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GAS–LIQUID CHROMATOGRAPHY OF VITAMIN D AS TRIMETHYLSILYL DERIVATIVES

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SUMMARY

Investigation of several column packing materials for the gas–liquid chromatographic analysis of vitamin D as the trimethylsilyl (TMS) derivatives showed that 4% OV-225 on High Performance Chromosorb W, 100–120 mesh, was the most suitable. The TMS derivatives were found to be unstable, but adequate reproducibility was achieved, using cholestane as the internal standard, by standardizing the time between silylation and injection.

INTRODUCTION

The routine measurement of vitamin D* in extracts of serum requires a gas–liquid chromatography (GLC) column which provides good separation of the vitamin from cholesterol and cholestane in as short a time as possible. Several methods have been published[1–8] for the GLC determination of vitamin D but we found none of these to be satisfactory. We have now investigated several column packing materials for separating the trimethylsilyl (TMS) derivatives of vitamin D, cholesterol, cholestane and dihydrotachysterol (DHT). In early experiments, with standards containing known amounts of vitamin D and cholestane, reproducibility of quadruplicate analyses was very poor. We therefore followed the silylation reaction by injecting aliquots of the reaction mixture over a 2-h period to ascertain the optimum time between reaction and injection.

EXPERIMENTAL

Materials

The instrument used was a Varian Aerograph 2100 with a hydrogen flame ionisation detector (FID) linked to a Varian Model 20 recorder. Peak areas were calculated using a Disc integrator, applying the trapezoid correction formula suggested by Disc Instruments Inc.[9] for baseline drift.

* Vitamin D refers here to both cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2).

All columns and packing materials were obtained from Varian Pty. Ltd. Pure crystalline DHT was obtained from Mann Research Labs. Inc., New York, U.S.A., and vitamins D_2 and D_3 from Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A. Cholesterol and α-cholestane were obtained from Ikapharm, Israel. All silylation reagents were manufactured by Pierce Chemical Co., Ill., U.S.A.

Preparation of columns

The columns used were of Pyrex glass, 2 mm I.D. To silanize them before packing, the columns were washed in concentrated hydrochloric acid, distilled water, methanol and finally chloroform. They were then soaked in a 10–20% solution of bistrimethylsilyl acetamide (BSA) in chloroform for at least 1 h, and oven dried (200–300°).

Silyl 8 was used to condition new columns after packing and to maintain their performance while in use.

Preparation of TMS derivatives

Samples were converted to their TMS derivatives by using the method of SWEELEY *et al.*[10], adapted for microgram quantities. A sample containing 80 μg of vitamin D and 40 μg of cholestane was dissolved in 50 μl of pyridine (silylation grade) in a small glass vial. Hexamethyldisilazane (HMDS) (4 μl) and trimethylchlorosilane (TMCS) (2 μl) were then added, and the sample was mixed for 30 sec with a Vortex mixer. This preparation was stored at 4° in the dark.

A new silylation reagent, N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) recently became available, providing a simpler method of silylation and giving a much smaller solvent tail[11]. Using this reagent, the sample was dissolved in pyridine (1 vol.) and MSTFA was added (0.1 vol.) to give a final vitamin concentration of 2–5 mg/ml. The sample was then mixed and stored as before.

General procedure

The air flow-rate was maintained at 300 ml/min at all times. The optimum hydrogen flow-rate for maximum FID response of each column was determined[12]. The carrier gas and air flow-rates were set, the flame was lit and the flow-rate of hydrogen was increased until the deflection of the recorder pen was over 100%. The hydrogen flow-rate was then reduced slowly and steadily with the hydrogen flow controller on the instrument until a maximum deflection was obtained (Fig. 1). The number of turns of the valve required to give this deflection was noted. This setting was used as the hydrogen flow-rate for that particular column.

A number of different column packings were tested. Cholesterol or cholestane was used as an internal standard for area measurement.

When they were chromatographed as their TMS derivatives, vitamins D_2 and D_3 split into two peaks, pyro- and isopyrovitamin D. Previous workers[1,3] have shown that conversion to these derivatives is complete, and that their ratio is constant for different amounts of vitamin. DHT gives only one peak when it is chromatographed as its TMS derivative. For quantitative measurement we used only the pyro-peak, which was the larger of the two under the conditions used and was eluted first.

