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THE GAS CHROMATOGRAPHY OF CALCIFEROL, DIHYDROTACHYSTEROL AND CHOLESTEROL

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SUMMARY

The gas chromatography of ergocalciferol, cholecalciferol, dihydrotachysterol$_2$, dihydrotachysterol$_3$ and cholesterol as the free sterols, the acetates and the trimethylsilyl ethers has been studied using SE-30 and OV-17 stationary phases. A separation of the trimethylsilyl ethers of pyrocholecalciferol, pyroergocalciferol and cholesterol was achieved on the OV-17 stationary phase. Using OV-17 also, a separation of the trimethylsilyl ethers of dihydrotachysterol$_2$ and dihydrotachysterol$_3$ was obtained: cholesterol trimethylsilyl ether was not separated from dihydrotachysterol$_2$ trimethylsilyl ether. It is feasible that gas chromatography can be used as the last step in separate methods to estimate either calciferol or dihydrotachysterol in serum.

INTRODUCTION

The aim of the work in this paper was to define gas chromatographic systems which could be used as the final stages in separate methods to estimate either ergocalciferol or dihydrotachysterol$_2$ in the sera of hypoparathyroid patients. These patients take, daily, between 0.25 mg and 5 mg of these substances to prevent hypocalcaemia and tetany.

Ziffer *et al.*[1] showed that two peaks were eluted from a gas chromatograph following the injection of pure calciferol. The first peak (approximately 70% of the total response) was identified as pyrocalciferol and the second peak was identified as isopyrocalciferol. When ergocalciferol and cholecalciferol were chromatographed on a column packing with SE-30 as the stationary phase, the resulting four peaks could not be completely resolved.

Other workers have converted calciferol into an isomeric form before gas chromatography, the advantage being that only a single peak was eluted from the gas chromatograph. Murray *et al.*[2] converted ergocalciferol and cholecalciferol to the corresponding isovitamins D and achieved a separation of the two forms on a column packing with a silicone oil stationary phase. Panalaks[3] also converted the calciferols

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to the isovitamins D and achieved a separation of the ergo- and chole-analogs on an OV-1 stationary phase. Nair and DeLeon[4] converted the ergo- and chole-analogs of calciferol trifluoroacetate into the corresponding 5,6-trans isomers and achieved a separation on an SE-52+XE-60 combined stationary phase. Sheppard *et al.*[5] converted the calciferols to the isotachysterol isomers and achieved separation of the ergo- and chole-analogs on a JXR column.

The production of two peaks for calciferol means that only about 70% of the total response can be measured in the larger peak. Also, when ergo- and cholecalciferol are chromatographed together, there is a problem of overlapping peaks. However, it seems to be agreed that. at constant injection temperature, the proportion of pyrocalciferol to isopyrocalciferol is constant (Vessman and Ahlén[6], Nair *et al.*[7]). On the other hand, when calciferol is converted to its isomers, the yield may be variable and less than quantitative. Also, when extracting calciferol from animal tissues, or serum, cholesterol is inevitably extracted with calciferol and the amount of cholesterol is relatively large compared to the amount of calciferol. Even if chromatographic separations (adsorption or partition) are used to separate cholesterol from calciferol before gas chromatography, it is possible that a small amount of cholesterol remains in the calciferol fraction. These are some of the points to be taken into account when exploring gas chromatographic systems to measure ergocalciferol and/or cholecalciferol extracted from biological systems.

The present study was to define gas chromatographic systems which gave useful separations for ergocalciferol, cholecalciferol and cholesterol without isomerisation of the calciferols before gas chromatography. The free sterols, the acetates and the trimethylsilyl ethers were studied.

The gas chromatography of dihydrotachysterol$_2$ gives a single peak (Nair *et al.*[7], Avioli and Sook Won Lee[8], Sheppard *et al.*[5]), but the simultaneous gas chromatography of the ergo- and chole-analogs has not been studied so far. In this work, the gas chromatography of dihydrotachysterol$_2$, dihydrotachysterol$_3$ and cholesterol was studied; the free sterols, the acetates and the trimethylsilyl ethers were used.

