Journal of Chromatography, 294 (1984) 460–465
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,655

Note

A rapid method for purification of 25-hydroxyvitamin D_2 from rabbit plasma by recycle high-performance liquid chromatography

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(First received January 17th, 1984; revised manuscript received February 10th, 1984)

In order to evaluate vitamin D in man, the concentration of 25-hydroxyvitamin D (25-OH-D) in plasma or serum is sometimes determined. Two forms of 25-OH-D occur naturally in human plasma, 25-OH-D_2 and 25-OH-D_3, which show similar biological effects on the mineral and skeletal metabolism of mammals. For the physiological assessment of 25-OH-D it is often necessary to measure 25-OH-D_2 and 25-OH-D_3 separately from one another. Therefore, some reports have appeared on the simultaneous determination of the two metabolites in plasma or serum[1–3]. However, there remains the important problem of an adequate source of authentic 25-OH-D_2 as a standard compound, because its synthesis has not yet been established. In order to solve this, we have purified 25-OH-D_2 generated *in vivo* from plasma of a rabbit which had received a large quantity of vitamin D_2, using successive preparative high-performance liquid chromatography (HPLC) with various columns[4]. The method yielded highly purified standard compound for HPLC assay, but it was rather complicated and time-consuming. Therefore, in this paper we describe a more rapid and convenient method using recycle HPLC, and the identification of the purified compound.

EXPERIMENTAL

Materials and reagents

Crystalline vitamin D_2 (Philips-Duphar, The Netherlands) was recrystallized from acetone–water (4:1), m.p. 115–116°C. Organic solvents of analytical grade were distilled before use.

Animals

Male rabbits weighing 4 kg were fed a normal diet (RC diet containing 200 I.U. of vitamin D_3 per 100 g; Oriental Yeast, Japan). Each was given 500,000 I.U. of vitamin D_2 in 0.1 ml of ethanol, intravenously. In order to obtain large amounts of 25-OH-D_2 generated *in vivo*, each rabbit was left for 48 h until the concentration of plasma 25-OH-D_2 was nearly maximal (Fig. 1). Blood was taken from the carotid artery and plasma was separated.

0021-9673/84/$03.00

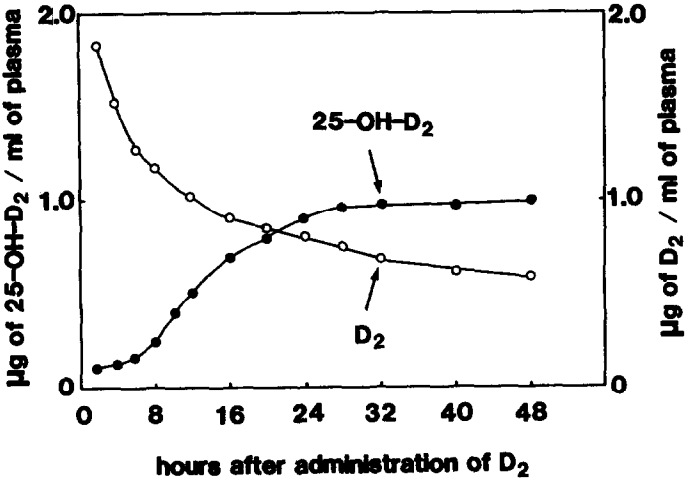

Fig. 1. Time course of rabbit plasma levels of Vitamin D_2 and 25-OH-D_2.

Isolation of the unsaponifiable matter from plasma

Alkaline saponification of rabbit plasma and isolation of the unsaponifiable matter were performed according to a previous method[4].

Equipment

Sample injector connected to a pre-column. A Rheodyne syringe-loading Model 7125 sample injector (Rheodyne., U.S.A.) was employed. A short stainless-steel tube (7.5 mm I.D. × 12 mm) packed with microparticulate Polygosil 60 (particle size 10 µm; Nagel, F.R.G.) was used as a pre-column connected to the sample injector (Fig. 2). This column was used as a clean-up pre-column prior to performance of recycle

