(OH)_2D_3$	11,38
1,25-$(OH)_2D_3$	TMSi	$(M - 90 - 90)^+$ 452	[26-$^2H_3$]-1,25-$(OH)_2D_3$	10
1,25-$(OH)_2D_3$	TMSi	$(M - 90)^+$ 542	[26-$^3H_2$]-1,25-$(OH)_2D_3$	13
D_3 25-OH-D_2 25-OH-D_3 24,25-$(OH)_2D_3$	TMSi	M^+ 456 and $(M - 90)^+$ 366 M^+ 544 M^+ 556 M^+ 632	D_2	7
D_3	TMSi	M^+ 456	[3,4-$^2H_2$]D_3	15
25-OH-D_3	TMSi	$(M - 90)^+$ 454	[26,27-$^2H_3$]25-OH-D_3	39
	TMSi	M^+ 468 (D_3) $(M - 90)^+$ 454 (25-OH-D_3)	[2,3,4,6,9,19-$^2H_8$]D_3 [26,27-$^2H_6$]25-OH-D_3	16[b]
25-OH-D_2 25-OH-D_3	TMSi	$(M - 90 - 15)^+$ 439 and 451	[26,27-$^2H_6$]25-OH-D_3	31,40,41
24,25-$(OH)_2D_2$ 25,26-$(OH)_2D_2$	nBBA/TMSi	$(M - 90 - 15)^+$ 461	[28-$^2H_3$]24,25-$(OH)_2D_2$ [26,27-$^2H_6$]24,25-$(OH)_2D_3$	

Abbreviations: TMSi, pertrimethylsilyl ethers; nBBA, cyclic *n*-butyl boronates; *t*-BDMSi, *tertiary*-butyldimethylsilyl ethers; HFB, heptafluorobutyrate ester; DHT, dihydrotachysterol; ITS, isotachysterol.
[a] Same internal standards as described in ref. 16.
[b] Reference 16 describes a method for the measurement of D_2 (see also ref. 18) and 1,25-$(OH)_2D_3$ in addition to those described here, but no values are given for these latter two analytes.

Table 3 Details of chromatographic methodology used in mass fragmentography methods for the determination of vitamin D and metabolites

Analyte	Column packing material	Column dimensions (diameter × length)	Reference
Conventional packed columns			
25-OH-D_3	1.5% SE30 on Chromosorb W (80–100 mesh)	2 mm × 2.5 m	8,9,36,39,40
D_3	1% FFAP on Gas Chrom (100–120 mesh)	2 mm × 2.0 m	33[a]
D_3	1.5% SE30 on Chromosorb W (80–100 mesh)	2 mm × 2.5 m	34,39
1,25-$(OH)_2D_3$	1.5% SE30 on Chromosorb W (80–100 mesh)	1.5 mm × 2.5 m	10
D_3,25-OH-D_2 25,OH-D_3 24,25-$(OH)_2D_3$	2% OV1 on Celite 545 (100–120 mesh)	4 mm × 2 m	7
24,25-$(OH)_2D_3$ 25,26-$(OH)_2D_3$	2% OV1 on Celite 545 (100–120 mesh)	5 mm × 2.5 m	11,12
25-OH-D_2,25-OH-D_3 24,25-$(OH)_2D_2$ 25,26-$(OH)_2D_2$ and other metabolites[43]	1% OV1 on Celite 545 (100–120 mesh)	5 mm × 1.5 m	31,40,41
Capillary WCOT columns			
25-OH-D_3 24,25-$(OH)_2D_3$ 1,25-$(OH)_2D_3$	OV101	0.35 mm × 25 m	35[b]
D_3,25-OH-D_3	SE30	0.29 mm × 25 m	15[b]
1,25-$(OH)_2D_3$	SUPEROX 4	0.25 mm × 4 m	13[c]
D_3,25-OH-D_3 1,25-$(OH)_2D_3$	OV101	0.32 mm × 25 m	43[a]
Polyhydroxylated 25-OH-D_3 metabolites	SE52	unknown × 15 m	51[d]

