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CHARACTERIZATION AND GAS CHROMATOGRAPHIC DETERMINATION OF ACTIVE PRINCIPLES OF BIOLOGICAL INTEREST IN PHARMACEUTICAL PRODUCTS (GASTRIC MUCOPROTEIN AND THIOCTIC ACID)

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SUMMARY

The assay of thioctic acid in pharmaceutical preparations and that of *l*-fucose in gastric mucoprotein are described here as two representative examples of the application of gas-liquid chromatography in analytical work with biologically active products, natural or synthetic.

In both these cases, gas-liquid chromatography gives qualitative and quantitative analytical results such as could not be obtained by traditional techniques; these are limited both in terms of specificity and sensitivity.

The quantitative results obtained are completely specific, reproducible and accurate, as required in quality control and assay of substances being prepared for therapeutic use.

INTRODUCTION

The application of gas-liquid chromatography in analytical work in the pharmaceutical field is of considerable importance. The assay of thioctic acid in pharmaceutical products and that of *l*-fucose in gastric mucoproteins used in the pharmaceutical industry are reported here as two highly representative examples of this method of analysis.

ASSAY OF THIOCTIC ACID IN PHARMACEUTICAL PRODUCTS

Thioctic acid (α-lipoic acid) occurs naturally both in the vegetable and animal kingdoms. The substance was first isolated in the crystalline state by REED *et al.*[1] in 1951 and called "α-lipoic acid" because of its behavior in fat solvents. Later, BROCKMAN *et al.*[2] identified its chemical structure as 6,8-dithiooctanoic acid in a cyclic form;

these same authors called the substance thioctic acid. In the following years, thioctic acid was made synthetically by various methods.

Pharmacological investigations carried out in view of the biochemical properties of thioctic acid revealed that the substance had liver-protecting qualities in various hepatic diseases and antitoxic properties in poisoning from various substances (*e.g.* mercuric chloride, arsenobenzene).

The quantitative assay of thioctic acid in compound formulations of some complexity is extremely difficult by means of the classical colorimetric and spectrophotometric methods. In pharmaceutical formulations with liver-protecting action, thioctic acid is usually associated with other active substances that are not always completely separable (*e.g.* liver extract, cysteine, methionine) and interference from such substances makes these analytical techniques unreliable. Further, because of the low specific extinction of thioctic acid (6.80 in methanol), one must work with relatively large quantities of the substance.

Our work is concerned with the more commonly used pharmaceutical products containing thioctic acid; these include tablets and injectable solutions. We have developed a quantitative method of analysis by gas chromatography after the substance has been extracted from the preparation and converted to its methyl ester[3]. Benzyl benzoate is used as an internal standard.

Thioctic acid is extracted with benzene from aqueous solutions or dispersions of the test product after slight acidification with hydrochloric acid. The benzene extracts are evaporated to a small volume; the extract is then esterified to methyl thioctate with anhydrous methanol–HCl (10% solution in methanol). Methylation is carried out in a refluxing apparatus in a water bath for about one hour. After the esterification, a suitable amount of benzyl benzoate (internal standard) is added; the solution is concentrated in vacuum and then diluted with ethyl ether. The ether solution is washed with a 5% solution of sodium bicarbonate in water, then filtered over anhydrous sodium sulfate; finally the solution is concentrated to a small volume in a stream of nitrogen. A standard solution of pure thioctic acid in methyl alcohol, which is then processed in exactly the same way as the test sample, is prepared in parallel. These two solutions, sample and standard, are examined by gas–liquid chromatography. Table I shows the details of the gas chromatographic technique.

The retention time of methyl thioctate relative to the internal standard, under the experimental conditions described, is 1.19.

TABLE I

ASSAY OF THIOCTIC ACID IN PHARMACEUTICAL PRODUCTS: EXPERIMENTAL CONDITIONS OF GAS CHROMATOGRAPHY

Apparatus	"Fractovap" Carlo Erba Model C
Detector	Flame ionization (FID)
Column (glass)	Length 80 cm; diameter 6 × 4 mm
Stationary phase	SE-52 5% on Gas-Chrom P 100–120 mesh, silanized
Column temperature	165°
Evaporator temperature	200°
Carrier gas	Nitrogen (25 ml/min)
Auxiliary gases	Hydrogen (20 ml/min), air (300 ml/min)
Chart speed	½ in./min
Introduction	~ 1 μl.