Once a suitable column had been chosen for the analysis of vitamin D, quadruplicate standards were chromatographed to obtain a relationship between the peak

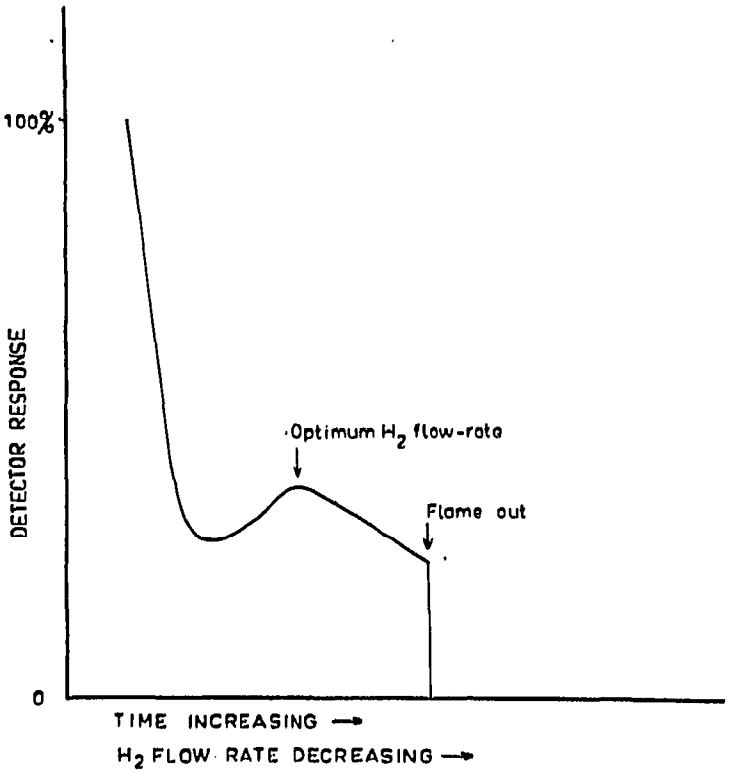

Fig. 1. Determination of optimum hydrogen flow-rate for maximum FID response.

area ratio and the weight ratio of the components. The area ratios for identical samples differed markedly. To ascertain the reason for this, several injections were made of the one sample. The first injection into the gas chromatograph, 0.5 μl, was made 5 min after preparation of the sample. Thereafter, 0.5-μl aliquots from the