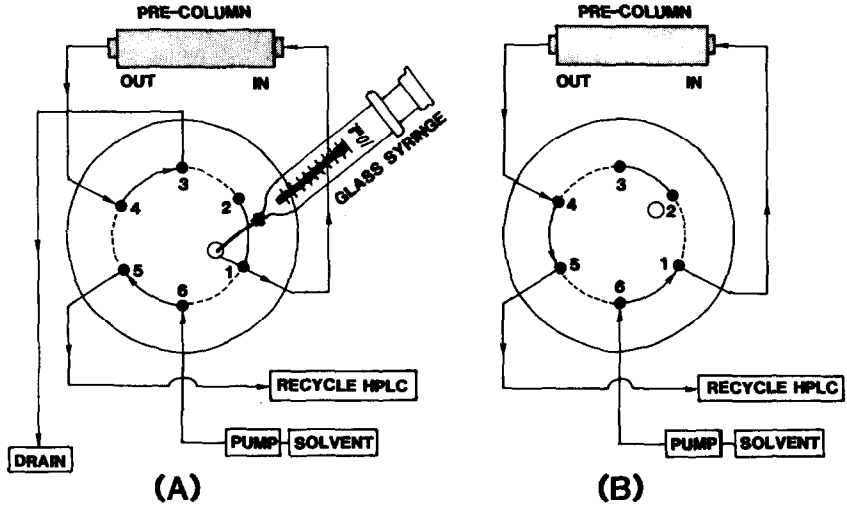

Fig. 2. Diagram of the sample injector and pre-column, showing loading (A) and injection (B) of sample solution.

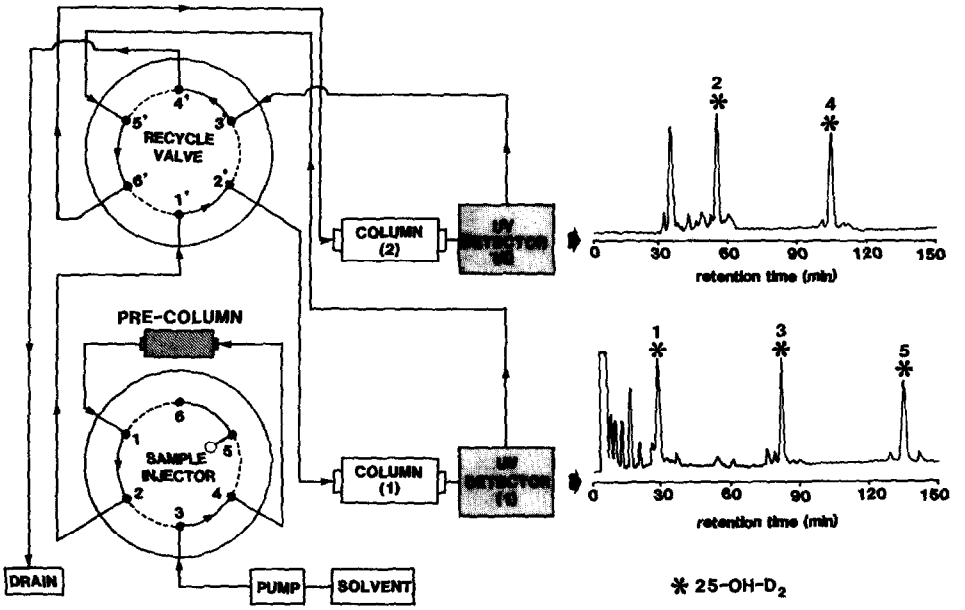

Fig. 3. Flow diagram of the recycle HPLC system and chromatograms of the unsaponifiable matter obtained from rabbit plasma. The figures on both chromatograms indicate the peaks corresponding to 25-OH-D_2 monitored with UV detectors 1 and 2 in series.

HPLC. It was possible to retain 25-OH-D_2 on the column selectively, whereas large amounts of less polar components were effectively eluted.

Recycle preparative HPLC. The recycle HPLC was performed on a Shimadzu-Dupont 841 high-performance liquid chromatograph (Shimadzu Seisakusho, Japan) using two kinds of stainless-steel silica columns in series: 300 mm × 8.0 mm I.D., packed with microparticulate 5 μm Nucleosil 5C (Nagel); 250 mm × 6.2 mm I.D., packed with microparticulate 5 μm Zorbax SIL (DuPont, Wilmington, DE, U.S.A.). These columns were arranged to form a recycle line with two UV detectors (Shimadzu UVD-1 and SPD-1 respectively, both operating at 254 nm, with 0.01 absorbance unit full scale) by use of a rotary six-port valve (as shown in Fig. 3). The solvent mixture of 2.5% isopropanol in *n*-hexane was used as a mobile phase and elution was carried out at a flow-rate of 2.2 ml/min (column pressure 80 kg/cm^2) at room temperature.

