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CAPILLARY GAS CHROMATOGRAPHIC STUDIES OF CHOLESTEROL BIOSYNTHESIS IN RATS TREATED WITH EGYT-1299

ÉVA TOMORI, EVA ORBÁN and ANDREA MADERSPACH
Institute for Drug Research, Szabadságharcosok utja 47–49, 1045 Budapest 4 (Hungary)

SUMMARY

Cholesterol and desmosterol concentrations were assayed by high-performance capillary gas chromatography. It was established that no toxic desmosterol accumulation occurred either in the serum or liver of rats receiving EGYT-1299 treatment. Desmosterol accumulation induced by triparanol was reduced by the agent at doses ten times lower than Atromid S; consequently its cholesterol lowering effect may be attributed to interference with cholesterol biosynthesis.

INTRODUCTION

Recent literature data[1] confirm the close relation, presumed for two decades, between high cholesterol levels in serum and myocardial infarction, coronary diseases and arteriosclerosis.

It was the objective of the present experiments to elucidate the mode of action of EGYT-1299 [bis (4-chlorophenoxy) acetyl urea] in the regulation of serum cholesterol levels. For this purpose, several methods are available; radioactive[2], colorimetric[3] and gas chromatographic[4,5]. In previous trials it has been established[6] that this reagent exhibits its hypolipidemic effect at lower doses than Atromid S.

In the present studies we needed to establish whether the cholesterol reducing substance is inducing desmosterol accumulation in the serum and liver of test animals, and to elucidate by means of the non-isotopic desmosterol suppression technique[7] whether cholesterol reduction is due to inhibition of cholesterol biosynthesis. The desmosterol (24-dehydrocholesterol) and cholesterol levels of biological samples were assayed by gas chromatography (GC) which is a most suitable method for the separation of these closely related substances whose structures differ only by a single double bond.

EXPERIMENTAL

Materials

Silylating reagents BSTFA and TMCS were supplied by Pierce (Rockford, Ill., U.S.A.); XE-60 and OV-17 stationary phases, the carrier Gas-Chrom Q (80–100 mesh), cholesterol and desmosterol standards by Applied Science Labs. (State

TABLE I

EXPERIMENTAL CONDITIONS OF GAS-LIQUID CHROMATOGRAPHY

Gas chromatograph, HP 5830A. Detector, FID. Carrier gas, nitrogen. Chart speed, 0.6 cm/min. Sample size, 1 μl.

Column	*Liquid phase*	*Flow-rate of carrier gas (ml/min)*	*Inlet pressure (p.s.i.)*				*Temperature (°C)*		*Injection system*
			Carrier gas	*Hydrogen*	*Air*	*Make up gas*	*Injection port*	*Column*	
6 ft. × 4.0 mm I.D. glass coil	A, 1% XE-60 on Gas-Chrom Q	45	—	28	24	—	240	190	—
6 ft. × 4.0 mm I.D. glass coil	B, 1% OV-17 on Gas-Chrom Q	45	—	28	24	—	260	240	—
50 ft. × 0.25 mm I.D. glass tubing	C, Pz-179	—	24	28	24	24	260	230	split 1/60
60 ft. × 0.25 mm I.D. glass tubing	D, OV-101	—	12	28	24	24	260	230, 1°/min to 270	glass solid injector

College, Pa., U.S.A.); cholestane by Sigma (St. Louis, Mo., U.S.A.); Atromid S by I.C.I. (Macclesfield, Great Britain). Triparanol was a gift from Richardson Merrel (Cincinnati, Ohio, U.S.A.) and EGYT-1299 was synthesized by Egyt Pharmaceutical Works (Budapest, Hungary). The petroleum ether used in the extractions had a b.p. of 30–50°. The glass capillary columns (50 ft. × 0.25 mm and 60 ft. × 0.25 mm) packed with the stationary phases PZ-170 and OV-101, respectively, were prepared by the Research Laboratory for Inorganic Chemistry, Hungarian Academy of Sciences (Budapest).