All methods used EI(+) and single focusing magnetic sector mass spectrometers (LKB 2091, except [a]LKB 9000, [b]VG Micromass 16F, or [c]Finnigan 1020, or Hitachi M-52).

are not readily adaptable to automation. Direct on-column injection onto a short (4-mm length) capillary column (0.25 m internal diameter Superox 4) has recently been used for the mass fragmentographic measurement of 1,25-$(OH)_2D_3$ in human plasma, but no significant increase in sensitivity was observed.[13]

Early methods for the measurement of vitamin D and its metabolites, which did not use stable isotopes as internal standards, share several disadvantages. The use of radiolabeled vitamin D standard to assess the efficiency of the extraction procedure[17,33] necessitated the addition of a second internal standard prior to mass fragmentography. Many workers describe instability problems and inconsistent recovery of metabolites through the various extraction stages when radiolabel alone is used, although silanization of all glassware should overcome this problem.

It is preferable to use an excess amount of a vitamin D analog as internal standard, which will both protect the low concentration of analyte during sample preparation and serve as a suitable internal standard for GC/mass fragmentography. Vitamin D_2 has been used for this purpose in a method developed for the measurement of vitamin D_3 in human serum.[34] This approach is not without problems since vitamin D_2 (which can be ingested from certain foods) may be present in the sample being analyzed. However, these investigators[34] were confident that low levels of circulating vitamin D_2 would not affect their results, as the amount of vitamin D_2 added to the serum sample was in such large excess. An alternative method for the measurement of vitamin D_3 in human plasma[33] used ^{14}C-labeled vitamin D_3 to assess recovery and then dihydrotachysterol$_2$ (DHT_2, a pseudo 1α-vitamin D_2 analog) as an internal standard for mass fragmentography. Dihydrotachysterol$_2$ is unsuitable as an internal standard during the extraction stages, as, unlike vitamin D, it does not bind to vitamin D-binding globulin, and therefore is unlikely to behave in the same way during extraction.

The availability of deuterium-labeled vitamin D standards has allowed the development of several methods for the determination of the majority of vitamin D metabolites circulating in plasma. Stable isotopes supposedly mimic the behavior of the natural metabolite and the assay procedure is greatly simplified, although separation of labeled and unlabeled compounds in various systems has been reported.[44] Care should be taken, however, in ensuring that the stable isotope is in a suitable position. Zagalak et al.[15] describe the use of [3,4-$^2H_2$]D_3 as an internal standard for the measurement of vitamin D_3. However, cholesterol has a molecular weight only two mass units higher than vitamin D_3, and its contamination of extracts could lead to inaccurate results. Although their method painstakingly removed cholesterol from the serum sample using alumina column chromatography and thin-layer chromatography, (TLC), followed by HPLC on silver nitrate impregnated silica, the use of an alternative internal standard would appear to be indicated. Zagalak et al.[15] also describe the preparation of [C2,2,3,4,4,6-$^2H_6$] vitamin D_3; this isotope would be a more sensible choice. The more deuterium atoms that can be incorporated into the internal standard, the less interference from the presence of natural isotopes in the unlabeled analyte. However, this has to be balanced by the fact that increasing numbers of deuterium atoms increased the likelihood that separation of labeled and unlabeled analyte may occur, particularly during GC. Adequate purification of the sample prior to GC/MS is, of course, important. The preparation of extracts for the determination of vitamin D_3 concentration especially needs to ensure the efficient removal of cholesterol from the sample. Although appropriate GC systems can resolve the two compounds, cholesterol circulates in such larger concentrations than vitamin D_3 (mg/ml versus ng/ml) that considerable contamination of the vitamin D_3 trace can occur if only a small fraction of the cholesterol remains. Careful selection of the ion to be monitored may avoid this problem.[35] Cholesterol can be removed by several methods, including digitonin precipitation of cholesterol,[33] TLC,[7] and HPLC.[41]

