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USE OF A LIQUID CHROMATOGRAPH IN LIPID CLASS SEPARATION

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SUMMARY

The advantages of monitoring lipid separation on a silicic acid column with a liquid chromatograph have been demonstrated and discussed.

The recovery yields of known synthetic lipid mixtures have been shown to be good and the machine has been used for monitoring the separation of lipid classes from normal human blood plasma and serum samples.

It has been shown that in general it is not advantageous to separate neutral lipids from phospholipids prior to complete fractionation, and that the analysis of plasma lipids is more meaningful than that of serum lipids.

INTRODUCTION

The use of silicic acid in the separation of lipids into their respective classes has been a widely used technique for many years[1–4]. In the past this has involved testing each fraction for cholesterol[5,6], fatty acids[7–9], phospholipids[10,11] and in some cases for glycerol residues[12] and ester linkages[13]. In order to ensure complete elution of each fraction it was often necessary to pass excess solvent through the column.

The results reported in this paper were obtained by coupling a silicic acid column to a Pye System 11 Liquid Chromatograph. The purpose of this unit is to detect any organic material eluted from the column and to record its presence on a chart recorder. Fig. 1 gives a diagrammatic representation of the chromatograph. A moving steel wire passes from the feed spool to the cleaner oven where any dirt or grease is removed from the wire. In the coating block it passes through the column eluent and becomes coated with it. The solvent is removed in the evaporator oven and the solute in the pyrolyser oven. From the pyrolyser oven the solute passes, in a carrier stream of inert gas into a hydrogen flame ionization detector from where a signal is passed through an ionization amplifier to a moving chart recorder.

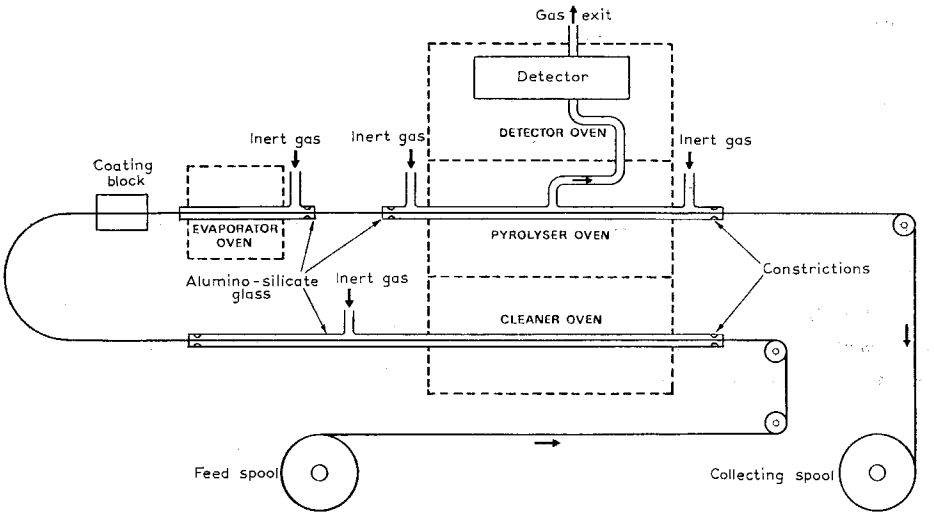

Fig. 1. Schematic diagram of liquid chromatograph.

EXPERIMENTAL

Materials

Silicic acid (Mallinckrodt) was washed several times with water followed by methanol and ether and allowed to dry at room temperature before being activated at 120° for 24 h. The silicic acid, now ready for use, was stored in a dry bottle with a tightly fitting cap. Columns were prepared immediately before use by pouring a suspension of silicic acid (1 g) in petroleum ether (b.p. 40–60°) into a 5 mm diameter column. The column was packed under pressure, and eluted with increasing concentrations of ether in petroleum ether and finally with methanol.

Analar grade solvents were used throughout. Biochemical-grade palmitic acid and cholesterol (B.D.H.) were used, and samples of cholesterol palmitate, tripalmitin, diolein, monopalmitin and L-α-lecithin (commercial grade) were obtained from Sigma Chemical Company.

Lipid extraction

Blood samples were processed immediately after withdrawal from the patient. Plasma samples had a 3.8 % citrate solution added to them (1 ml/10 ml blood) while serum samples were allowed to clot by standing at 37° for 1 h in the absence of citrate solution. Red cells were removed by centrifugation (3,500 r.p.m., 15 min).

