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Note

High-performance liquid chromatography of vitamin D: enhanced ultraviolet absorbance by prior conversion to isotachysterol derivatives

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There has been a steadily increasing use of high-performance liquid chromatography (HPLC) in the assay of vitamin D in biological specimens[1]. HPLC is now routinely used for the purification of vitamin D and its metabolites prior to quantitation by ligand binding assay[2,3]. It has also been applied recently to the simultaneous analysis of a number of metabolites of vitamin D in human plasma[4] by the use of ultraviolet (UV) detection for quantitation. Until relatively recently, UV monitoring of HPLC effluent has been carried out with fixed-wavelength detectors at 254 nm (ref. 3). The use of variable-wavelength detectors slightly increases the sensitivity by selection of UV maxima. This communication describes the simple and rapid formation of isotachysterol derivatives[5] prior to HPLC which enhances the sensitivity by a factor of two.

MATERIALS AND METHODS

Vitamin D_3 (D_3) was obtained from Koch-Light Labs. (Colnbrook, Great Britain); 25-hydroxyvitamin D_3 (25-OH-D_3) was a generous gift from Dr. N. Eve (Roussel Laboratories, Wembley Park, Great Britain) and Dr. J. A. Campbell (Upjohn, Kalamazoo, MI, U.S.A.), and 24,25-dihydroxyvitamin D_3 (24,25-OH-D_3) was from Dr. N. T. Pollitt (Roche Products, Welwyn Garden City, Great Britain). Other reagents used were as specified by Seamark *et al.*[5] and were analytical reagent grade whenever possible. Solvents for HPLC were obtained from Rathburn, Peebleshire, Great Britain. HPLC was carried out with a Model 750/03 pump, a Rheodyne 7125 injection valve, and a Model SF770 variable-wavelength (190–700 nm) detector (Schoeffel Instruments) all supplied by Aplied Chromatography Systems, Luton, Great Britain. A Zorbax-SIL (5 μm, 250 × 0.46 mm, DuPont (U.K.), Hitchin, Great Britain) column was eluted with a solvent system hexane-isopropanol (9:1)[5]. UV spectra were recorded on a SP1700 spectrophotometer (Pye-Unicam, Cambridge, Great Britain).

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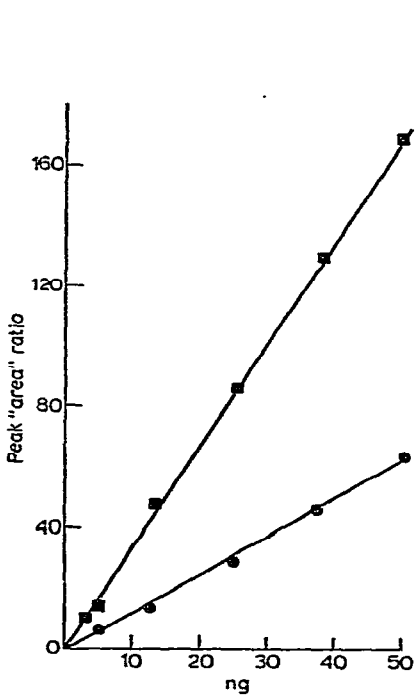
Aliquots of D_3, 25-OH-D_3, and 24,25-OH-D_3, containing amounts of each compound ranging from 5–50 ng, were pipetted into 1-dram vials (4 cm height × 1.3 cm diameter, FBG-Trident, Bristol, Great Britain). Carrier vitamin D_2 (200 ng) was added to tubes containing 25-OH-D_3 and 24,25-OH-D_3. The solvent was removed in a vacuum oven at 37°C and isotachysterol derivatives formed by the method of Seamark *et al.*[5]. Chloroform (75 μl) was added to the *seco*-steroid residue and hydrochloric acid gas was blown into the tube. The tube was stoppered and incubated at 0°C for 5min. The residual solvent was then evaporated under a gentle stream of nitrogen. Equal amounts of the internal standard (500 ng of 1α,25-dihydroxyvitamin D_3) were added to each tube, the contents of which were injected into the HPLC sample valve. The effluent was monitored with the UV detector set at 290 nm.