The first full description of a mass fragmentographic procedure for the measurement of a vitamin D metabolite in plasma using stable isotope dilution was published in 1976.[9] The chemical preparation of [26-$^2H_3$]-25-OH-D_3 was described and was used as the internal standard in the determination of 25-OH-D_3 concentration in 2.5 ml serum. After solvent extraction and column chromatography, pertrimethylsilyl ether derivatives were formed.[45] A fragment ion of low mass was monitored during mass fragmentography (m/z 131 and 134). This fragment represents C-26, C-27 and the derivatized C-25 hydroxyl group and thus retains the deuterium label. Although it would have been preferable to monitor an ion fragment of higher mass, it was reported that these were too low in intensity.

A subsequent publication from the same group[42] described the measurement of the formation of 25-OH-D_3 in liver homogenates using a mass fragmentographic procedure based on that outlined above, but with formation of the 25-trimethylsilyl-3-*t*-butyldimethylsilyl ether derivative. The use of this mixed derivative added further specificity to the assay previously described.[9] The new derivative fragmented to give high mass ions of good intensity, allowing the molecular ion of the analyte and internal standard (m/z 586 and 589) to be monitored during mass fragmentography. The use of these ions produced much cleaner chromatograms and previously reported contamination problems were overcome. This assay procedure was successfully applied in several subsequent studies.[46]

Björkhem et al.[10] also described a method for the measurement of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in plasma by isotope dilution mass fragmentography. 1,25-$(OH)_2D_3$, the calcium homeostatic hormone, circulates at levels of 20 to 60 pg/ml in normal humans. To measure these low levels accurately, 20 ml plasma or serum was required. Using [26-$^2H_3$]-1,25-$(OH)_2D_3$, prepared biosynthetically, as internal standard, plasma samples were extracted with organic solvent and subjected to extensive chromatography, including two HPLC steps. The 1,25-$(OH)_2D_3$ fraction

Table 4 Details of derivatives and ions monitored during single ion monitoring of vitamin D and vitamin D metabolites

Analyte	Internal standard	Derivative	Ion monitored
D_2		3-TMSi	m/z 363 $[M - 90 - 15]^+$
D_3		3-TMSi	m/z 351 $[M - 90 - 15]^+$
	$(^2H_6)D_3$	3-TMSi	m/z 356 $[M - 90 - 15]^+$
25-OH-D_2		3,25(TMSi)$_2$	m/z 451 $[M - 90 - 15]^+$
25-OH-D_3		3,25(TMSi)$_2$	m/z 439 $[M - 90 - 15]^+$
	$(^2H_6)$25-OH-D_3	3,25(TMSi)$_2$	m/z 445 $[M - 90 - 15]^+$
24,25-$(OH)_2D_2$		24,25-nBBA-3-TMSi	m/z 461 $[M - 90 - 15]^+$
	$(^2H_3)$24,25-$(OH)_2D_2$	24,25-nBBA-3-TMSi	m/z 464 $[M - 90 - 15]^+$
24,25-$(OH)_2D_3$		24,25-nBBA-3-TMSi	m/z 449 $[M - 90 - 15]^+$
25,26-$(OH)_2D_2$		25,26-nBBA-3-TMSi	m/z 461 $[M - 90 - 15]^+$
	$(^2H_6)$24,25-$(OH)_2D_3$	24,25-nBBA-3-TMSi	m/z 455 $[M - 90 - 15]^+$
25,26-$(OH)_2D_3$		25,26-nBBA-3-TMSi	m/z 449 $[M - 90 - 15]^+$
	$(^2H_5)$25,26-$(OH)_2D_3$	25,26-nBBA-3-TMSi	m/z 454 $[M - 90 - 15]^+$
1,25-$(OH)_2D_3$		1,3,25(TMSi)$_3$	m/z 501 $[M - 131]^+$
	$(^2H_6)$1,25-$(OH)_2D_3$	1,3,25(TMSi)$_3$	m/z 507 $[M - 131]^+$