Preparation of serum and liver samples

Cholestane internal standard (174 μg) was added to 1 ml of serum or 0.5 g of liver. Serum samples were saponified for 1 h at 37°, and liver samples for 2 h at 100° with 15 ml of ethanolic KOH [10 *N* KOH–dry ethanol (1:1)]. Since both total cholesterol and desmosterol levels had to be detected saponification was controlled by thin-layer chromatography. The extracts were spotted on Kieselgel G plates (20 × 20 cm), and the plates developed in the solvent system hexane–diethyl ether–formic acid (120:30:3). Visualization was carried out with a 0.06% $FeCl_3$ solution (glacial acetic acid–sulphuric acid–96% ethanol). After hydrolysis, the serum and liver samples were extracted with petroleum ether (3 × 15 ml), and the petroleum ether phase concentrated to 10 ml. An aliquot of this concentrate was placed into a tightly plugged PTFE vessel having silylated walls. The organic layer was evaporated under a current of nitrogen, and the residue silylated with a mixture of BSTFA and TMCS (80 μl × 5 μl) for 30 min at 60°. An aliquot (1 μl) of this sample was injected into the evaporator, or, in the case of a capillary column, on the glass solid injector.

Gas–liquid chromatography

Experimental conditions are summarized in Table I.

Calibration curves and quantitation

A calibration curve was determined on an OV-101 glass capillary column (D), and the detector responses studied for both steroids at the experimental conditions

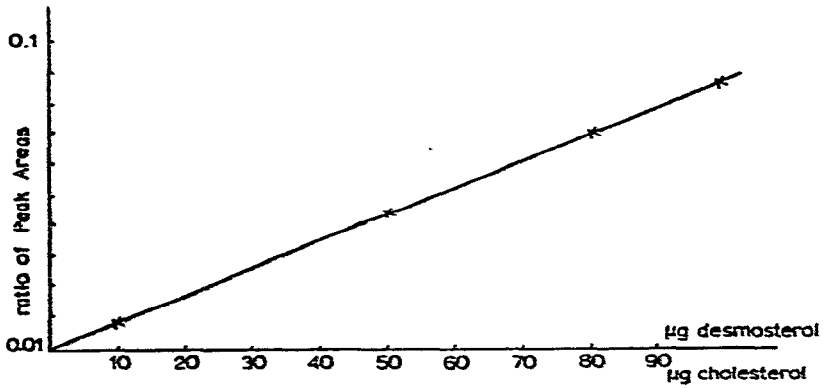

Fig. 1. Standard calibration curve for an OV-101 capillary column (D). Experimental conditions as in Table I. Ratio of peak areas = peak area of desmosterol or cholesterol to peak area of internal standard cholestane.

applied (Fig. 1). The linear responses obtained enabled quantitative assays of biological samples. Similar linear responses were achieved when columns A, B and C were employed.

RESULTS AND DISCUSSION

Preliminary runs were carried out on stationary phases of different selectivities for the quantitative assay of cholesterol and desmosterol in biological samples to determine the best experimental conditions. The studies were then extended to glass capillary columns. The main characteristics of both packed and capillary columns are summarized in Table II.

TABLE II

RELATIVE RETENTION OF STEROL TMS ETHERS USING CHOLESTANE AS REFERENCE SUBSTANCE, PEAK RESOLUTION VALUES AND NUMBER OF EFFECTIVE THEORETICAL PLATES

Retention times of cholestane (min): on column A, 4.55; column B, 6.76; column C, 7.07; column D, 12.06. $t_{1,2}$ = Relative retention = (sterol TMS t_2)/(cholestane t_1). R_s = Peak resolution. N' = Number of effective plates per metre for cholesterol TMS ether sample.

Compound	*Liquid phase*											
	Packed columns						*Capillary columns*					
	A			*B*			*C*			*D*		
	$t_{1,2}$	R_s	N'	$t_{1,2}$	R_s	N'	$t_{1,2}$	R_s	N'	$t_{1,2}$	R_s	N'
Cholesterol	2.04			2.36			1.71			1.70		
3-TMS		1.21			1.66			7.90		4.2		
Desmosterol	2.48			2.85			2.11			1.80		
3-TMS			800			850			886			800
Cholestane	1.00			1.00			1.00			1.00		

All data for the OV-101 capillary column in Table II were measured at an isothermal column temperature of 240°. In column C the linear flow-rate was 30.24 cm/sec, and in column D 20.43 cm/sec. Analysis time was proportionally reduced at higher flow-rates, as was the number of theoretical plates. Nonetheless, peak resolution values, listed in Table II, were still satisfactory and the utilization of higher flow-rates not only resulted in shorter analysis times but also allowed the analysis of high-boiling sterol TMS ethers at relatively low temperatures.

