High-pressure liquid chromatography: separation of the metabolites of vitamins D_2 and D_3 on small-particle silica columns

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Abstract The high-pressure liquid chromatographic separation of all of the known metabolites of vitamin D_2 and vitamin D_3 found in biological fluids has been achieved. This technique has been successfully applied to the analysis of vitamin D mixtures, purification of vitamin D metabolites, and identification of radioactive peaks. Some theoretical bases for the observed resolutions are suggested.

Supplementary key words 1,25-dihydroxyvitamin D_3 · 25-hydroxyvitamin D_3

Adsorption chromatography was one of the first chromatographic techniques applied to the separation of vitamin D and its metabolites (1–3). Though its resolving powers are very good, it suffers from the difficulties of requiring extremely polar solvents to elute tightly adsorbed metabolites and large particle size for reasonable elution rates. Hence, adsorption chromatography was superseded by the milder liquid–gel chromatography described by Holick and DeLuca (4). Using Sephadex LH-20, these workers were able to isolate and identify several metabolites from biological fluids as well as routinely assay radioactive metabolites in analytical studies.

Occasionally, Celite liquid–liquid partition columns have been used to resolve metabolites of vitamin D_3 that are difficult to separate, such as 1,25-$(OH)_2D_3$ from 25,26-$(OH)_2D_3$ (5, 6). However, these procedures are time consuming, require large amounts of solvent, and are difficult to reproduce, which has limited their application in this field.

Recent advances in commercially available instrumentation and column packing have enabled the development of high-pressure liquid chromatography and its application to the separation and determination of fat-soluble vitamins. Under the pressures generated in this technique, gel particles cannot be used, thus requiring the development of more stable and hardier column packings.

A packing used earlier in the development of high-pressure liquid chromatography consisted of octadecylsilane bonded to glass beads (ODS–Permaphase; Du Pont Instruments, Wilmington, Del.). Separation of vitamin D compounds on this material is by reversed-phase liquid–liquid partition chromatography, and the system has the ability to partially resolve vitamins D_2 and D_3 by virtue of their differential solubility in methanol–water mixtures (Du Pont Methods Bulletin 820M10, 1972). Matthews et al. (7) later demonstrated the resolution of a limited number of synthetic vitamin D compounds on the ODS–Permaphase support and suggested its usefulness in analysis of radioactive metabolites in lipid extracts. However, resolution of 1,25-$(OH)_2D_3$ from 25,26-$(OH)_2D_3$ is minimal on this system.

Williams (8) recently reported the application of a small porous silica column packing to the resolution of synthetic vitamin D compounds. Except for these reports, no systematic definitive study of the separation of all the vitamin D_3 and D_2 compounds known to be present in biological fluids has been published. It is the purpose of this paper to illustrate the ability of high-pressure liquid chromatography to separate virtually all of the known metabolites of vitamins D_2 and D_3 found in biological fluids and also to point out some of the structural differences that bring about these observed resolutions.

Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 25-OH-D_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_2$, 24,25-dihydroxyvitamin D_2; 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1α-OH-D_2, 1α-hydroxyvitamin D_2; 24-OH-D_3, 24-hydroxyvitamin D_3; 24-OH-D_2, 24-hydroxyvitamin D_2; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3.

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EXPERIMENTAL METHODS

General procedures

All solvents were of analytical grade and redistilled (Skellysolve B doubly redistilled, 67–69°C) before use. Ultraviolet spectra were obtained with a Beckman DB-G recording spectrophotometer.

High-pressure liquid chromatography

High-pressure liquid chromatography was performed on a Du Pont 830 LC apparatus fitted with a Waters U-6-K injection port (Waters Associates, Milford, Mass.). Using such a system, injections could be made at pressures of 3000–4000 psi without stop-flow procedures. The best resolution was achieved using two 25 cm × 2.1 mm ID Zorbax-Sil columns in series. Solvent systems used were 1–20% isopropanol in Skellysolve B, and normal operating pressures of 3000–4000 psi gave flow rates between 0.4 and 0.8 ml/min. Detection was by a UV monitor at 254 nm with a maximum sensitivity of 0.01 absorbance units.

Column chromatography

Sephadex LH-20 columns (1 × 60 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) prepared and developed in chloroform–Skellysolve B 65:35 were used as described by Holick and DeLuca (4). Hydroxyalkoxypropyl Sephadex columns (1 × 60 cm) prepared and developed in chloroform–Skellysolve B 10:90 were used as described by Jones, Schnoes, and DeLuca (9).