Abbreviations: TMSi, Trimethylsilyl ether; nBBA, cyclic *n*-butyl boronate ester.
Data from ref. 41, with permission of John Wiley & Sons Ltd.

isolated was converted into the pertrimethylsilyl ether derivative and mass fragmentography was performed monitoring the $(M - 90 - 90)^+$ ion (m/z 452 and 455). The monitoring of base peak fragments m/z 131 and 134 was also investigated. Although monitoring these ions was more sensitive, extensive contamination was a problem; thus, the more specific high mass ions were selected. Little progress has been made in the 10 years since this publication to reduce the sample volume necessary or the simplicity of the assay. This possibly may be achieved once the problems associated with the use of capillary columns for the estimation of vitamin D metabolites have been overcome, and may also require the use of more sensitive mass spectrometry systems, perhaps using negative ion chemical ionization (provided suitable and stable electron capture derivatives can be formed).

The majority of mass fragmentographic assays described for vitamin D metabolites during the 1980s have been developed in our laboratory. Using chemically synthesized deuteriated internal standards,[14] fully evaluated methods for the measurement of 24,25-$(OH)_2D_3$ (ref. 12), 25,26-$(OH)_2D_3$ (ref. 11), 25-OH-D_3, 25-OH-D_2, 24,25-$(OH)_2D_2$, and 25,26-$(OH)_2D_2$ (ref. 31) in human plasma have been described. In these assays, the analyte is converted, after extensive purification, into either the pertrimethylsilyl ether derivative (25-OH_2-D_2 and 25-OH-D_3) or the *n*-butyl boronate ester-3-trimethylsilyl ether derivative (24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, 24,25-$(OH)_2D_2$, and 25,26-$(OH)_2D_2$) before GC/MS. *n*-Butyl boronate esters will form across vicinal or 1,3 *cis*-hydroxyl groups, and their formation improves the specificity of the assay by providing mass fragments of high mass to charge ratio. Other alkyl boronate esters can also be formed, but provide no particular advantages over the *n*-butyl boronate derivative. Methyl boronates can be formed across the C-24,C-25 but not, surprisingly, across C-25,C-26. Phenyl and *n*-butyl boronates, on the other hand, can be formed across both C-24,C-25 and C-25,C-26.[11]

The mass spectrometer used during the development of these assays only allowed selected ion monitoring of three ions simultaneously; therefore, no more than two metabolites could be analyzed at once. Modern machines equipped with data handling facilities would, however, be able to generate a "profile" of vitamin D metabolites from a single GC run, although the more ions monitored simultaneously, the less sensitive each assay becomes. The extraction and purification procedure devised for such a multiple assay is given in Figure 7. This is also the method used to prepare samples for the measurement of individual metabolites. Table 4 shows the derivatives used, the ions monitored, and the internal standards used during mass fragmentography. This method illustrates how it is possible to overcome the problems associated with lack of suitable deuteriated standard. In the assay developed for 25,26-$(OH)_2D_2$, no deuteriated standard was available; thus, hexadeuterated 24,25-$(OH)_2D_3$ was used as it co-eluted in the HPLC system used prior to GC/MS. Pentadeuterated 25,26-$(OH)_2D_3$, which might have seemed the obvious choice, although available, was not used because it was separated from 25,26-$(OH)_2D_2$ during HPLC. Typical mass fragmentograms of standard material are shown in Figure 8, along with the ions monitored and the differences in the structures of the different metabolites. On injection onto the GC column, B ring closure occurs and vitamin D and its metabolites isomerize into two forms, pyro and isopyro, which separate during chromatography. Thus, two peaks are obtained from each sample.