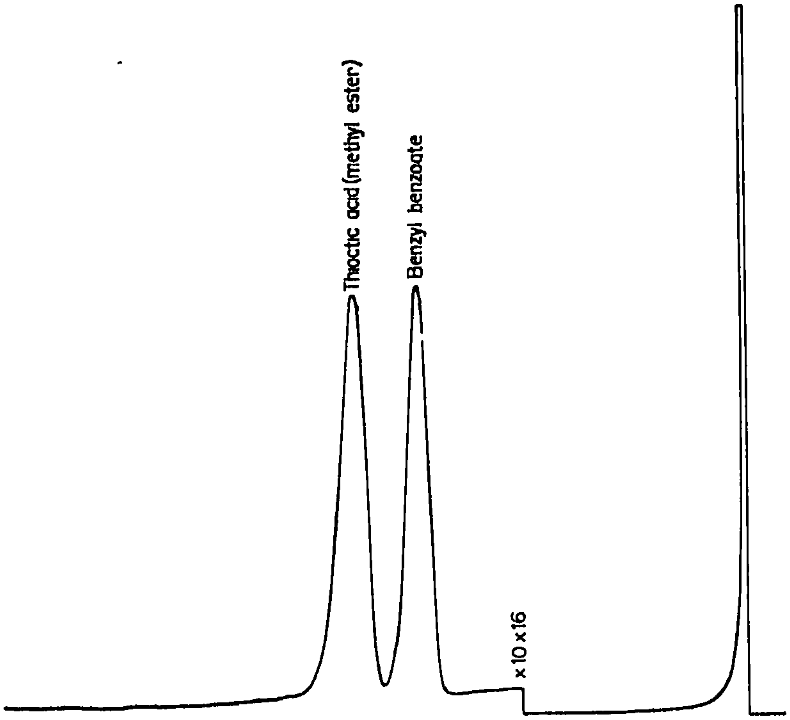

Fig. 1. Chromatogram of thioctic acid from injectable solutions. Internal standard: benzyl benzoate.

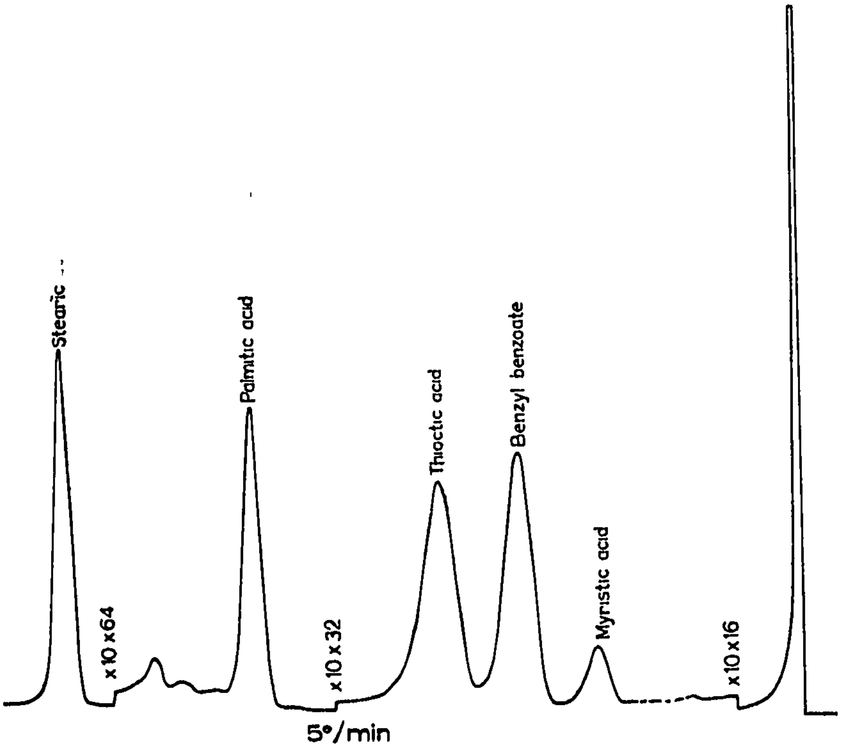

Fig. 2. Chromatogram of thioctic acid extracted from tabletted products. Internal standard: benzyl benzoate. Fatty acids contained in the tablet excipient are also visible .

The areas of the methyl thioctate and benzyl benzoate peaks are measured from the gas chromatographic tracings. By comparison of the areas of the sample and standard methyl thioctate, with reference to benzyl benzoate, the amount of thioctic acid contained in the aliquot of the test product can be calculated.

Figs. 1 and 2 show the chromatograms obtained. The analysis of the ampoule product shows two peaks, one for methyl thioctate and one for benzyl benzoate. In the chromatogram from the tablet product, in addition to the said two peaks, one can also see peaks due to myristic, palmitic and stearic acids from the tablet excipients which consist of saturated fatty acids; these peaks, however, do not interfere with those of methyl thioctate and benzyl benzoate.