Chemicals

Certain reference compounds were obtained commercially in crystalline form: vitamin D_3 from Philips-Duphar, Amsterdam, The Netherlands; vitamin D_2 from General Biochemicals, Chagrin Falls, Ohio; and 25-OH-D_3 from the Upjohn Co., Kalamazoo, Mich. 25-OH-D_2 and 1,25-$(OH)_2D_2$ were prepared by the methods of Suda et al. (10) and Jones et al. (9). 1α-OH-D_3 and 1,25-$(OH)_2D_3$ were prepared in this laboratory as previously described (11, 12). 1α-OH-D_2, 24,25-$(OH)_2D_3$, and 25,26-$(OH)_2D_3$ were also synthesized in this laboratory (13–15). 24-OH-D_3 was synthesized recently by Ikekawa et al. (16). 24-OH-D_2 (peak IVa of Suda et al. [10]) and 24,25-$(OH)_2D_2$ were both isolated from pig plasma, and their purification and identification will be described in a separate communication.[2]

RESULTS

Zorbax-SIL is a small-particle silica column packing that has strong adsorptive affinity for the hydroxyl group(s) of vitamin D and its metabolites. **Figs. 1** and **2** illustrate the resolution of vitamin D and its metabolites. Although it is difficult to devise a single solvent system that will elute 1,25-$(OH)_2D_3$ in a convenient time and yet will

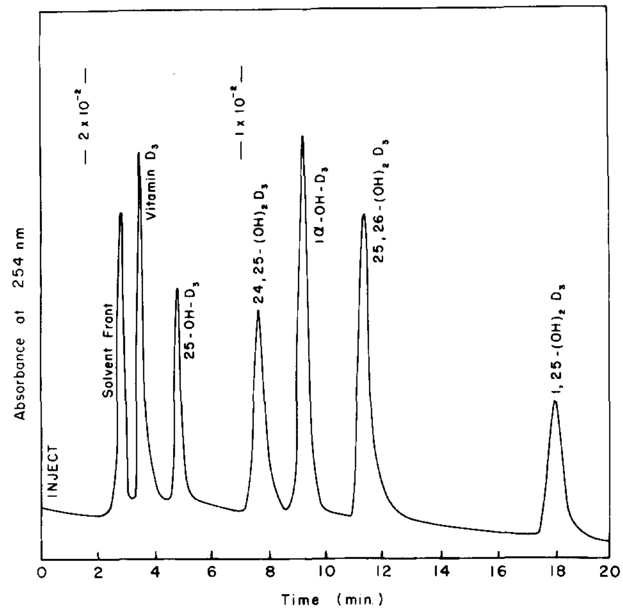

Fig. 1. High-pressure liquid chromatography of vitamin D_3 and its metabolites. A mixture of 40 ng of vitamin D_3, 30 ng of 25-OH-D_3, 25 ng of 24,25-$(OH)_2D_3$, 40 ng of 1α-OH-D_3, 40 ng of 25,26-$(OH)_2D_3$, and 25 ng of 1,25-$(OH)_2D_3$ were injected in 10 μl of 10% isopropanol in Skellysolve B using a U-6-K injector (Waters). With 10% isopropanol in Skellysolve B at 3000 psi pressure and two Zorbax-SIL (Du Pont) (2.1 mm × 25 cm) columns in series, a flow rate of 0.5 ml/min was achieved.