Dihydroxylated vitamin D metabolites have the same molecular weight, but *n*-butyl boronate ester-3-trimethylsilyl ether derivatives of 24,25-$(OH)_2D$ and 25,26-$(OH)_2D$ separate under the GC conditions used.[12] 1,25-$(OH)_2D$ does not form an *n*-butyl boronate ester derivative, as the 1α and 3β hydroxyl groups are too far apart for a reaction to take place. In the methodology described by our group, using a single focusing magnetic sector mass spectrometer with electronic MID,

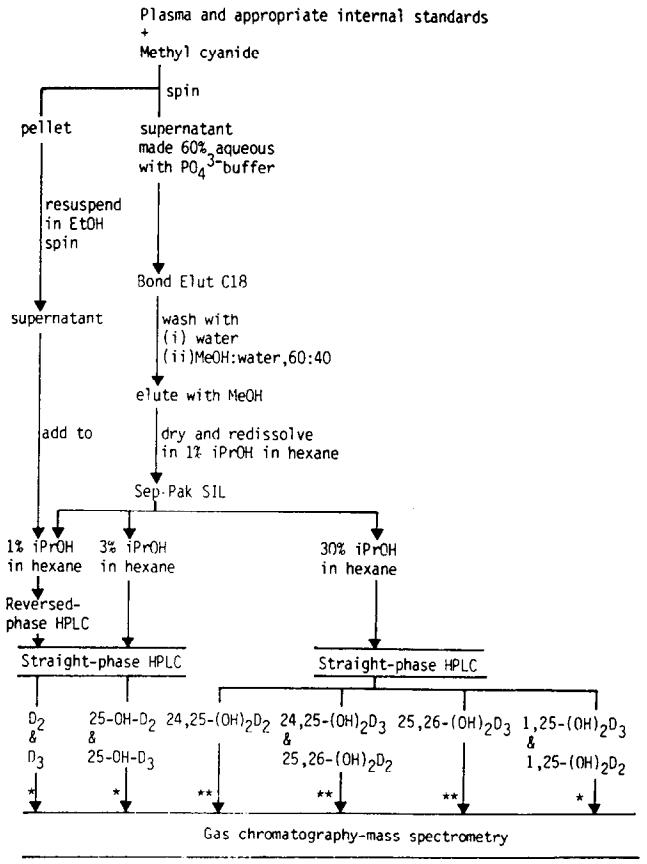


Figure 7 Proposed scheme for the extraction and purification of vitamins D_2 and D_3 and eight metabolites from plasma/serum prior to GC/MS. *Formation of pertrimethylsilyl ethers. **Formation of cyclic *n*-butyl boronate-3-trimethylsilyl ether. EtOH, ethanol; MeOH, methanol; iPrOH, isopropanol. It would be possible to collect 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 25,26-$(OH)_2D_2$ in a single fraction and quantitate together. (Reprinted from ref. 5 with permission from the copyright holder, Butterworth-Heinemann.)

each metabolite is measured separately using the appropriate internal standard; this requires HPLC separation prior to derivatization. Using a modern mass spectrometer with computerized data handling, it should be possible to measure metabolites without prior HPLC in a single GC run. The use of fewer internal standards may be possible using this approach. Selective timing of the monitoring of ions may be necessary to detect metabolites in low concentration, but most data-handling systems are capable of this.

All the methods developed so far have used conventional packed GC columns. As mentioned above, increased sensitivity could be achieved using capillary columns if the injection and adsorption problems encountered could be overcome. Recent experiences using a simple "bench top" quadrupole mass selective detector (HP5970), linked to a capillary GC column and equipped with extensive data acquisition and manipulation facilities, have provided encouraging results. Sensitive selected ion monitoring is possible, with as little as 15 pg derivatized standard 1,25-$(OH)_2D_3$ being detected easily. There is a minimum detectable limit (2 × background) of approximately 3 pg derivatized standard (Figure 9), and 25-OH-D levels are easily measured in 2 ml plasma (Figure 10).