MATERIALS AND METHODS

All solvents were redistilled and dried over anhydrous sodium sulphate. Acetate derivatives were formed by the reaction of acetic anhydride in a mixture of dry tetrahydrofuran and dry pyridine, and were recovered from the reaction mixture in the usual way. The acetates were recrystallised from methanol or from a methanol:acetate = 1:1 mixture. TMSi-ether* derivatives were prepared by the action of BSA in hexane. Up to 1.0 mg of sterol was dissolved in 1 ml of hexane and 0.2 ml of BSA was added. The mixture was heated in a stoppered tube at 50° for 45 min and then evaporated to dryness, at 50°, by a stream of nitrogen. The residue was dissolved in a measured volume (usually 1 ml) of hexane:HMDS = 98:2 mixture.

A Beckman GC-4 analytical gas chromatograph was used; it was fitted with dual flame ionisation detectors for use with dual columns. Two column packings were

* The abbreviations used in this paper are: TMSi-ether = trimethylsilyl ether; OAc = acetate; OH = free sterol; HMDS = hexamethyldisilazane; BSA = bis(trimethylsilyl)acetamide; D_2 = ergocalciferol; D_3 = cholecalciferol; DHT_2 = dihydrotachysterol$_2$; DHT_3 = dihydrotachysterol$_3$; f.s.d. = full scale deflection.

used: 2.5% SE-30 on 100–120 mesh Chromosorb G (Perkin Elmer Co. Ltd.) and 1% OV-17 on 100–120 mesh silanised Gas Chrom Q. The latter column packing was made by the filtration technique (Horning *et al.*[9]). Before making the packing, it was found necessary to reflux the support for 3 days in 10% HMDS in 40–60° petroleum ether over nitrogen in order to reduce the adsorption of sample components on the column packing during gas chromatography. The two types of columns used were specified as (i) 5 ft. × 3 mm i.d. 2.5% SE-30 on 100–120 mesh Chromosorb G and (ii) 10 ft. × 2 mm i.d. 1% OV-17 on 100–120 mesh silanised Gas Chrom Q.

D_2, D_3, cholesterol, DHT_2 and DHT_3 were chromatographed as the free sterols, the acetates, and the TMSi-ethers at temperatures between 210 and 230° using nitrogen flow-rates between 60 and 80 ml/min. The samples were introduced into the gas chromatograph either by on-column injection or by the use of a solid sampler (Evans[10]).

RESULTS

The per cent of pyro-compound formed from the injection of calciferol was found to be the same for the ergo- and chole-forms of calciferol when the injection temperature was maintained between 240 and 250°; this was also true for the TMSi-ethers (Table I).

TABLE I

PROPORTION OF PYRO- AND ISOPYRO-CALCIFEROL PRODUCED BY INJECTION AT AN INLET TEMPERATURE OF 240°–250° (% ± S.D.), MEASURED BY TRIANGULATION AND WEIGHING
$n = 6$ in each case.

Parent compound	*Per cent of pyro-compound formed*
D_2–OH	75.7 ± 3.1
D_3–OH	74.8 ± 2.4
D_2–TMSi-ether	73.5 ± 2.4
D_3–TMSi-ether	74.4 ± 2.8

For all the compounds chromatographed, the OV-17 column could be run at a lower temperature than the SE-30 column to give the same retention times (at the same flow-rates), even though the OV-17 column was twice as long as the SE-30 column. The OV-17 column gave the most useful separation when D_2–TMSi-ether, D_3-TMSi-ether and cholesterol–TMSi-ether were chromatographed; this column separated pyrocholecalciferol–TMSi-ether, pyroergocalciferol–TMSi-ether and cholesterol–TMSi-ether (Fig. 1). This could not be achieved as the free sterols, or the acetates, on either column. The OV-17 column also gave the most useful separation for the dihydrotachysterols; this column separated the two forms as the TMSi-ethers. However, cholesterol–TMSi-ether could not be completely separated from DHT_2–TMSi-ether (Fig. 2). Retention data for the OV-17 column are given in Tables II and III. Retention data for the SE-30 column are given in Table IV. The response of the detector to pyroergocalciferol–TMSi-ether and pyrocholecalciferol–TMSi-ether (measured as peak heights) for known amounts of D_2 and D_3, respectively, is shown in Fig. 3. A known amount of recrystallised cholesterol–OAc was added as a standard measure of detector response when unknown amounts of the calciferols were chromatographed. The response graph for the dihydrotachysterols (Fig. 4) was obtained in a similar way.