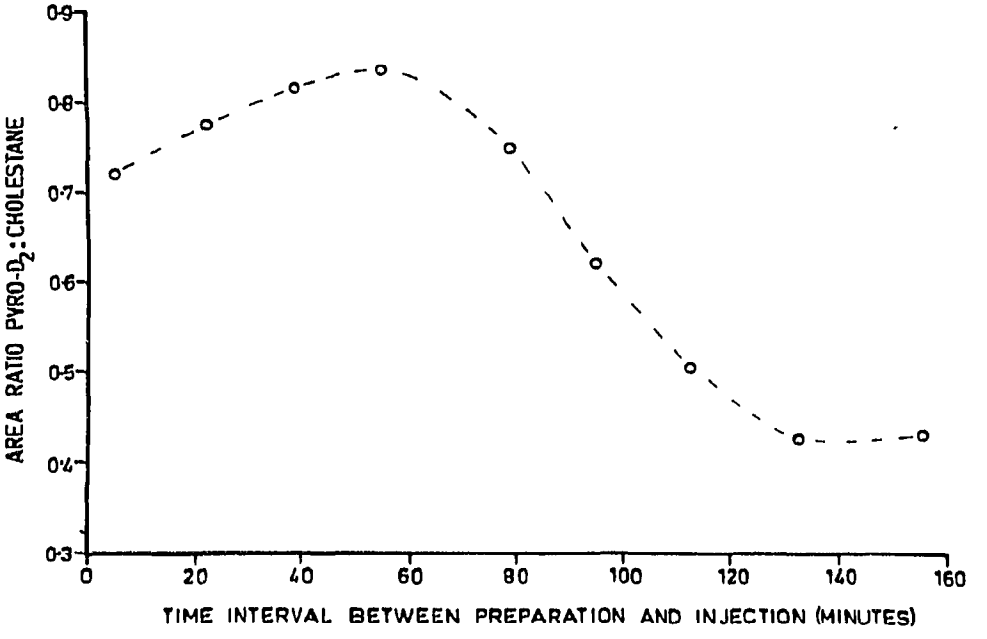

Fig. 2. Variation of peak area ratio with time. Peaks measured represent the TMS derivatives of pyrovitamin D_3 and cholestane in a 2.09:1 w/w mixture of vitamin D_3 and cholestane. Gas chromatograph conditions are described in the text.

same sample were injected at approximately 20-min intervals. The areas of the pyrovitamin D and cholestane peaks were measured, corrected for baseline drift and expressed as a ratio for each chromatogram. A graph was plotted (Fig. 2) of area ratio against time.

TABLE I

RELATIVE RETENTION TIMES OF THE TMS DERIVATIVES OF VITAMIN D[a]

Stationary phase	*Relative retention times*					
	Vitamin D_3		*Vitamin D_2*		*DHT*	*Cholesterol*
	Pyro	*Isopyro*	*Pyro*	*Isopyro*		
3% BDS[b]	0.58	1.12	0.67	1.31	0.84	1.00
3% SE-52[b]	0.64	1.00	0.72	1.11	0.84	1.00
4% OV-225[c] (183-cm column)	1.39	2.17	1.56	2.47	1.66	2.13
4% OV-225[c] (243-cm column)	1.41	2.25	1.62	2.62	–	2.11

[a] Conditions for each column are described in the text.
[b] Retention time relative to cholesterol.
[c] Retention time relative to cholestane.

RESULTS

Column packing materials

The following packing materials and operating conditions were used.

(A) 3% of butanediol succinate (BDS) on Aeropak 30, 100–120 mesh. Column, 152 cm × 2 mm I.D. Column temperature 190°; injector and detector temperatures 220°. Carrier gas flow-rate 60 ml/min.

Fairly good separation was obtained by using this column (Table I). However, the retention time of cholesterol was almost 1½ h, so the analysis was very slow and the peaks were poorly shaped.

(B) 3% of SE-30 on Aeropak 30, 100–120 mesh. Column, 152 cm × 2 mm I.D. Column temperature 220°; injector and detector temperatures 250°. Carrier gas flow-rate 55 ml/min.

This column gave shorter retention times than that packed with BDS, but the separation of cholesterol and cholestane from the vitamins was unsatisfactory for quantitative work.

(C) 3% of SE-52 on Aeropak 30, 100–120 mesh. Column, 152 cm × 2 mm I.D. Column temperature 220°; injector and detector temperatures 300°. Carrier gas flow-rate 85 ml/min.

The retention times on this column were rather high (cholesterol 60 min), but the separation of cholesterol from the vitamins was excellent, and the peaks were of good shape. The peak for cholestane was on the solvent tail so this could not be used as an internal standard. However, the column was highly successful in the analysis of pharmaceutical samples[13], where cholesterol was used as an internal standard (Table I).

(D) SE-52/XE-60 on Chromosorb W, acid-washed, DMCS-treated, 80–100 mesh. Column, 183 cm × 2 mm I.D. Column temperature 215°; injector temperature 250°; and detector temperature 280°. Carrier gas flow-rate 50 ml/min.

This column was recommended by NAIR *et al.*[3] but we found it to be no improvement over the SE-52 column.

(E) 4% of OV-225 on High Performance Chromosorb W, acid-washed, DMCS-treated, 100–120 mesh. Column, 183 cm × 2 mm I.D. Column temperature 230°; injector and detector temperatures 300°.

OV-225 was recommended by Varian Pty. Ltd.[14], who used 1.5% of OV-225 on a 183-cm column. We found that separation with this phase composition was inadequate for quantitative work but that by increasing the OV-225 level to 4% very good results were obtained (Table I). The retention time for vitamin D was quite short (20 min) and the cholestane was well separated from the solvent tail. However, when a biological extract was chromatographed the solvent tail was larger and interfered with the peaks.