Use of stable isotope-labeled vitamin D in studies of metabolism

Vitamin D is extensively metabolized in the side chain, producing a large number of polyhydroxylated metabolites. One such metabolite is the 23,26-lactone of 25-hydroxyvitamin D_3, which is formed in vivo after administration of large amounts of vitamin D_3. The mechanism of formation of this metabolite was the subject

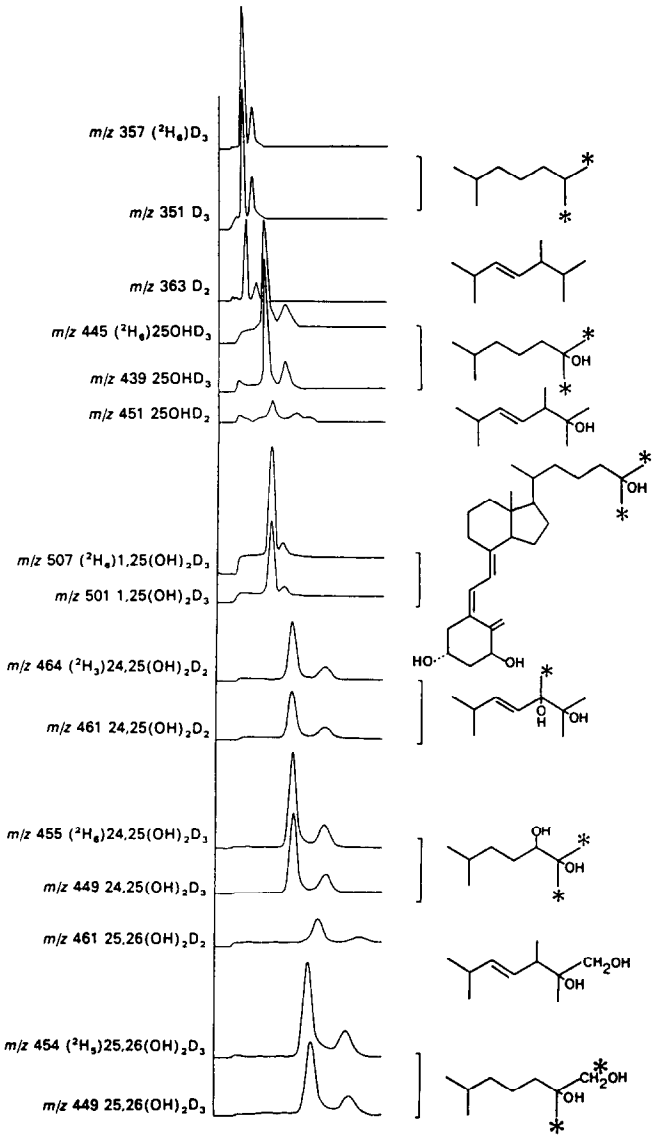


Figure 8 Selected ion chromatograms of vitamins D_2 and D_3 and some of their metabolites. Side-chains of underivatized molecules are indicated at the right-hand side of each trace (as are the ions monitored in each channel). Hydroxyls at C-1 and C-3 are derivatized as trimethysilyl ethers and C-25 as a trimethylsilyl ether or as a cyclic *n*-butyl boronate when a further vicinal hydroxyl is present (i.e., in 24,25- and 25,26-dihydroxy metabolites). The asterisks (*) indicates the positions at which deuterium label is attached. (Reprinted from ref. 41, with permission of John Wiley & Sons, Ltd.)