Fig. 3 shows the calibration curve constructed by assaying various amounts of thioctic acid by gas chromatography relative to constant amounts of internal standard. It can be seen that the curve follows a linear course over fairly wide limits.

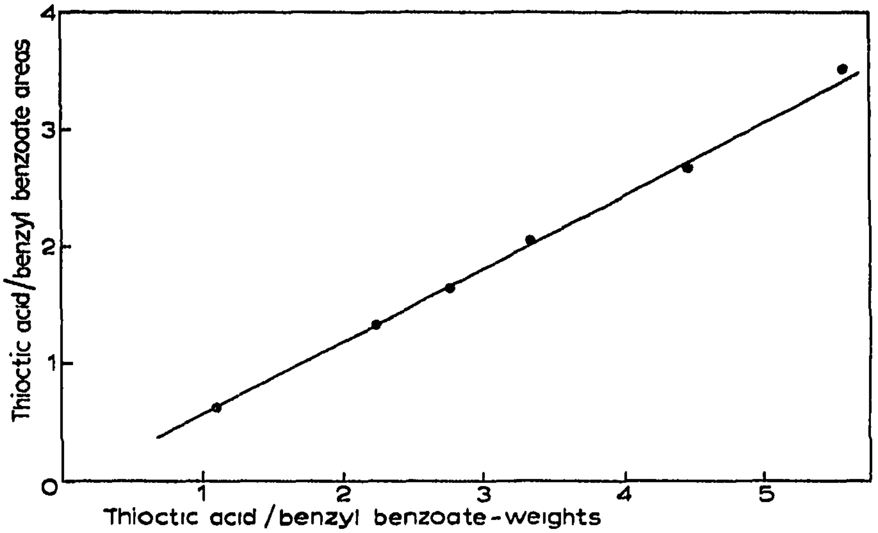

Fig. 3. Calibration curves for thioctic acid with an internal standard of benzyl benzoate.

ASSAY OF *l*-FUCOSE CONTAINED IN GASTRIC MUCOPROTEIN

The mucoproteins are extracted by suitable methods from the gastric mucosa of pigs and are used in the preparation of pharmaceutical products and of special powdered milk for infant feeding. The biological activities of mucoproteins are multifarious; it will suffice to mention their activity as an "intrinsic factor" (or binding factor) facilitating the intestinal absorption of vitamin B_{12}, their liver-protecting and fat-dispersant activity, and their action in improving the utilization of protein nitrogen.

From the analytical point of view it is very expedient to characterize this mucoprotein in terms of a particular carbohydrate it contains, namely *l*-fucose.

After an acidic hydrolysis of the polysaccharides the monosaccharides produced were usually identified and estimated by paper and column chromatography and by colorimetric reactions which are not very sensitive or specific.

TABLE II

ASSAY OF *l*-FUCOSE IN GASTRIC MUCOPROTEIN: EXPERIMENTAL CONDITIONS OF GAS CHROMATOGRAPHY

Apparatus	"Fractovap" Carlo Erba Model C
Detector	Flame ionization (FID)
Column (glass)	Length 2 m; diameter 6 × 4 mm
Stationary phase	SE-30 1% on Gas-Chrom P 100–120 mesh, silanized
Column temperature	Programmed from 150° to 220° (3.5° /min)
Evaporator temp.	290°
Carrier gas	Nitrogen (25 ml/min)
Auxiliary gases	Hydrogen (20 ml/min), air (300 ml/min)
Chart speed	½ in./min

The application of gas chromatography to carbohydrate analysis has made it possible to investigate the carbohydrate composition of glycoproteins both qualitatively and quantitatively[4–7].

Our aim in the present investigation was the quantitative assay of *l*-fucose in the mucoprotein; accordingly, we deliberately omitted assaying or even separating the other component sugars. We hydrolyzed the glycoproteins with methanol, then converted the methyl glycosides to TMS derivatives. The assay was carried out with *d*-sorbitol as internal standard. Table II shows the experimental conditions for gas chromatography.