Lipids were extracted from plasma or serum by a modification of the method of FOLCH *et al.*[14]. To ensure complete extraction the MeOH–CCl_3H mixture was refluxed for 5 min.

After extraction the lipid solution was evaporated to dryness, taken up in petroleum ether (*ca.* 1 ml) and stored at 4° for not more than two days.

Lipid assays

Cholesteryl derivatives. Cholesterol and cholesteryl esters give a green coloration with acetic anhydride in the presence of concentrated sulphuric acid[5]. Acetic anhydride (2 ml) was added to samples of cholesteryl compounds (0.1–2.0 μmole) in chloroform (5 ml) followed by concentrated sulphuric acid (0.1 ml). The colour was allowed to develop in the dark and measured at 630 mμ. The colour formed was found to be unstable and gave an optimum absorbance after 12–15 min. The most convenient method of obtaining reproducible results was by making repeated absorbance measurements until a consistent decrease was observed. The maximum absorbence value was recorded in each case and the method calibrated against a standard cholesterol solution.

Fatty acids. Glycerides (0.05–1.00 μmole) were hydrolysed as follows. The solutions were evaporated to dryness and then taken up in an ethanol–water (94:6, v/v) mixture (5 ml) containing two pellets of KOH (*ca.* 150 mg) and refluxed for 1 h.

After cooling and neutralising (HCl) the free fatty acid was extracted from the aqueous layer with hexane[7], and determined colorimetrically by the method of DUNCOMBE[8].

Phospholipids. Phospholipids (0.5–5.0 μmole) were completely hydrolysed and the inorganic phosphate liberated was determined by the method of FISKE AND SUBBAROW[15]. Hydrolysis was carried out by evaporating the sample to dryness in a Pyrex tube, adding 10 *N* H_2SO_4 (0.5 ml) and heating strongly for 5–10 min. It was found advisable to cover the open end of the tube with an inverted funnel connected to a water pump, as considerable fuming occurred. Any charred material was removed by adding a few drops of 20 vol. H_2O_2 and reheating. This was repeated until, after prolonged heating, the solution remained colourless.

For the FISKE AND SUBBAROW determination it was necessary to use two molybdate solutions, one containing 2.5 % ammonium molybdate in 5 *N* H_2SO_4 and the other containing 2.5 % ammonium molybdate in 3 *N* H_2SO_4. The former was used with aqueous solutions of the sodium phosphate standard and the latter with hydrolysed phospholipids. All colorimetric measurements were made with a Unicam S.P. 500 spectrophotometer.

Thin layer. The purity of fractions from silicic acid columns was checked by thin-layer chromatography on Kieselgel G plates developed in petroleum ether–ether–acetic acid (85:15:1, v/v)[16] and sprayed with 2′,7′-dichlorofluorescein.

Liquid chromatograph

The parameters of the liquid chromatograph were set as shown below.

Oxygen free nitrogen flow rate: through detector 30 ml/min, through cleaner 20 ml/min, and through evaporator 5 ml/min
Hydrogen flow rate for burner: 30 ml/min
Air flow rate for burner: 500 ml/min
Detector temperature: 350°
Cleaner temperature: 600°
Evaporator temperature: 50°
Wire speed: minimum (*ca.* 2 in./sec)
Chart speed: 3 in./h
Amplifier sensitivity: 5 × 10

RESULTS

Recovery of synthetic lipid mixtures

The purity of each component of the mixture was checked quantitatively and by thin-layer chromatography. At least 99 % purity was obtained with all samples except tripalmitin and lecithin. Dipalmitin was found to contain some free fatty acid and this was allowed for in calculating recovery yields.

Neutral lipids. A mixture of cholesteryl palmitate, tripalmitin, diolein, monopalmitin, cholesterol and palmitic acid in petroleum ether (0.5 ml) was eluted from a silicic acid column with increasing concentrations of ether in petroleum ether. The elution chromatogram is shown in Fig. 2a. Table I shows the order of elution of each fraction from the column and its recovery yield.