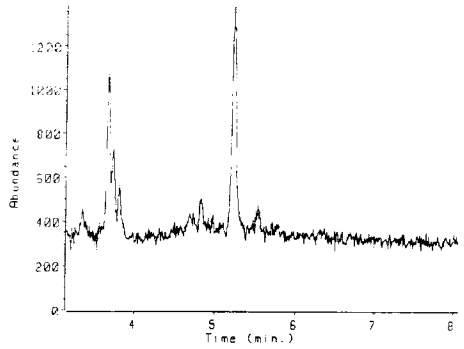


Figure 9 Selected ion monitoring of the (M − 90 − 90)$^+$ ion produced from 15 pg 1,25-$(OH)_2D_3$, pertrimethylsilyl ether derivative (m/z 452), using capillary GC and the Hewlett-Packard 5970 mass-selective detector.

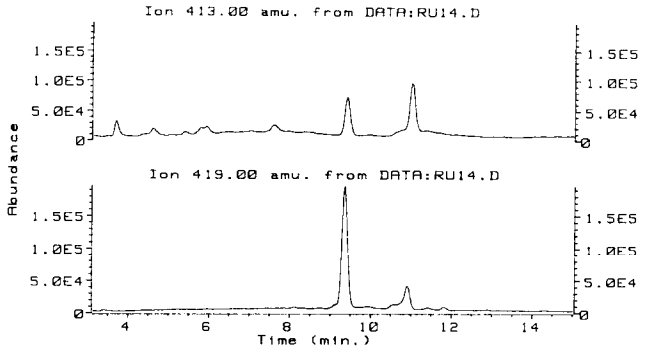


Figure 10 Mass fragmentography using 25-hydroxyvitamin D_3 in a plasma extract. After addition of 100 ng of [$^2H_6$]-25-OH-D_3, plasma (0.8 ml) was extracted and purified on Bond-Elut C-18 and Sep-Pak SIL cartridges as previously described.[31] Pertrimethysilyl ethers were formed and an aliquot of the final extract was chromatographed on an HP1 (Hewlett-Packard line equivalent of OV101) wall-coated chemically bonded capillary column (0.25 mm internal diameter × 25 m). The ion monitored was (M − 131)$^+$ (m/z 413 for the analyte and m/z 419 for the hexadeuterated internal standard). The pyro peak for the analyte and internal standard are both relatively uncontaminated, whereas the iso-pyro peak of the analyte is contaminated with an unknown peak. This plasma sample contained 36 ng/ml 25-OH-D_3.

of some discussion until the suggestion that the 23,26-lactol was an intermediate.[47] A recent study[48] of the metabolism of 25-hydroxydihydrotachysterol$_3$ confirmed this pathway of side-chain metabolism, and all the suggested metabolites were isolated from and identified in the perfused rat kidney. Experiments were carried out using both labeled (deuterated) and unlabeled 25-hydroxy-DHT_3, and the use of the deuterium-labeled substrate was of considerable importance in confirming the structures assigned to both the lactone and lactol. The deuterium-labeled 25-hydroxy-DHT_3 was labeled on C-26 and C-27, incorporating six deuterium atoms, and was derived from deuterated 25-hydroxy-tachysterol$_3$ obtained as a by-product of B ring opening of 25-hydroxy-7-dehydrocholesterol$_3$ by UV light.[14] 25-Hydroxytachysterol$_3$ can be reduced with lithium in liquid ammonia[49] to produce, among other products, the 25-hydroxy-DHT_3 compound. With deuterium labeling on C-26, it is possible to predict (see Figure 11) that the lactol and lactone produced in vitro from such labeled 25-hydroxy-DHT_3 should give mass spectra containing, for example, molecular ions only 4 or 3 amu, respectively, higher than the unlabeled compounds, rather than the 6 amu difference between the substrates. This indeed occurred, and provided additional support for the structural assignments made on the basis of mobility in HPLC systems and mass spectra obtained from unlabeled metabolites.

Summary

It is clear that the best mass fragmentographic assays for vitamin D metabolites in human body fluids are those based on isotope dilution using the appropriate deuteriated-labeled metabolite as an internal standard. The difficulty that all workers in this area have experienced primarily relates to the lack of available standards, both unlabeled and labeled, although deuterium-labeled standards are particularly difficult to acquire. It has therefore been necessary to produce the required labeled standards using synthetic organic chemistry. However, the use of in vitro and in vivo biosynthetic systems for further conversion of synthetic labeled standards to more polar deuterated metabolites should not be ignored.