Gas chromatography–mass spectrometry (GC–MS). GC–MS was performed on a Hitachi Super Mass M-003 gas chromatograph–mass spectrometer in electron impact mode. A glass column (100 cm × 0.3 cm I.D.) packed with 3% OV-17 Gas-Chrom Q (80–100 mesh) was operated at 290°C with a helium flow-rate of 30 ml/min. The separator temperature, ionizing current and ionizing voltage were 320°C, 60 μA and 25 eV, respectively. When a peak was observed on a chromatogram, the mass spectra and mass chromatograms were recorded.

Ultraviolet (UV) absorption spectrum. A Hitachi 323 automatic spectrophotometer (Hitachi Seisakusho, Japan) was used to record the UV spectrum of the purified 25-OH-D_2.

Procedure

Elimination of components less polar than 25-OH-D_2 by a pre-column. The pre-column was presaturated with 10 ml of the starting solvent (0.4% isopropanol in *n*-hexane). The unsaponifiable matter obtained as above was dissolved in 10 ml of the starting solvent and applied with a glass syringe to the valve of the sample injector set in the load line (Fig. 2A). By this procedure, the whole volume of a sample solution entered into the pre-column from port 4 and the batch eluate came out from port 6 via port 1. Large amounts of less polar components including cholesterol, carotenoids and vitamin D_2 were discarded from the pre-column. On the other hand, 25-OH-D_2 and other materials having similar polarities were effectively trapped in the pre-column. The 25-OH-D_2 thus retained was easily eluted and was subjected to recycle HPLC by changing the valve of the sample injector from the load line to the injection line (Fig. 2B). When the procedure was repeated, the pre-column could be used again.

Recycle HPLC. After setting the valve of the sample injector to the injection line (Figs. 2 and 3), 25-OH-D_2 was eluted from the pre-column by 2.5% isopropanol in *n*-hexane and subjected to recycle HPLC with the same solvent. In the recycle system (Fig. 3), the eluate eluted from the pre-column enters first into column 1 through ports 1′ and 2′. It then passes into column 2 through ports 5′ and 6′ via the UV detector (1) which monitors the elution profile of column 1. The elution profile of column 2 was also monitored with UV detector 2. The eluate from column 1 can be switched to the drain through ports 3′ and 4′. However, if the eluate is to be repeatedly recycled, the recycle valve should be changed from positions 3′ and 4′ to 3′ and 2′. (This change can easily be performed by valve switching.) Then, the eluate from column 2 is repeatedly applied to column 1 and the eluate can be collected at any time from port 4′ by returning the valve to its previous position as shown by the solid line in Fig. 3.

RESULTS AND DISCUSSION

The proposed system was very effective in removing large amounts of com-

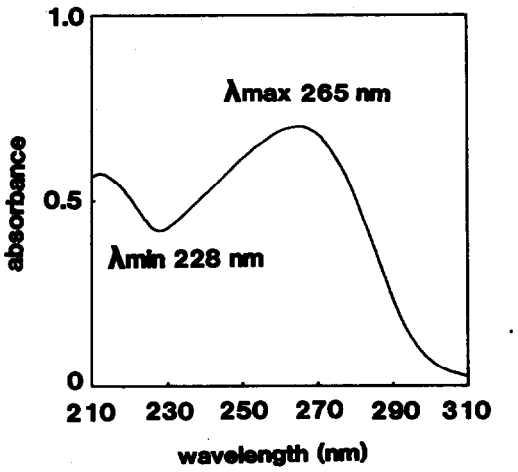

Fig. 4. UV absorption spectrum of purified 25-OH-D_2.