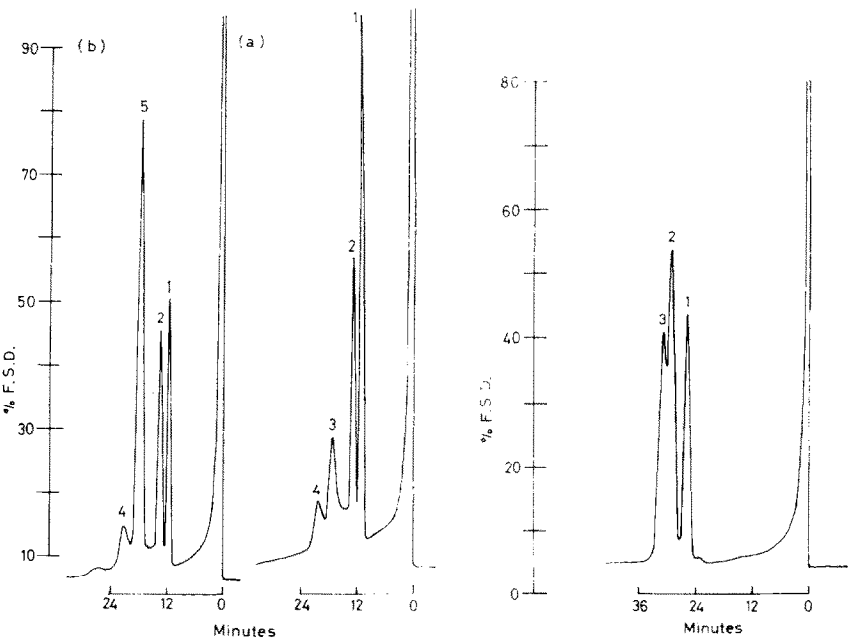

Fig. 1. Gas chromatography of D_2–TMSi-ether, D_3–TMSi-ether and cholesterol–TMSi-ether. Column: 10′ × 2 mm i.d. 1% OV-17 on silanised Gas Chrom Q. Carrier gas: nitrogen at 80 ml/min. Temperatures: inlet 250°, column 215°, detector line 260°, detector 290°. Attenuation: 2×10^2 (10^{-10} amps f.s.d.). Sampling: on-column injection. (a) D_3–TMSi-ether (0.5 μg) + D_2–TMSi-ether; (b) as (a) but with cholesterol–TMSi-ether added.
1 = pyro-D_3–TMSi-ether; 2 = pyro-D_2–TMSi-ether; 3 = isopyro-D_3-TMSi-ether; 4 = isopyro-D_2–TMSi-ether; 5 = 4-cholesterol–TMSi-ether.

Fig. 2. Gas chromatography of DHT_2–TMSi-ether, DHT_3–TMSi-ether and cholesterol–TMSi-ether. Column: 10′ × 2 mm i.d. 1% OV-17 on silanised Gas Chrom Q. Carrier gas: nitrogen at 60 ml/min. Temperatures: inlet 240°, column 203°, detector line 260°, detector 290°. Attenuation: 2×10^2 (10^{-10} amps f.s.d.). Sampling: on-column injection. 1 = DHT_3–TMSi-ether (0.3 μg); 2 = DHT_2–TMSi-ether; 3 = cholesterol–TMSi-ether.

Response graphs were obtained at various attenuation settings of the amplifier; all settings gave straight line graphs. In Figs. 5 and 6 are shown one example each of the chromatograms used to obtain the response graph.