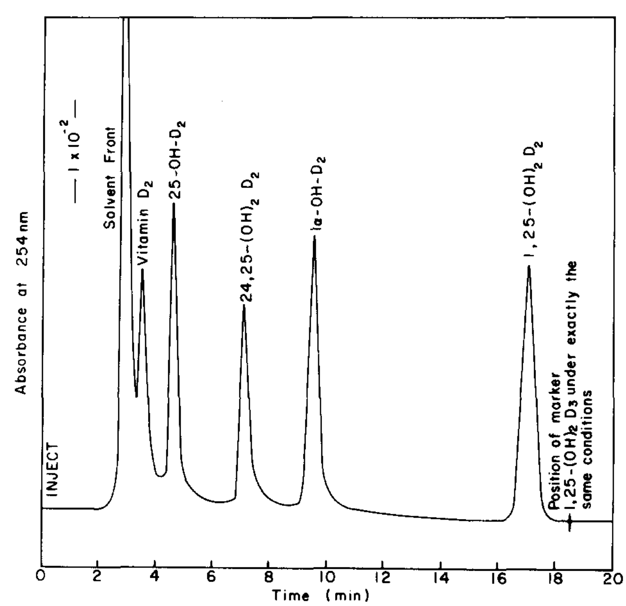

Fig. 2. High-pressure liquid chromatography of vitamin D_2 and its metabolites. A mixture of 12 ng of vitamin D_2, 25 ng of 25-OH-D_2, 20 ng of 24,25-$(OH)_2D_2$, 35 ng of 1α-OH-D_2, and 45 ng of 1,25-$(OH)_2D_2$ was injected in 10 μl of 10% isopropanol in Skellysolve B. Chromatography was carried out as described in Fig. 1.

[2] Jones, G., H. K. Schnoes and H. F. DeLuca. In preparation.

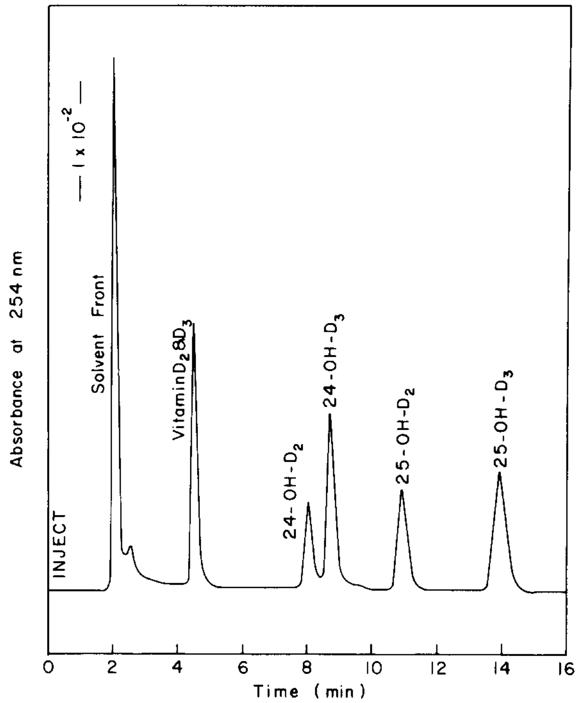

Fig. 3. High-pressure liquid chromatography of vitamins D_3 and D_2, 25-OH-D_3, 25-OH-D_2, 24-OH-D_3, and 24-OH-D_2. 10 ng of vitamin D_3, 6 ng of vitamin D_2, 19 ng of 25-OH-D_3, 13 ng of 25-OH-D_2, 16 ng of 24-OH-D_3, and 7 ng of 24-OH-D_2 were applied to the column in 10 μl of 2.5% isopropanol in Skellysolve B using a U-6-K injector. With 2.5% isopropanol in Skellysolve B at 4000 psi pressure and two Zorbax-SIL (2.1 mm × 25 cm) columns in series, a flow rate of 0.70 ml/min was achieved.

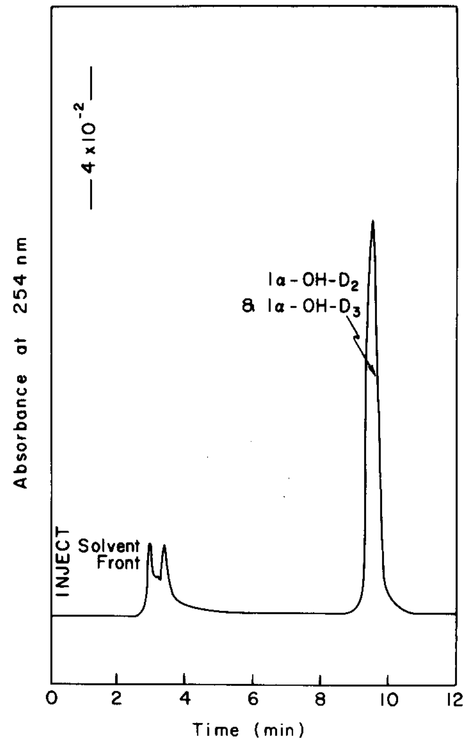

Fig. 4. High-pressure liquid chromatography of 1α-OH-D_3 (70 ng) and 1α-OH-D_2 (55 ng). The chromatographic procedure was described in Fig. 1.