TABLE I

ELUTION OF A LIPID MIXTURE FROM A SILICIC ACID COLUMN

Eluent (%)		*Component eluted*	*Input concentration* (*μmoles*)	*Recovery* (*μmoles*)	*Yield* (%)
Ether	*Petroleum ether*				
1	99	Cholesteryl palmitate	1.412	1.39	98.5
4	96	Tripalmitin	0.315	0.313	93.4
10	90	Palmitic acid	0.075	0.073	99.4
		Cholesterol	0.168	0.165	97.4
25	75	Diolein	0.506	0.483	98.3
100	—	Monopalmitin	0.913	0.910	99.5

Lecithin. Fig. 2b is the elution pattern of lecithin. Its recovery yield from silicic acid was determined separately by phosphorus determinations before and after elution from the columns because it contained a number of impurities which would have interfered with the neutral fractionation. The recovery yield was 93.5 %. In order to achieve complete elution of lecithin from the column it was necessary to use 100 % methanol as the solvent.

Separation of normal human blood plasma lipids

Lipids were extracted from two identical samples of normal human blood plasma (5 ml) and fractionated by different methods.

Method A. Silicic acid (1 g) was added to the lipid extract in chloroform (3 ml) and shaken for several minutes. The chloroform and silicic acid were separated by centrifugation and the chloroform decanted off. The silicic acid was washed several times with more chloroform and these washings were added to the original chloroform extract which was evaporated to dryness, taken up in petroleum ether (0.5 ml) and fractionated on a silicic acid column in the usual way (Fig. 2c). The phospholipids, adsorbed by silicic acid from the chloroform extract, were eluted with methanol.

Method B. The lipid extract was eluted from a silicic acid column without prior separation of the phospholipids, Fig. 2d.

The results of the two methods are compared in Table II. Tables III gives the order of elution of the lipid classes.

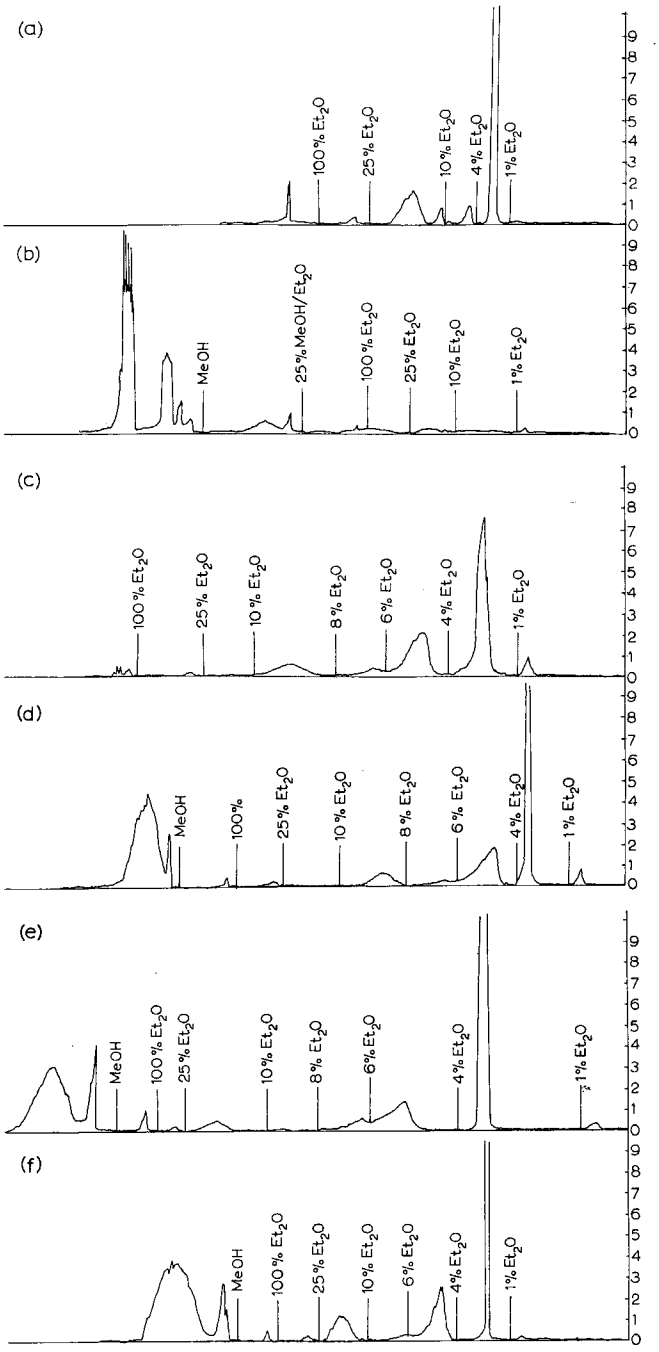

Fig. 2. Liquid chromatograph elution patterns. (a) Separation of synthetic neutral lipid mixture; (b) lecithin; (c) separation of lipids from normal human blood plasma by Method A; (d) separation of lipids from normal human blood plasma by Method B; (e) separation of lipids from normal human blood plasma; (f) separation of lipids from normal human blood serum. 1 cm represents 1 in. on the original chart. Direction of elution is from right to left.