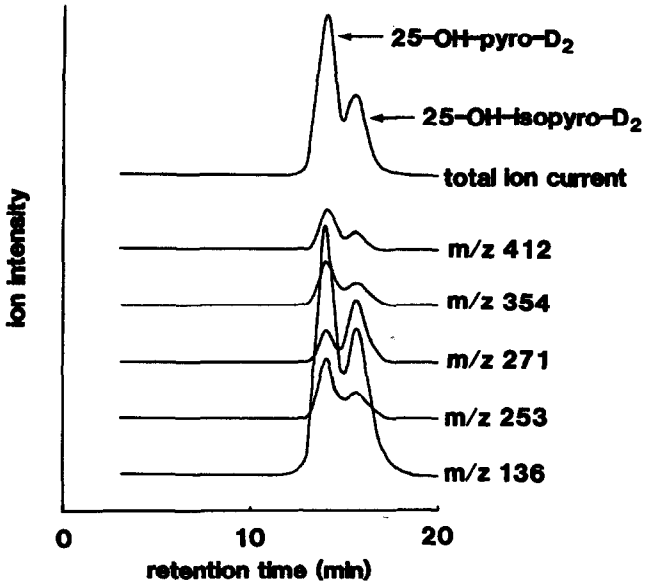

Fig. 5. Mass chromatograms of purified 25-OH-D_2.

ponents less polar than 25-OH-D_2. Recently, the Sep-Pak cartridge (Waters Associates, Milford, MA, U.S.A.) has been widely used as a convenient clean-up tool[5–9]. We have also demonstrated that this cartridge is very useful and time-saving when eliminating less polar concomitants and have used it for the determination of vitamin D and 25-OH-D in various foods, feeds and pharmaceuticals[10]. The pre-column used in the present work is similar to the Sep-Pak silica cartridge in respect of batch elution of 25-OH-D_2 and other less polar components present in rabbit plasma. In our system, however, we wanted to use the pre-column not only for clean-up but also to load a large volume of sample solution directly onto the HPLC column. Use of the

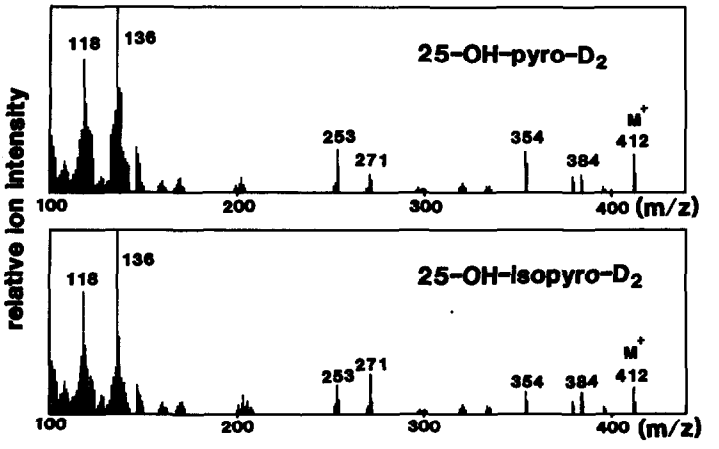

Fig. 6. Mass Spectra of 25-OH-pyro-D_2 and 25-OH-isopyro-D_2 derived from purified 25-OH-D_2.

sample injector connected to the pre-column enabled both aims to be fulfilled. As shown in Fig. 3, a clear single peak corresponding to 25-OH-D_2 was observed on the recycle chromatogram at about 150 min after starting the recycle system.

The UV spectrum of the purified 25-OH-D_2 showed a characteristic maximum at 265 nm and a minimum at 228 nm, derived from the *cis*-triene structure, as shown in Fig. 4. The purified 25-OH-D_2 was also subjected to GC–MS analysis in order to confirm its purity. As shown in Fig. 5, the two peaks corresponding to 25-hydroxypyrovitamin D_2 and 25-hydroxyisopyrovitamin D_2 as the two characteristic thermal isomers of 25-OH-D_2 were observed on the chromatogram, and mass spectra corresponding to the two peaks were completely identical to those of the authentic standard (Fig. 6). From these results, we conclude that the 25-OH-D_2 obtained by our recycle HPLC system was completely purified and could be used as an authentic standard for HPLC assay of 25-OH-D_2. Our method yielded about 30 μg of pure 25-OH-D_2 from 50 ml plasma of a rabbit which had received 500,000 I.U. of vitamin D_2. The yield of 25-OH-D_2 is sufficient for routine HPLC assay. We suggest that the described system will be useful for purification of other lipophilic compounds.

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