DISCUSSION

In this study it has been shown that it is not necessary to convert vitamin D to an isomer prior to gas chromatography to obtain a useful separation. Pyrocholecalciferol–TMSi-ether, pyroergocalciferol–TMSi-ether and cholesterol–TMSi-ether can be separated and the peak heights of the pyrocholecalciferol–TMSi-ether and pyroergocalciferol–TMSi-ether can be used to measure the amount of the corresponding calciferol. It has been shown, also, that the ratio of pyrocalciferol–TMSi-ether to isopyrocalciferol–TMSi-ether is constant between the injection temperature range of 240–250° for both forms of calciferol; also, the value of the ratio is the same for both forms of calciferol. These results provide a basis for a method to estimate the calciferols, the method being completed by a method of extraction and purification and a method to

TABLE II

RETENTION TIMES RELATIVE TO PYRO-D_3–TMSi-ETHER ON A COLUMN OF 10′ × 2 mm i.d. 1% OV-17 ON 100–120 MESH SILANISED GAS CHROM O

Pyro-D_3–TMSi-ether = 14.5 min at 210° and 60 ml/min of N_2.
Pyro-D_3–TMSi-ether = 11.0 min at 215° and 80 ml/min of N_2.
Cholestane = 7.0 min at 215° and 80 ml/min of N_2.

Compound	*Retention time*
pyro-D_3–TMSi-ether	1.00
pyro-D_2–TMSi-ether	1.17
isopyro-D_3–TMSi-ether	1.55
isopyro-D_2–TMSi-ether	1.85
cholesterol–TMSi-ether	1.56
pyro-D_3–OAc	2.10
pyro-D_2–OAc	2.47
isopyro-D_3–OAc	2.76
isopyro-D_2–OAc	3.46
cholesterol–OAc	2.84
pyro-D_3–OH	1.69
pyro-D_2–OH	1.97
isopyro-D_3–OH	1.98
isopyro-D_2–OH	2.43
cholesterol–OH	3.10

TABLE III

RETENTION TIMES RELATIVE TO DHT_3–TMSi-ETHER AND DHT_3–OAc ON A COLUMN OF 10′ × 2 mm i.d. 1% OV-17 ON 100–120 MESH SILANISED GAS CHROM Q.

DHT_3–TMSi-ether = 17.6 min at 210° and 60 ml/min of N_2.
DHT_3–TMSi-ether = 25.7 min at 203° and 60 ml/min of N_2.
DHT_3–OAc = 23.8 min at 225° and 60 ml/min of N_2.

DHT_3–TMSi-ether	= 1.00	DHT_3–OAc	= 1.00
DHT_2–TMSi-ether	= 1.14	DHT_2–OAc	= 1.11
cholesterol–TMSi-ether	= 1.19	cholesterol–OAc	= 1.19

TABLE IV

RETENTION TIMES RELATIVE TO PYRO-D_3–TMSi ETHER ON A COLUMN OF 5′ × 3 mm i.d. 2.5% SE-30 ON 100–120 MESH CHROMOSORB G.

Pyro-D_3–TMSi-ether = 14.5 min at 230° and 80 ml/min of N_2.
Cholestane = 9.5 min at 230° and 80 ml/min of N_2.

Pyro-D_3–TMSi-ether	= 1.00
Pyro-D_2–TMSi-ether	= 1.12
Isopyro-D_3–TMSi-ether	= 1.46
Isopyro-D_2–TMSi-ether	= 1.62
Cholesterol–TMSi-ether	= 1.54

estimate losses during these processes. A complete method of estimation will be given in a subsequent paper, but two possible ways to estimate losses during extraction and purification are discussed here. The first is to add a known amount of radioactively-labelled calciferol to the sample before extraction begins and then to count a portion of the extract prior to gas chromatography. If both forms of calciferol are to be estimated, then different isotopes should be used for each form (*e.g.* [^{14}C]ergocalciferol and [^{3}H]cholecalciferol), unless it is shown that the same losses of the two forms occur irrespective of the relative proportions of each. Since the gas chromatography systems