resolve vitamin D from the solvent front of the column, 10% isopropanol in Skellysolve B (using 3000 psi pressure) provides a reasonable compromise. Obviously, an increase in the number of hydroxyl groups on the vitamin D molecule increases the interaction with the silica adsorbent as reflected by increased retention. The 1α-hydroxyl apparently interacts much more strongly with the silica than do the side-chain hydroxyls. This is best illustrated by the retention of the dihydroxylated 1α-OH-$D_{(2 \text{ or } 3)}$ compounds over the trihydroxylated 24,25-$(OH)_2D_{(2 \text{ or } 3)}$ compounds. Thus, high-pressure liquid chromatography on silica allows for a dramatic resolution of the naturally made 1,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ in normal lipid extracts, a resolution impossible on conventional Sephadex LH-20 column chromatography (4) or ordinary silicic acid column chromatography (2). However, the interaction between the side-chain hydroxyls and the silica is more than adequate to provide an impressive separation of 24,25-$(OH)_2D_3$ from 25,26-$(OH)_2D_3$ and a separation of 25-OH-D_3 from vitamin D_3.

Of some importance is the resolution of vitamin D_2 compounds from vitamin D_3 compounds. The silica columns do not permit the resolution of vitamin D_2 from vitamin D_3 (**Fig. 3**) or 1α-OH-D_2 from 1α-OH-D_3 (**Fig. 4**), suggesting that the side chain without hydroxyls does not interact significantly with the silica. However, the introduction of hydroxyls on the side-chain positions of 24 or 25 permits a clear resolution of the vitamin D_2 and D_3 analogs (Fig. 3 and **Fig. 5**). A partial separation of 24,25-$(OH)_2D_2$ from 24,25-$(OH)_2D_3$ is also achieved (**Fig. 6**). In all cases the D_2 analog elutes before its corresponding D_3 analog. These results suggest that the methyl group on C-24 must shield or reduce the interaction of either the 24-OH or the 25-OH, with the silica making such compounds less tightly held than their D_3 counterparts.

Base-line resolution of vitamin D, 24-OH-D, and 25-OH-D is achieved only by use of a less polar solvent system (2.5% isopropanol is Skellysolve B), as depicted in Fig. 3. Again, side-chain hydroxylation is necessary to provide a significant effect of the 24-methyl group on the interaction with the silica adsorbent.

To illustrate the analytical usefulness of this system for biological materials, Figs. 7 and 8 have been included. **Fig. 7** represents the radioactivity and absorbance profiles of a blood plasma extract of vitamin D-deficient rats given two 5-IU doses of 26,27-^{3}H-labeled 25-OH-D_3 36 and 12 hr before being killed. The extract was first chromatographed on a Sephadex LH-20 column (1 × 60 cm) using a solvent system of chloroform–Skellysolve B 65:35 (4), the 1,25-$(OH)_2D_3$ region was combined with standard nonradioactive 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ compounds, and an aliquot was applied to the high-pressure liquid column.

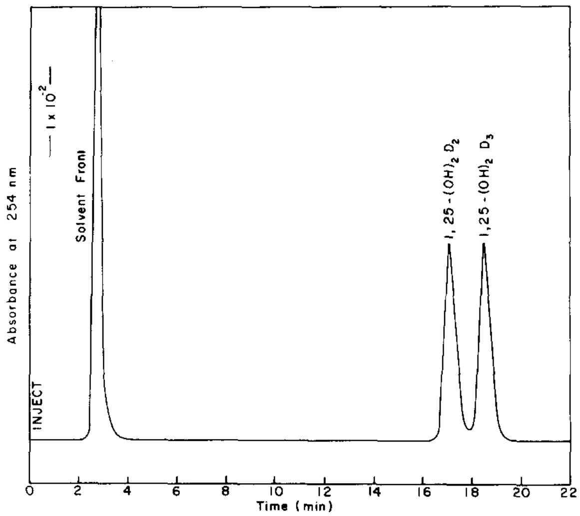

Fig. 5. High-pressure liquid chromatographic separation of 1,25-$(OH)_2D_3$ (43 ng) and 1,25-$(OH)_2D_2$ (40 ng). Experimental conditions were as in Fig. 1.

Note that the presence of other tissue lipids did not change the resolution or the elution position of the metabolites appreciably.