Methanolysis is carried out with anhydrous methyl alcohol in an ampoule containing about 100 mg of mucoprotein, in the presence of *p*-toluenesulfonic acid, for 12 h, at 80°–85°, with continuous agitation. A suitable amount of *d*-sorbitol is then added to the solution containing the methyl glycosides; the mixture is transferred to a 10-ml vial and evaporated until dry in a stream of nitrogen. The residue is desiccated for 6 h under vacuum, then redissolved in 2 ml of anhydrous pyridine and treated with 1 ml of hexamethyldisilazane and 0.5 ml of trimethylchlorosilane. The

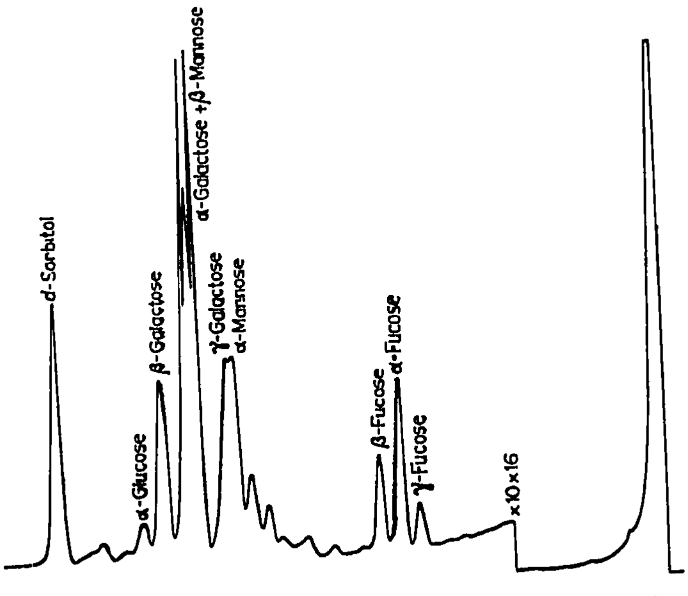

Fig. 4. Chromatogram of mucoprotein monosaccharides as methyl glycoside TMS ethers. Internal standard: *d*-sorbitol.

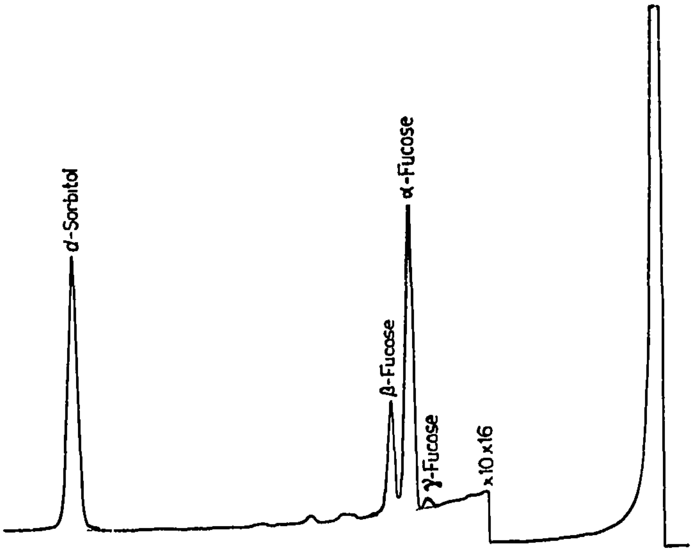

Fig. 5. Chromatogram of the standard *l*-fucose (as methyl glycoside TMS ether) together with the internal standard.

reaction mixture so obtained is shaken vigorously for at least 1 min; after 10–15 min it is ready for chromatography. A known amount of standard *l*-fucose (approximately 3 mg for 100 mg of mucoprotein assayed) is processed in parallel.

Fig. 4 is a gas chromatographic tracing showing the saccharide content of the mucoprotein. Each monosaccharide is represented by two or three peaks, corresponding to the anomeric forms, α, β, and γ (as methylglycoside TMS ethers). The peak of the internal standard (*d*-sorbitol) is also visible.

Fig. 5 shows a gas chromatogram of the standard *l*-fucose with the internal standard added.

Quantitative assay is carried out by calculating the sums of the areas of the *l*-fucose peaks in the sample and standard chromatograms. The percentage fucose contained in the mucoprotein being tested is calculated by comparing the values obtained, with reference to the *d*-sorbitol added.

Table III shows the results obtained on the testing of 5 specimens of mucoprotein. In addition to each *l*-fucose value, the corresponding protein nitrogen value is shown. An inversely proportionate relationship between the two values is apparent.

TABLE III

l-FUCOSE VALUES (BY GLC) AND CORRESPONDING PROTEIN NITROGEN VALUES OBTAINED IN THE TESTING OF SOME MUCOPROTEINS

Sample	*l-Fucose (%)*	*Nitrogen (%)*
1	4.00	7.59
2	3.80	8.35
3	3.30	8.54
4	2.95	9.03
5	4.90	7.12

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