Aliquots of vitamin D_3, 25-OH-D_3, and 24,25-OH-D_3 were mixed with the same amount of internal standard (500 ng) and subjected, without isomerisation, to HPLC, monitoring the effluent at 264 nm.

Peak "area" ratios (peak "area" of vitamin D/peak "area" of internal standard × amount of internal standard) were calculated. "Area", a quantity proportional to peak area, was calculated by multiplying retention time by peak height[6]. To correct for the differing absorption of the internal standard at the different wavelengths used,



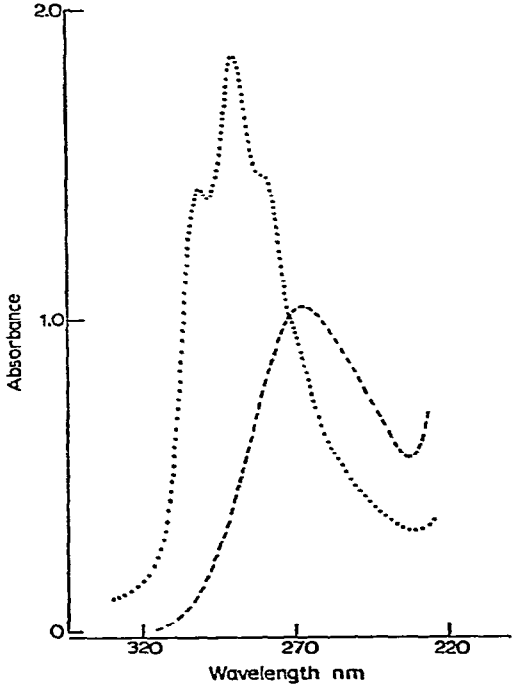


Fig. 1. Relationship between mass and peak "area" ratio before and after isotachysterol formation. D_3, 25-OH-D_3, and 24,25-OH-D_3 measured at 264 nm (●); isotachysterol$_3$, 25-OH-isotachysterol$_3$ and 24,24-OH-isotachysterol$_3$ measured at 290 nm (■). See text for definition of peak "area" ratio.

Fig. 2. The UV absorption spectra of equimolar amounts of D_3 (– – –) and isotachysterol$_3$ (· · ·), measured in hexane–isopropanol (9:1).

a UV spectrum of 1α,25-OH-D_3 in hexane–isopropanol (9:1) was obtained, and a factor relating the UV absorption at 290 nm to that at 264 nm was calculated. Peak "area" ratios were then corrected by using this factor. Standard curves for D_3, 25-OH-D_3, and 24,25-OH-D_3 before isomerisation, monitored at 264 nm, and after isomerisation, monitored at 290 nm, were obtained and are illustrated in Fig. 1.

DISCUSSION

Plasma levels of the majority of the so-far recognised metabolites of D_3 are sufficiently high to enable physico-chemical determination by HPLC with UV detection, using reasonable (3–5 ml) volumes of plasma[3]. The concentrations of 24,25-OH-D_3, and 25,26-OH-D_3 are, however, almost at the minimum detectable limit. Any procedure which can enhance the sensitivity of UV detection is therefore of interest. It is clear from an examination of the UV spectra of equimolar concentrations of D_3 and its isotachysterol$_3$ isomer (see Fig. 2), that the isotachysterol$_3$ has an enhanced absorption at 290 nm in comparison to the absorption of D_3 at its maximum, 264 nm. Most fixed-wavelength detectors monitor at 254 nm, which is not the maximum for any of the vitamin D metabolites examined here. Prior conversion to isotachysterol and use of a variable-wavelength detector enhance the sensitivity of detection by a factor of two.

The isomerisation of vitamin D derivatives, not containing a 1α-hydroxyl, has been carefully evaluated by Seamark *et al.*[5], who showed quantitative conversion down to 100 ng levels. Below this it was necessary to add carrier amounts (200 ng) of vitamin D_2 to all the vitamin D metabolites examined with the exception of D_3 itself which gave quantitative conversion at 10 ng even in the absence of added carrier D_2 (ref. 5). For the vitamin D metabolites examined here, conversion to isotachysterol isomers has not affected the separation in the HPLC system used.

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