For the assays performed on glass capillary columns, a variety of sample introduction systems were employed: in column C the split system, and in column D the glass solid injector designed by Van den Berg and Cox[8]. Although in preliminary experiments both systems provided nearly identical results, the glass solid injector was considered more advantageous since in this case the entire sample could be submitted to analysis. Due to the high boiling points of the compounds, no losses occurred during evaporation. By evaporating the silylating agent the column also became more durable. The desmosterol content could be detected with high sensitivity by applying the glass solid injector, and the limit of desmosterol detectability in biological samples was 1 ng. All the following assays were carried out on a glass capillary column lined with OV-101, and by use of the glass solid injector.

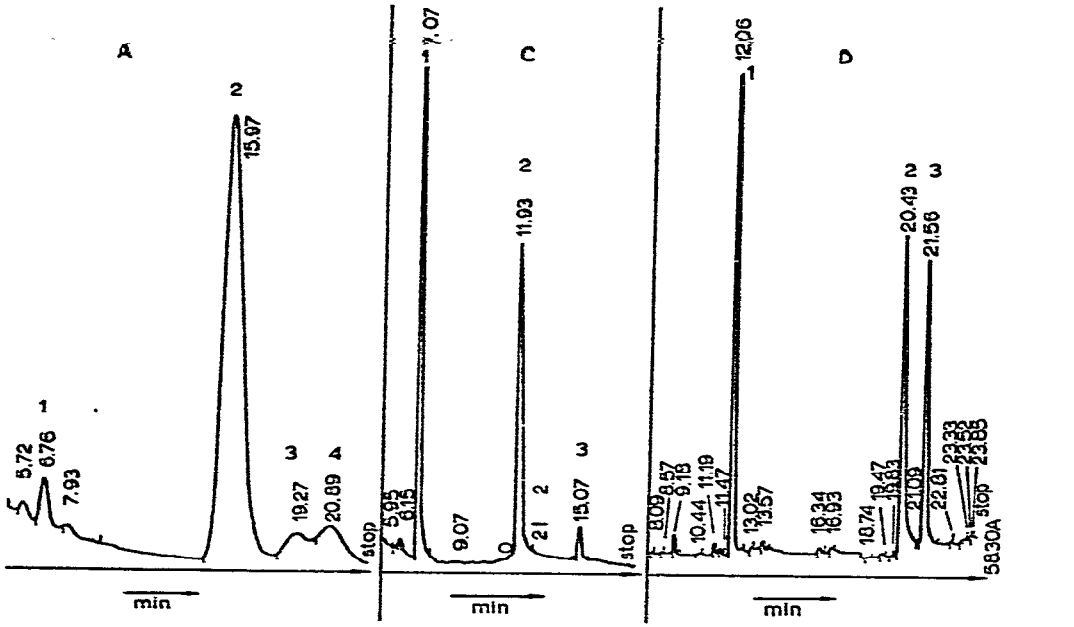

Fig. 2. Gas–liquid chromatogram of a rat serum sample after extraction. Peaks: 1 = cholestane; 2 = cholesterol TMS; 3 = desmosterol TMS; 4 = unidentified substance. Experimental conditions for assays carried out on columns A, C and D are as in Table I.

Fig. 2 shows chromatograms obtained with packed and glass capillary columns which yielded a variety of information about biological samples at nearly identical periods of time.