Fig. 8 represents a profile from an extract of liver homogenate from vitamin D-deficient chicks incubated with 3α-^{3}H-labeled vitamin D_2 according to the procedure of Tucker, Gagnon, and Haussler (17) and prepurified on

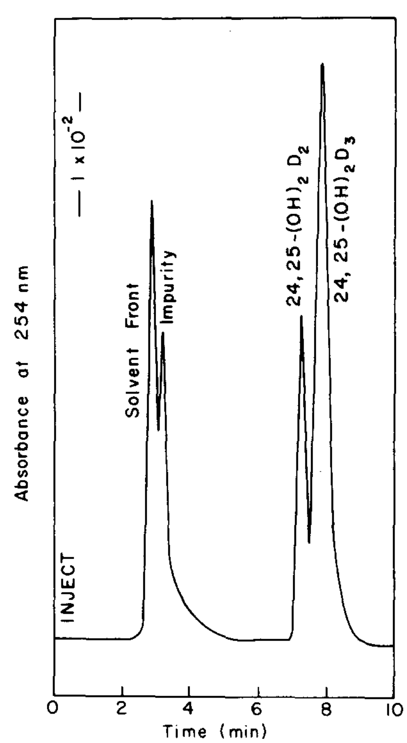

Fig. 6. High-pressure liquid chromatographic separation of 24,25-$(OH)_2D_3$ (58 ng) and 24,25-$(OH)_2D_2$ (25 ng). Experimental conditions were as in Fig. 1.

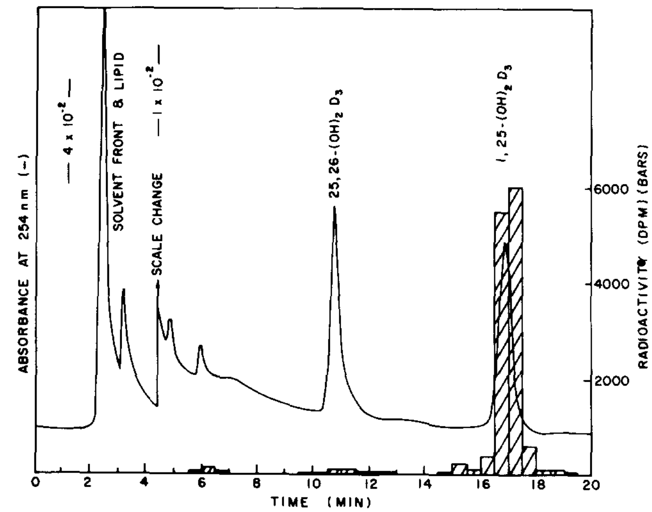

Fig. 7. High-pressure liquid chromatography of 26,27-^{3}H-labeled 1,25-$(OH)_2D_3$ present in the lipid extract of plasma from vitamin D-deficient rats given two 5-IU doses of 26,27-^{3}H-labeled 25-OH-D_3 36 and 12 hr before being killed. The profile represents an aliquot of the 1,25-$(OH)_2D_3$ region from Sephadex LH-20 column chromatography of the plasma extract mixed with synthetic 25,26-$(OH)_2D_3$ (40 ng) and 1,25-$(OH)_2D_3$ (40 ng). Experimental conditions were as in Fig. 1.

hydroxyalkoxypropyl Sephadex (1 × 60 cm; 10% chloroform in Skellysolve B; Ref. 9). Aliquots of the 25-OH-D_2 region were then chromatographed with marker vitamin D_2, 24-OH-D_2, and 25-OH-D_2.

The application of this separation technique to the isolation of compounds in preparation for identification has already been reported in the isolation and identification of 1,25-$(OH)_2D_2$ (9).

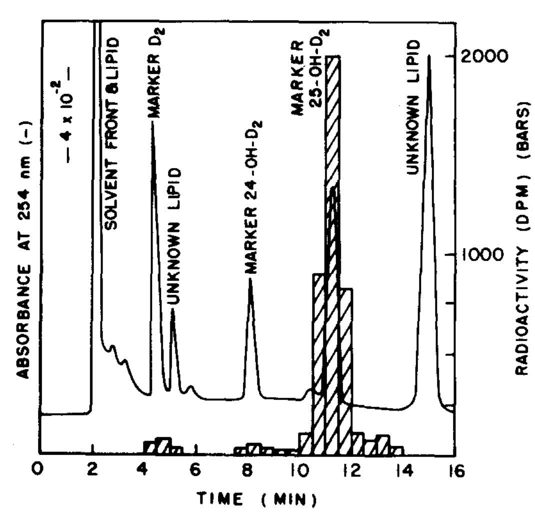

Fig. 8. High-pressure liquid chromatography of 3α-^{3}H-labeled 25-OH-D_2 present in the lipid extracts of liver homogenates from vitamin D-deficient chicks, prepared and incubated with 3α-^{3}H-labeled vitamin D_2 (9) by the method of Tucker et al. (17). The profile represents an aliquot of the 25-OH-D_2 region from hydroxyalkoxypropyl Sephadex column chromatography of the liver extract mixed with vitamin D_2 (100 ng), 24-OH-D_2 (50 ng), and 25-OH-D_2 (120 ng). Experimental conditions were as in Fig. 3.