(F) 4% of OV-225, as Phase (E), but in a 243 cm × 2 mm I.D. column. This column gave excellent results (Table I and Fig. 3). The retention time for cholestane was $6\frac{1}{2}$ min, compared with $2\frac{1}{2}$ min on the 6-ft column.

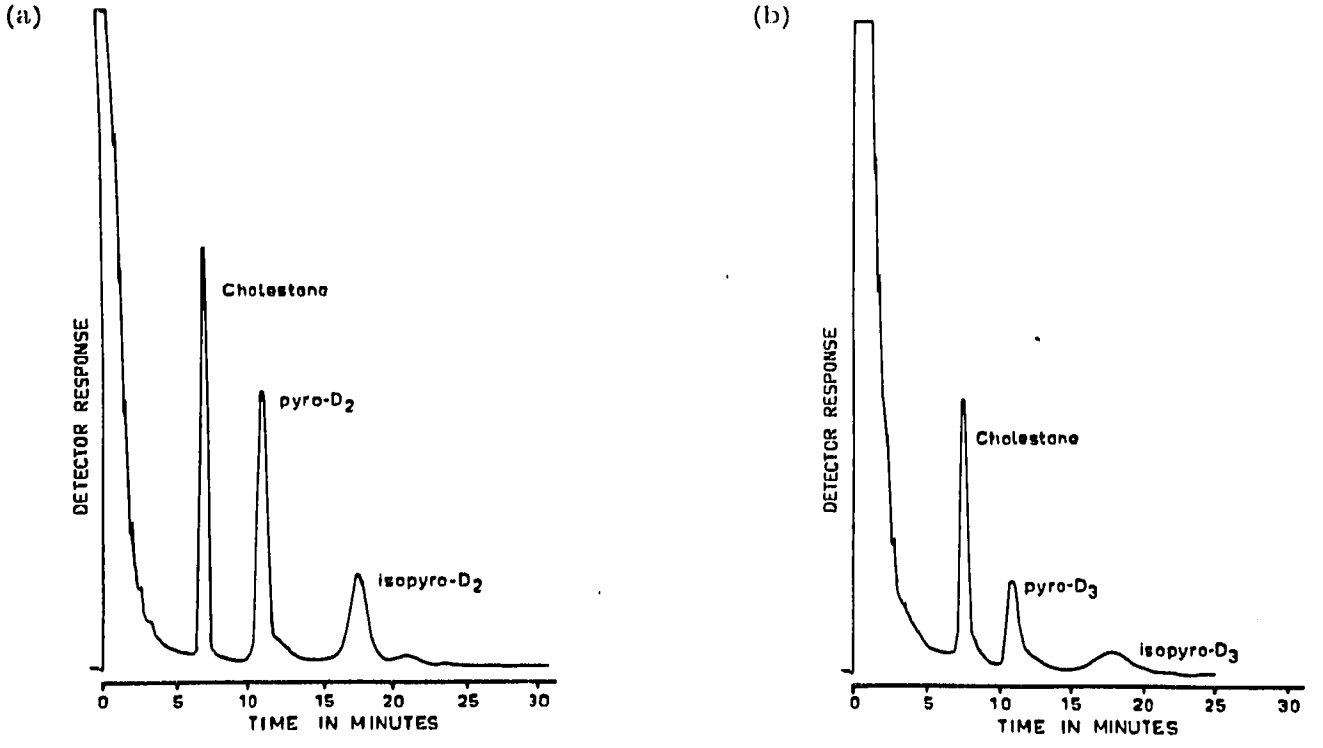

Fig. 3. Chromatograms of TMS derivatives of (a) vitamin D_2 and (b) vitamin D_3 with cholestane on 243 cm × 2 mm I.D. column packed with 4% of OV-225. Conditions are described in the text.

