CHROM. 3811

Gas–liquid chromatography of ergocalciferol and cholecalciferol in nanogram quantities

In the determination of ergocalciferol and cholecalciferol by the gas–liquid chromatographic method (MURRAY, DAY AND KODICEK[1]) the vitamin is first converted to isotachysterol (KOBAYASHI[2]) and submitted to thin-layer chromatography on silica gel to remove the majority of the cholesterol, and then subjected to gas–liquid chromatography. Isotachysterol, unlike vitamin D, gives only one peak at the temperature of the gas–liquid chromatograph. A means of increasing the sensitivity of this method so as to allow the measurement of physiological levels of the vitamin has been sought. The possibility of using halo-esters and their detection by electron capture only became possible with the recent introduction of ^{63}Ni electron-capture detectors for high-temperature work. Tritium electron-capture detectors were subject to contamination due to condensation, and subsequent loss of sensitivity, when used with these high-boiling compounds at the detectors' maximum operating temperature of 225°. The choice of esters in this present study follows work on steroid hormones by EXLEY AND CHAMBERLAIN[3] and the increased sensitivity to heptafluorobutyrates compared to other halo-esters.

Experimental

Reagents. Heptafluorobutyric anhydride (Koch-Light) was dispensed into small sealed ampules and kept at +4°.

Isotachysterol$_2$ and isotachysterol$_3$ were prepared from ergocalciferol and cholecalciferol, respectively, by the method of MURRAY *et al.*[1].

[1-^{3}H]Cholecalciferol was prepared by Dr. P. BELL, of this laboratory, according to the method of CALLOW, KODICEK AND THOMPSON[4].

Esterification. Heptafluorobutyric anhydride, 0.1 ml, was added to isotachysterol in 1 ml tetrahydrofuran in ice and left for 1 h. The reaction was stopped by the addition of 10 ml of water and the ester extracted with 1 ml petroleum ether 40–60°. The petroleum ether extract was washed once with water, then 2% $NaHCO_3$ and again with water. The petroleum ether extract was diluted to the appropriate concentration for application by Hamilton syringe to the gas–liquid chromatograph.

Gas–liquid chromatography. F. & M. Model 402 gas–liquid chromatograph allowing the alternate use of flame ionisation and ^{63}Ni electron-capture detectors was used. Glass columns, 4 ft. × 3 mm I.D., were packed with 3.8% methyl silicone gum rubber W-98 on Diatoport S 80/100 mesh (Hewlett-Packard Ltd.). The carrier gas was argon for the flame ionisation detector and 5% methane in argon for the electron-capture detector.

Thin-layer chromatography. Samples were run on thin-layer plates of Silica Gel G (E. Merck) with 0.25 mm thickness. Chloroform was used as the developing solvent and the spots were visualised by U.V. absorption at 256 mμ.

Results

The esterification of steroids is usually carried out at 20° or, as in the case of testosterone, at even more elevated temperatures. However, gas–liquid chromato-

TABLE I

RELATIVE RETENTION TIMES FOR CHOLESTEROL, ISOTACHYSTEROL AND THEIR HEPTAFLUOROBUTYRATE ESTERS

Temperature of column 220° and of flash heater 250°. The retention time of 5α-cholestane was 5 min at a flow rate of 60 ml/min.

Compound	*Free alcohol*[a]	*Heptafluorobutyrate ester*[a]		*Heptafluorobutyrate ester*[b]
5α-Cholestane	1.00	1.00	0.58	—
Cholesterol	1.90	1.71	1.00	1.00
Isotachysterol$_2$	2.98	2.04	1.19	1.14
Isotachysterol$_3$	2.71	1.88	1.10	1.11

[a] Detected with the flame ionisation detector at 250°.
[b] Detected with the ^{63}Ni electron-capture detector at 290°.

graphy with flame ionisation detection showed that isotachysterol in the presence of heptafluorobutyric anhydride is broken down at temperatures of 20–70° to give a number of products. When the product formed at 0° was subjected to gas–liquid chromatography using the flame ionisation detector it gave neither the breakdown products seen after esterification at higher temperatures nor a peak of unesterified isotachysterol. Only one main peak was observed indicating that the reaction had proceeded to completion without untoward breakdown of isotachysterol.

The product formed in the presence of heptafluorobutyric anhydride at 0° was shown to be isotachysterol heptafluorobutyrate by the following criteria. The U.V. absorption maxima at 278, 298 and 301 mμ of the product were identical to that of the original isotachysterol indicating that the chromophore was unchanged. The I.R. spectra of the product showed the presence of a strong carbonyl band at 1780 cm^{-1}, while the absence of a band above 3400 cm^{-1} indicated that there was no free hydroxyl group. Also there were other similarities in the position of the bands in the I.R.

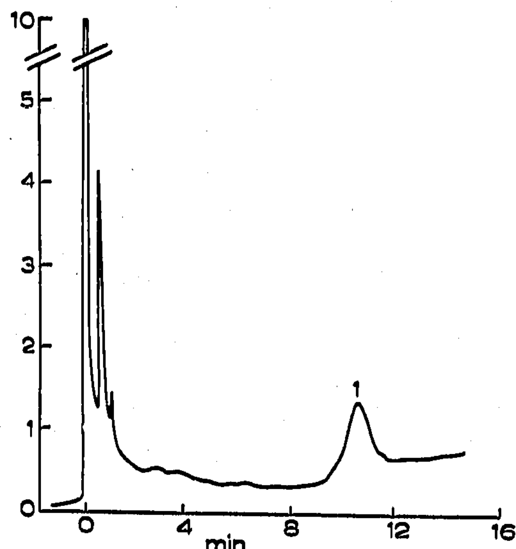

Fig. 1. Gas–liquid chromatogram of 5 ng of isotachysterol$_3$ heptafluorobutyrate (peak 1) with electron-capture detector. Attenuation × 16. Pulse interval 150 μsec.

spectra, of the product and of testosterone heptafluorobutyrate[5]. Thin-layer chromatography of the radioactive product prepared from [1-^{3}H]isotachysterol$_3$ using [1-^{3}H]-cholecalciferol as starting material, gave a single spot of radioactivity at an R_F value of 0.82, which distinguished it from unesterified isotachysterol with an R_F value of 0.47. After saponification at room temperature a substance was recovered which co-chromatographed with isotachysterol.

The average yield of isotachysterol$_3$ heptafluorobutyrate at 0°, using the radioactive dilution technique with [1-^{3}H]isotachysterol$_3$ prepared from [1-^{3}H]cholecalciferol, was 90% (range 86–94%).

The behaviour of the esters of isotachysterol$_2$, isotachysterol$_3$ and cholesterol on gas–liquid chromatography are shown in Table I. The effect of the ester group on the retention time of cholesterol was small, being of the same order as that found by EXLEY AND CHAMBERLAIN[3] for steroid hormones, whereas the esters of isotachysterol$_2$ and isotachysterol$_3$ showed a more noticeable shift. The retention times are given relative to either 5α-cholestane in those chromatographs in which the flame ionisation detector was used or relative to cholesterol heptafluorobutyrate for the electron-capture detector.

Fig. 1 shows the gas–liquid chromatogram using electron-capture detection of 5 ng of isotachysterol$_3$ heptafluorobutyrate. The increased sensitivity towards the heptafluorobutyrate esters allows thus the estimation of at least 10 ng (0.4 I.U.) of ergocalciferol and cholecalciferol, respectively. The level of detection is about 20 times better than that with a flame ionisation detector for isotachysterol and permits the determination of vitamin D, so far only in pure form, in quantities which occur in natural sources.

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