DISCUSSION

The present report demonstrates a powerful chromatographic system for the separation of all the known metabolites of either vitamin D_2 or vitamin D_3. Furthermore, by appropriate manipulation of the solvent mixtures it is possible to separate all the known metabolites of vitamin D_3 from their respective vitamin D_2 counterparts. This, therefore, represents an important advance in technology of vitamin D chromatography that permits unequivocal identification of metabolites of vitamin D, the purification of metabolites in preparation for identification (9), and possible analysis of metabolites in blood and tissue (see Figs. 7 and 8). We have not yet applied tissue extracts directly to these analytical columns. However, a single prepurification step through Sephadex LH-20 permits analysis by this high-pressure liquid system. In such cases, the resolution and elution position remain unchanged from that achieved with pure compounds (Figs. 7 and 8).

The presently described method utilizes fine-particle silica as an adsorbent for superior resolution and high pressure to achieve reasonable flow rates. The separations probably depend largely upon the interaction between the hydroxyl groups on the vitamin D molecules and the silica. There is a rough correlation between the number of hydroxyl groups and elution position, illustrating the more hydroxyls, the more tightly held is the compound. However, the position of the hydroxyl on the molecule is also of great importance. This is best illustrated by the fact that 1α-OH-D_3 (a synthetic analog of 1,25-$(OH)_2D_3$), which is a dihydroxy compound, is more tightly held than 24,25-$(OH)_2D_3$, a trihydroxy compound. The strong interaction of the 1α-OH group undoubtedly is responsible for the fact that 1α,25-$(OH)_2D_3$ is held tightly to the column and elutes very late in the profile. This interaction is also responsible for the impressive and highly desirable separation of 25,26-$(OH)_2D_3$ from 1,25-$(OH)_2D_3$. This separation is not achieved on silicic acid column chromatography (2) or Sephadex LH-20 chromatography (4). It has been achieved by laborious Celite liquid–liquid partition chromatography (6, 18), a laborious silicic acid-impregnated paper method (19), and a reversed-phase high-pressure liquid chromatographic method using ODS–Permaphase as a support (7). However, in each case the separation achieved does not approach that obtained by the currently described procedure. This separation is of great importance to accurate measurement of ^{3}H-labeled 1,25-$(OH)_2D_3$ levels of tissue and blood samples.

Separation of the vitamin D_2 metabolites or analogs from their corresponding vitamin D_3 counterparts in the present technique requires the presence of a hydroxyl on C-25 or C-24. Thus, vitamin D_2 or 1α-OH-D_2 cannot be separated from their vitamin D_3 counterparts on these silica columns by the present techniques. Likely, the methyl group on the C-24 must decrease the interaction of either the 24-OH or the 25-OH, with the silica resulting in an earlier elution than the corresponding vitamin D_3 analogs. Thus, the ODS–Permaphase is superior to the silica columns in the resolution of vitamin D_2 from vitamin D_3 (Du Pont Methods Bulletin 820M10, 1972). It is not known, however, if this superiority holds for the hydroxylated compounds. Because the ODS–Permaphase separation of vitamin D_2 from vitamin D_3 depends on a slight solubility difference of the two in the eluting solvent, the introduction of hydroxyls may be so dominant as to minimize this solubility difference. However, only experimental examination of this will permit such conclusions to be made.

The importance of 1,25-$(OH)_2D_3$ in biology and medicine is well known (20), and its measurement is of great benefit not only for research investigators but also for diagnostic purposes. In addition, it may be advantageous in medicine to measure not only 1,25-$(OH)_2D_3$ but also all of the known metabolites of vitamin D_3. The present procedure provides a convenient and effective separation of the metabolites, and if a sensitive measurement technique specific for the vitamin D compounds can be found, this can now become a reality. At the very least, the high-pressure liquid chromatographic procedure can provide a highly effective purification necessary for those metabolites measured by the competitive binding technique (21–23). JLR

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