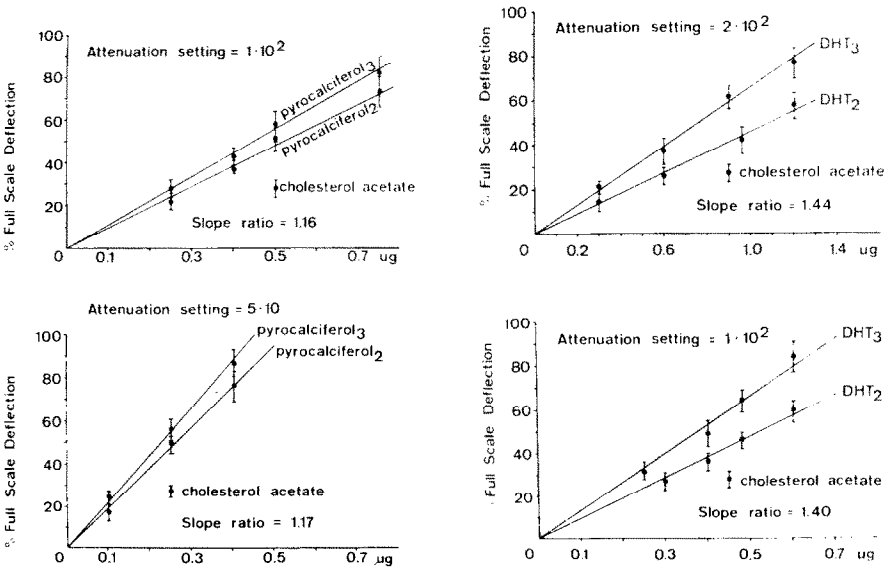

Fig. 3. Response graphs for pyro-D_3–TMSi-ether (marked pyrocalciferol$_3$) and pyro-D_2–TMSi-ether (marked pyrocalciferol$_2$). The peak height (% f.s.d.) is plotted against the weight of D_3 and D_2 (as the free hydroxyl compounds). Cholesterol–OAc was added as a standard of detector response. Column: 10′×2 mm i.d. 1% OV-17 on. 100–120 mesh silanised Gas Chrom Q. Carrier gas: nitrogen at 60 ml/min. Temperatures: inlet 240°, column 210°, detector line 260°, detector 290°. Attenuation: as shown. Sampling: solid sampling.

Fig. 4. Response graphs for DHT_3–TMSi-ether (marked DHT_3) and DHT_2–TMSi-ether (marked DHT_2). The peak height (as % f.s.d.) is plotted against the weight of DHT_3 and DHT_2 (as the free sterols). The conditions for gas chromatography are the same as given for Fig. 3.

would give the most accurate results when the weights of ergocalciferol and cholecalciferol were approximately equal, in practice it is probable that only one form of labelled calciferol need be used. The second possibility is to use the "internal standard" method as described by Murray *et al.*[2]. It should be noted that the concept of internal standard, as used by Murray *et al.*[2], is different from the usual concept of internal standard used in quantitative gas chromatography. The usual concept of internal standard is discussed by Horning *et al.*[11]. In the method of Murray *et al.*[2], a known amount of cholecalciferol was added to the sample when ergocalciferol was to be measured (and *vice versa*). After extraction, purification and conversion to the isovitamins D, gas chromatography separated the ergo- and chole-forms of the isovitamins D. The unknown weight was calculated from the peak areas by simple proportion. The method assumes that the ratio of the two forms (ergo- and chole-) during gas chromatography is the same as the ratio in the original sample. This is true if there is no separation of the two forms prior to gas chromatography and all processes give the same yield for both forms. These conditions seem to have been met in the method devised by Murray *et al.*[2] when, after addition of the known weight of one of the forms of calciferol, the weights of ergocalciferol and cholecalciferol in the original sample were approximately equal. The same principle can be employed in a method which uses the gas chromatographic systems described in this paper, since the proportion of pyrocompound formed on cyclisation is the same for both forms of calciferol. It is also important that Hanewald *et al.*[12] have shown that the isomerisation rates of cholecalci-

