SEPARATION OF HYDROXYLATED DERIVATIVES OF VITAMIN D_3 BY HIGH SPEED LIQUID CHROMATOGRAPHY (HSLC)

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1. Introduction

The recent upsurge of interest in the metabolism of vitamin D_3 (cholecalciferol) has relied partly on the use of partition chromatography on Sephadex LH-20 as an analytical tool to separate metabolites [1]. This system is simple but it is relatively slow and resolution is poor.

We present here a method for the separation of hydroxylated derivatives of vitamin D_3 using high speed liquid chromatography. First, resolution is orders of magnitude greater: metabolites unresolved on Sephadex LH-20 are separated. Second, an analysis is complete in under 30 min. Third, fractions may also be collected for further studies, e.g. determination of radioactivity. Fourth, continuous monitoring of the column effluent by UV absorption is possible.

Further, this technique allows co-chromatography of authentic synthetic compounds with experimentally produced radioactive metabolites to facilitate identification. Using this approach we have confirmed previously the identity of the major metabolite of 25-hydroxycholecalciferol (25-HCC) under conditions of calcium deprivation to be 1,25-dihydroxy–cholecalciferol (1,25-DHCC) by its co-elution with a synthetic sample [2].

2. Experimental

2.1. *Bio-synthesis of metabolites of 25-HCC*

The methods for the preparation of chick kidney homogenates, incubation of 25-HCC in these homogenates and the extraction of metabolites from them have been previously described [3].

2.2. *Separation of metabolites on Sephadex LH-20*

The extracts were chromatographed on columns (20 × 1.5 cm) of Sephadex LH-20 (Pharmacia AB, Uppsala, Sweden) using chloroform-hexane (13:7, v/v) collecting the eluate directly into scintillation vials positioned on an LKB 3401B rotating fraction collector (LKB Produkter AB, Stockholm, Sweden). The solvent was removed by evaporation and the radioactivity determined by liquid-scintillation counting.

2.3. *High speed liquid chromatography*

A Du Pont 830 liquid chromatograph fitted with a gradient elution accessory was employed for this work. Separations were carried out in a steel tube (500 mm × 2.1 mm I.D.) packed with octadecyl–silane permanently bonded to glass beads (ODS-Permaphase, Du Pont De Nemours, Wilmington, Delaware, USA) kept at 40°C. Samples, (1–10μl) in methanol, were injected through a silicone septum into the high pressure line to the separation column and eluted by a linear gradient of methanol concentration in water. Flow rates of 1 ml per min were achieved with a pressure drop of 700 p.s.i. over the column. The absorption of the effluent was recorded continuously at 254 nm in an 8 μl volume flowcell. For determination of radioactivity, fractions of the effluent were collected in a microfraction collector (TDC 80, Gilson, Villiers-le-Bel, France). The fractions were then separately washed into scintillation vials with methanol and dried before the determination of radioactivity by liquid-scintillation counting.

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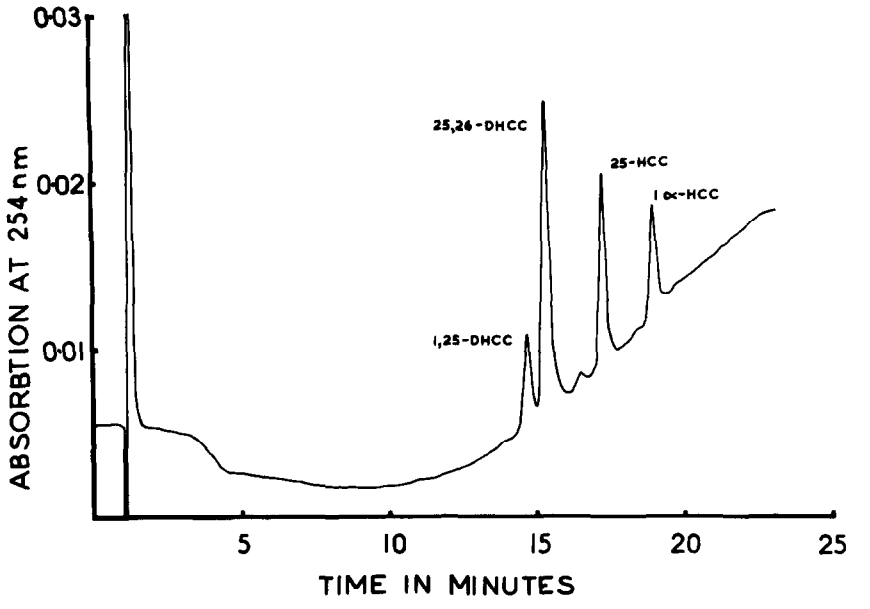