If the required standards are available, it is possible to develop mass fragmentographic methods for the measurement of the major metabolites of vitamin D in human plasma that give precise and accurate results. These assays are not designed for routine use, but rather as reference procedures against which other less rigorous analytic techniques can be evaluated. A simple mass fragmentographic method for the measurement of plasma levels of 1,25-$(OH)_2D_3$, the major calcium homeostatic hormone, in reasonable volumes of plasma has not yet been developed, although one mass fragmentographic assay, albeit requiring 20 ml plasma,

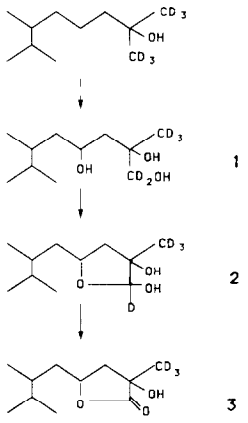


Figure 11 Predicted loss of deuterium atoms from the side chain of 25-hydroxy-[26,27-$^2H_6$]dihydrotachysterol$_3$ during the in vitro formation of the 25-hydroxyvitamin D_3-23,26-lactone in the isolated perfused rat kidney. (See ref. 48 for details of experimental procedure.)

has been described; this method has recently been used[13] to validate the calf thymus radio receptor assay for 1,25-$(OH)_2D_3$ ref. 50.

The assays already developed will be greatly improved in terms of sensitivity and probably specificity when GC with capillary column is used. However, there appear to be injection difficulties to overcome. Vitamin D_2 and its metabolites are more difficult to measure than vitamin D_3, but the concentration of vitamin D_2 and its metabolites in human plasma is very low in the United Kingdom and only assumes significance in countries where foodstuffs are supplemented with vitamin D_2.

High-performance liquid chromatography/MS also offers an alternative approach to the measurement of vitamin D and its metabolites in human body fluids. However, no such assay has yet been described. It is unlikely that HPLC/MS will, in its present form, provide sufficient sensitivity for the measurement of 1,25-$(OH)_2D_3$ in human plasma, although it may be useful for 25-OH-D_3. Further developments in interfacing the HPLC and MS may, however, overcome this lack of sensitivity. High-performance liquid chromatography/MS has the advantage that excellent HPLC systems for the separation of the majority of vitamin D metabolites have been developed, and the direct injection of the solute into the MS obviates the necessity for derivatization. It is, however, not yet clear how specific such a procedure will be.

In our view, it is very important that laboratory personnel setting up assays for vitamin D and its metabolites think very carefully about the specificity of their assay and institute both internal and external quality-assurance schemes. Otherwise, results obtained may be of little value and it may not be possible to compare results with those obtained from other laboratories. Good external quality-assurance schemes use GC/MS target values, wherever possible, to enable participating laboratories to gauge the accuracy of their methodology. Some HPLC methods have purported to test the efficacy of purification procedures used by demonstrating that the final extract gives an apparently homogeneous peak with a mass spectrum identical to the analyte. However, Holmberg et al.[36] have clearly demonstrated that such procedures give a false sense of security and that the proper evaluation of such methods requires comparison of individual results obtained by both the method under evaluation and by mass fragmentography. It is essential for the provision of such mass fragmentographic values that labeled and unlabeled standards continue to be available so that suitable mass fragmentographic procedures can be carried out.

Acknowledgments

We are grateful to the UK Medical Research Council for grants to D.N.K. (for support of the Steroid Reference Collection) and to D.N.K., H.L.J.M., and D.J.H.T. (project grant), to the Wellcome Trust, National Kidney Research Fund, and Duphar Pharmaceuticals Ltd. for grants to H.L.J.M.

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