In the first set of experiments groups of CFY male rats (ten rats each) were treated orally for 10 days with EGYT-1299, Atromid S and triparanol at the doses listed in Tables III and IV. On the eleventh day the animals were bled to death, their livers removed and the cholesterol and desmosterol levels of the serum and liver determined (see Tables III and IV). It is well known that triparanol reduces cholesterol levels by inhibiting Δ^{24}-dehydrocholesterol reductase which catalyzes the conversion of desmosterol into cholesterol[9]. Since desmosterol cannot enter the

TABLE III

CHOLESTEROL AND DESMOSTEROL EXTRACTED FROM RAT SERUM AND ASSAYED ON AN OV-101 GLASS CAPILLARY COLUMN

Experimental conditions as in Table I. Ten test animals.

Compound	*Dose (mg/kg)*	*Cholesterol (μg/ml)*	*S.D.*	*Desmosterol (μg/ml)*	*S.D.*
Control		1138	111.5	—	
EGYT-1299	30	714	159*	—	
Atromid S	300	642	159.8*	—	
Triparanol	25	475	100.9**	406	104.8

* $p < 0.001$.
** $p < 0.01$.

TABLE IV

CHOLESTEROL AND DESMOSTEROL EXTRACTED FROM RAT LIVER AND ASSAYED ON AN OV-101 GLASS CAPILLARY COLUMN

Experimental conditions as in Table I. Ten test animals.

Compound	*Dose (mg/kg)*	*Cholesterol (μg/mg)*	*S.D.*	*Desmosterol (μg/mg)*	*S.D.*
Control	—	2850	114.4	—	
EGYT-1299	30	2500	381.4	—	
Atromid S	300	2510	367	—	
Triparanol	25	1296	116*	1066	152.6

* $p < 0.001$.

organism from outside its assay is suitable for the differentiation of cholesterol originating from internal and external sources. According to the results in Tables III and IV, free serum cholesterol levels are reduced by EGYT-1299 and Atromid S by 37 and 43%. Similarly to Atromid S, no desmosterol accumulation was induced either in the serum or in the liver by EGYT-1299.

In the second set of trials triparanol was administered in combination with both EGYT-1299 and Atromid S. The experiments were carried out to confirm that EGYT-1299, similarly to Atromid S, is exerting its cholesterol reducing effect by interfering with cholesterol biosynthesis. According to the desmosterol suppression technique[7], the serum desmosterol level may be used as an index of endogenous sterol production. This was verified as follows. In rats treated with triparanol,

TABLE V

CHOLESTEROL AND DESMOSTEROL EXTRACTED FROM RAT SERUM AND ASSAYED ON AN OV-101 GLASS CAPILLARY COLUMN

Experimental conditions as in Table I. Ten test animals.

Compound	*Dose (mg/kg)*	*Cholesterol (μg/ml)*	*S.D.*	*Desmosterol (μg/ml)*	*S.D.*
Triparanol	25	475	109	406	104.8
Triparanol + EGYT-1299	25 + 30	299	100.4*	202	46**
Triparanol + Atromid S	25 + 300	297	69.7*	206	32.4**

* $p < 0.01$.
** $p < 0.001$.

TABLE VI

CHOLESTEROL AND DESMOSTEROL EXTRACTED FROM RAT LIVER AND ASSAYED ON AN OV-101 GLASS CAPILLARY COLUMN

Experimental conditions as in Table I. Ten test animals.

Compound	*Dose (mg/kg)*	*Cholesterol (μg/mg)*	*S.D.*	*Desmosterol (μg/mg)*	*S.D.*
Triparanol	25	1296	116	1066	152.6
Triparanol + EGYT-1299	25 + 30	1442	138.8	1012	189.2
Triparanol + Atromid S	25 + 300	1176	277	880	116.4

which inhibits the desmosterol–cholesterol conversion step, cholesterol biosynthesis was simultaneously blocked by cholesterol feeding, when no desmosterol could be detected in the serum of the animals. The data in Table V show that desmosterol serum levels are lowered by the combined treatment of EGYT-1299 + triparanol compared to controls receiving only triparanol, while those of Table VI demonstrate that no significant change is induced in the liver compared to those of the controls.

It was shown on the basis of data assayed in the serum that EGYT-1299 is exerting its cholesterol reducing effect by interfering with cholesterol biosynthesis prior to the desmosterol–cholesterol conversion step.

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