Fig.1. High speed liquid chromatography of a mixture af 1,25-DHCC, 25,26-DHCC, 25-HCC an 1α-HCC on ODS-Permaphase using a linear solvent gradient from water to methanol, increasing the gradient by 5% v/v per min. Compounds were detected by their absorption at 254nm.

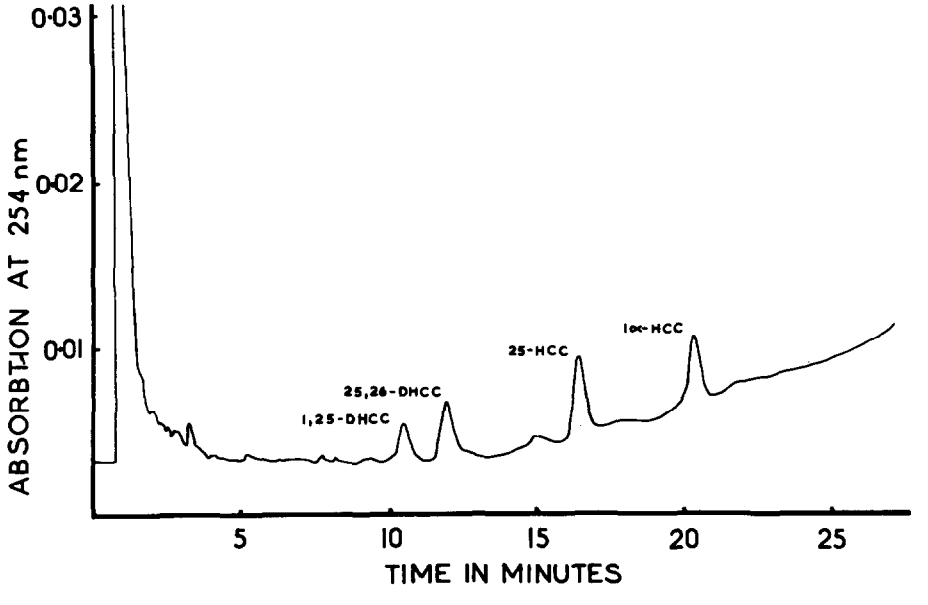

Fig. 2. High speed liquid chromatography on ODS-Permaphase of the same mixture as used in fig. 1 but with a restricted, shallower gradient. The compounds were eluted with a linear solvent gradient from methanol–water, 3:7, v/v to metanol–water, 8:2, v/v and increasing the gradient by 2% v/v per minute. Compounds were detected by their absorption at 254nm.

2.4. *Solvents*

Chloroform and methanol were of Analar grade. Water was glass-distilled. Hexane was the petroleum fraction B.P. 62–68° C (Shell Mex and B.P. Ltd., Hemel Hempstead, Herts., UK).

3. Results and discussion

The chromatogram in fig.1 shows the elution profile of a mixture of 25-HCC, 1,25-DHCC, 25,26-DHCC and 1α-HCC separated on ODS-Permaphase using a linear solvent gradient from water to methanol. The concentration of methanol in water increased at a rate of 5% v/v, per min. The ultraviolet absorption peaks of the compounds are sharp and completely resolved.

Greater separation of the peaks may be achieved by restricting the solvent gradient to the range 30–80% (v/v) methanol in water and using a shallower gradient (increasing by 2%, v/v, per minute), (see fig. 2). The concomitant peak broadening, however, results in a reduction of sensitivity (minimum detectable quantity).

The quantities of compounds used in fig. 1 range from 10 ng (1,25-DHCC) to 50 ng (25-HCC) wich are well within the normally encountered in physiological experiments. This method thus allows the possibility of optical detection of vitamin D_3 metabolites produced in in vitro experiments, in addition to the normally employed radioactive methods.