Silylation procedure

It can be seen from Fig. 2 that the peak area ratio of pyro-vitamin D to cholestane reached a maximum approximately 50 min after preparation of the sample. When the sample was not refrigerated after preparation, this maximum occurred earlier and a lower yield resulted. Diminution of the vitamin D peak was also much faster when the sample was not refrigerated. As the size of the pyro-vitamin D peak decreased, a fourth peak with a retention time of 4.6 relative to cholestane appeared on the chromatogram (Fig. 4). This presumably represents a

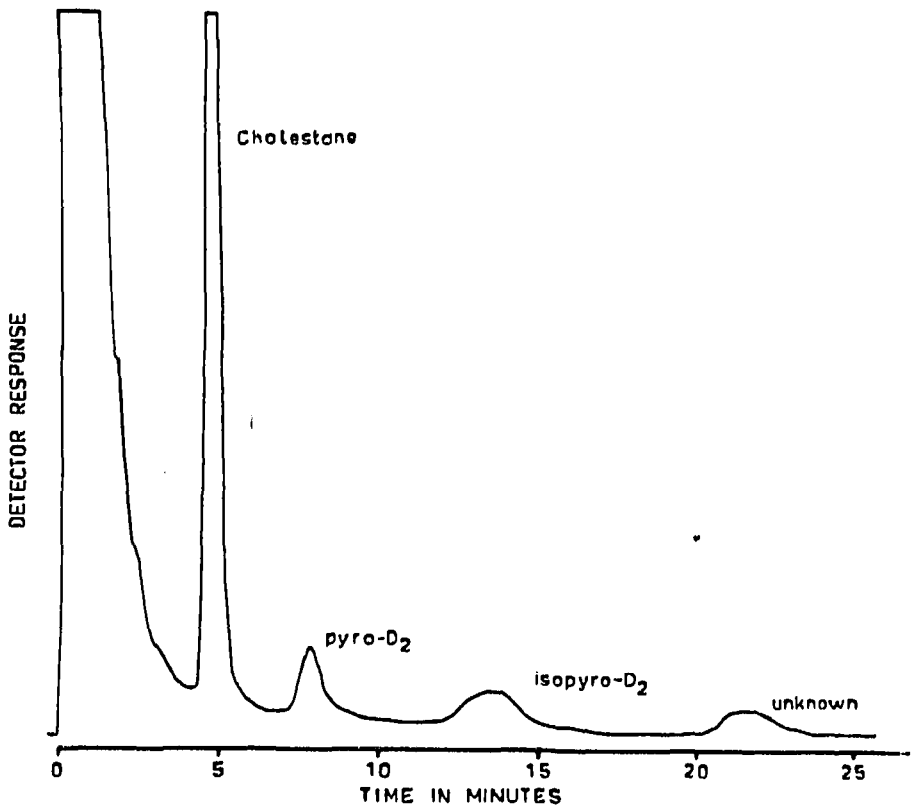

Fig. 4. Gas chromatogram of a mixture containing TMS derivatives of vitamin D_2 and cholestane, injected one day after preparation. Conditions of gas chromatography are given in the text.

breakdown product of vitamin D, as the size of the peak increases as that of vitamin D decreases.

This experiment was repeated five times and a qualitatively and quantitatively similar curve was obtained each time.

The shape of the time-decay curve can be explained by assuming firstly that vitamin D is slower than cholestane to react with the silylation reagents and secondly that the TMS derivative of vitamin D breaks down, whereas that of cholestane remains stable. The ratio of the two derivatives therefore would increase to a maximum as the vitamin D reacted and then decrease as the TMS-vitamin D broke down. By allowing all samples to develop for the same length of time after preparation, good agreement for quadruplicate samples was obtained (Table II).

TABLE II

AREA RATIO OF PYROVITAMIN D AND CHOLESTANE PEAKS IN FOUR IDENTICAL SAMPLES

Sample number	*Area ratio*	*Weight ratio*
1	0.92	2.09
2	0.95	2.09
3	0.93	2.09
4	0.95	2.09

CONCLUSIONS

The new stationary phase OV-225 gave improved results in the GLC determination of vitamin D. With a column packed with 4% of OV-225, the separation of the TMS derivatives of vitamin D from that of the internal standard cholestane was good, and the retention times were short. This makes it very useful for the routine analysis of serum extracts of vitamin D.

The TMS derivatives of vitamin D are highly unstable, even under refrigeration. Quantitative analyses based on the formation of these derivatives were found to be valid only if the time interval between preparation and GLC injection was standardized.

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