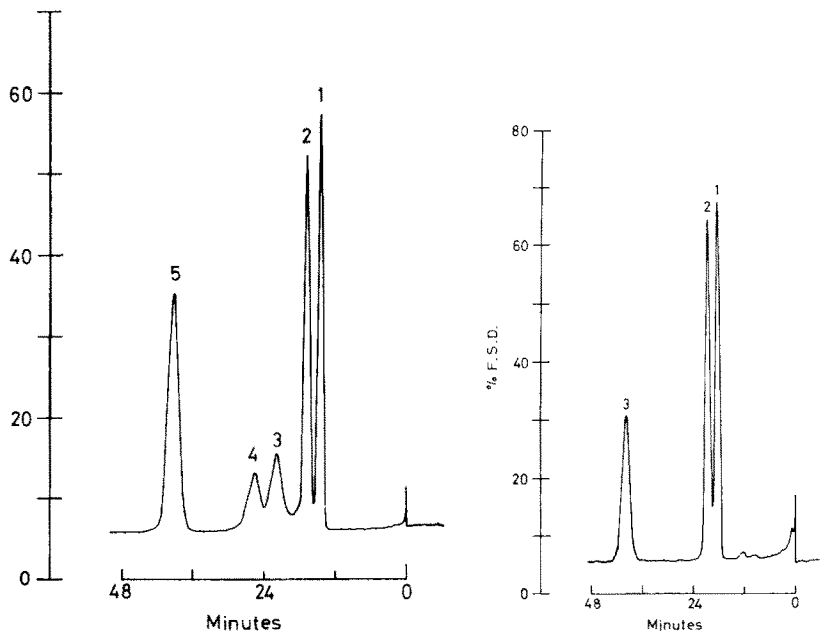

Fig. 5. Gas chromatography of D_3–TMSi-ether, D_2–TMSi-ether and cholesterol–OAc. The conditions for gas chromatography are as given in Fig. 3. Attenuation: 5×10 (2.5×10^{-11} amps f.s.d.). 1 = pyro-D_3–TMSi-ether (0.25 μg D_3); 2 = pyro-D_2–TMSi-ether (0.25 μg D_2); 3 = isopyro-D_3–TMSi-ether; 4 = isopyro-D_2–TMSi-ether; 5 = cholesterol–OAc (0.25 μg).

Fig. 6. Gas chromatography of DHT_3–TMSi-ether, DHT_2–TMSi-ether and cholesterol–OAc. The conditions for gas chromatography are as given for Fig. 3. Attenuation: 1×10^2 (5×10^{-11} amps f.s.d.). 1 = DHT_3–TMSi-ether (0.5 μg); 2 = DHT_2–TMSi-ether (0.5 μg); 3 = cholesterol–OAc (0.45 μg).

ferol to precholecalciferol and of ergocalciferol to preergocalciferol are the same, since some loss of calciferol can take place by isomerisation followed by adsorption chromatography. Thus, the "internal standardisation" method of Murray *et al.*[2] can be applied to this system, with the proviso that the weights of ergocalciferol and cholecalciferol in the original sample are approximately equal.

For the gas chromatography of the dihydrotachysterols, the OV-17 column also gave shorter retention times and greater resolution than the SE-30 column. The ergo- and chole-forms of dihydrotachysterol–TMSi-ether could be almost completely separated by the OV-17 column, but cholesterol–TMSi-ether was not well separated from DHT_2–TMSi-ether. The results suggest that a method for the estimation of DHT_2, based on this gas chromatography system, can be devised only if very small amounts of cholesterol–TMSi-ether remain in the sample prepared for gas chromatography and contribute a negligible proportion to the measurement of the peak height of the DHT_3–TMSi-ether. A method of extraction and purification which satisfies this condition will be given in a subsequent paper. Since radioactively labelled dihydrotachysterol is not commercially available, the "internal standardisation" method of Murray *et al.*[2] has to be used to correct for losses during extraction and purification. This is not a serious limitation since only DHT_2 is used in the treatment of hypoparathyroidism and, hence, DHT_3 can be used as the "internal standard".

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