A comparison of the results obtained when the extract of a chick kidney homogenate incubation using tritiated 25-HCC as substrate is chromatographed both on Sephadex LH-20 and by HSLC is shown in fig. 3. Sephadex LH-20 chromatography separates the extract into two broad radioactive peaks. The same sample on HSCL also gives two radioactive peaks. They are however extremely sharp and well separated.

It is clear from these results that HSLC is a valuable tool in the analysis of vitamin D_3 metabolites. The resolution of this method gives more confidence to the identification and homogeneity of detected peaks. It is known, for example, that 25,26-DHCC coelutes with 1,25-DHCC during partition chromatography on Sephadex LH-20 [1]. Reference to figs. 1 and 2 shows that these two compounds are well separated by HSLC and no confusion could occur. Moreover the use of a gradient elution device gives a flexibility for difficult separations not easily achieved in the partition chromatography on Sephadex LH-20 presently in use.

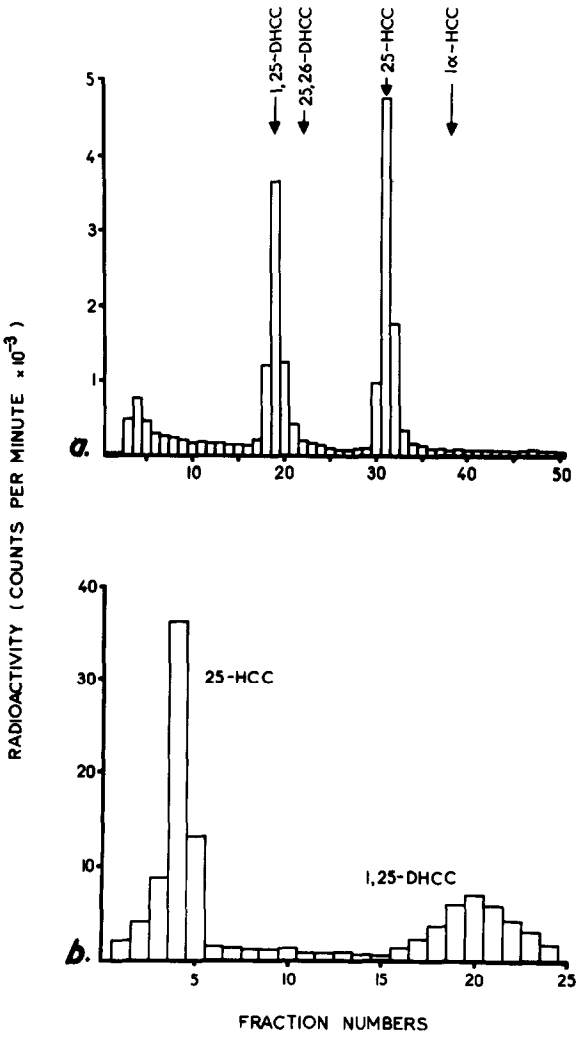

Fig.3. A comparison of the separation of the radioactive products af an incubation of tritiated 25-HCC in a chick kidney homogenate by HSLC (a) and by Sephadex LH-20 (b). The vertical arrows indicate the elution positions of synthetic derivatives of cholecalciferol. The ODS-Permaphase column was eluted with a linear solvent gradient from methanol–water, 3:7, v/v to methanol–water, 8:2, v/v. The Sephadex LH-20 column was eluted by chloroform-hexane, 13:7, v/v. Compounds were detected by collecting fractions and determining their radioactivity.

The high speed of analysis, under 30 min per run, is very attractive and fractions may be easily collected (e.g. for determination of radioactivity). In all instances where a comparison has been possible between the UV peak and the radioactivity profile of a vitamin D_3 derivative the resultant peaks have had the same width and elution position (see for example ref. [2]) The ability to repetitively inject samples into an automatic instrument is a great advantage over the preparation and operation of many columns eluted with volatile organic solvents, the need for lengthy runs and the use of many fraction collectors as is necessary with analyses in Sephadex LH-20.

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