TABLE II

COMPARISON OF NORMAL BLOOD PLASMA LIPIDS

Fraction	*Method A* (*μmoles/ml*)	*Method B* (*μmoles/ml*)
Cholesteryl esters	2.60	2.89
Triglycerides	1.17	1.42
Free fatty acids	0.163	0.166
Free cholesterol	0.889	0.942
Diglycerides	0.110	0.108
Monoglycerides	0.200	0.222
Phospholipids	4.06	5.10

TABLE III

ORDER OF ELUTION OF BLOOD PLASMA LIPIDS FROM A SILICIC ACID COLUMN

Eluent			*Fraction(s) eluted*
Petroleum ether (%)	*Ether* (%)	*Methanol* (%)	
99	1	—	Cholesteryl esters
96	4	—	Triglycerides
94	6	—	FFA/triglycerides
92	8	—	Cholesterol
90	10	—	
75	25	—	Diglycerides
—	100	—	Monoglycerides
—	—	100	Phospholipids

Comparison of normal human blood plasma and serum lipids

Lipid extracts were prepared from plasma and serum samples (5 ml) of normal human blood. The two samples were eluted from a silicic acid column according to Method B, Fig. 2e and f. The results are compared in Table IV. The order of elution was the same as that shown in Table III except that free cholesterol was only eluted from the column when the ether content of the eluent reached 10 %.

TABLE IV

COMPARISON OF NORMAL BLOOD PLASMA AND SERUM LIPIDS FROM THE SAME SOURCE

Fraction	*Plasma* (*μmoles/ml*)	*Serum* (*μmoles/ml*)
Cholesteryl esters	2.59	1.18
Triglycerides	1.51	1.69
Free fatty acids	0.271	0.177
Free cholesterol	1.06	1.08
Diglycerides	0.324	0.273
Monoglycerides	0.301	0.595
Phospholipids	3.98	2.81

DISCUSSION

The recovery yields (Table I) obtained by separating a synthetic mixture of lipids of known concentration demonstrate that the loss of material through monitoring the silicic acid column with a liquid chromatograph is negligible. The time lag between a sample entering the coating block and its detection on the chart recorder is of the order of 5 sec, with the result that the passing of unnecessary volumes of eluent down the column is reduced to a minimum. Secondly, no material is lost in carrying out colorimetric, or other tests, in order to locate the eluted fractions. Fig. 2 demonstrates that 5 ml of plasma is an adequate quantity for the detection of the major lipid classes. With synthetic lipid mixtures complete class resolution was achieved (Fig. 2a). However, in the case of the plasma and serum samples (Fig. 2c–f) some overlap between triglycerides and free fatty acids was observed which was probably due to the presence of unsaturated triglycerides present in blood plasma, as these tend to be eluted after the more saturated compounds[17]. Quantitative estimations of these components were made by carrying out fatty acid determinations, by the DUNCOMBE method[8], before and after hydrolysis. It was found that in some cases free cholesterol was eluted with 8 % ether in petroleum ether whereas at other times 10 % ether was needed. Slight variations in the solvent composition could account for this as solvents were made up immediately before use to avoid loss of one component through evaporation. However, as there is no indication of other lipids being eluted at this solvent polarity it is advisable to elute free cholesterol with 10 % ether in petroleum ether.

In Table II a comparison has been made between two methods of fractionation. In Method A, the phospholipids were separated from the neutral components before fractionation. In Method B they were not. In the latter case this group is retained on the column for the longest period of time. It is apparent that when the two-stage fractionation is carried out there is some loss of material particularly in the phospholipid fraction. This is presumably due to losses of small quantities of solvents and silicic acid during transference from one vessel to another. A similar comparison was made by BATES[18], who made the same observations. The advantage of removing the phospholipids prior to fractionation is that these compounds are subject to autoxidation which could occur through remaining on a silicic acid column for prolonged periods of time unless special precautions are taken[17]. If the phospholipid fraction is required for further investigation it is probably advantageous to adopt Method A, although a certain loss in yield would be involved.

A detailed analysis of blood plasma lipids is frequently of more value if it is accompanied by a protein analysis. Protein analysis is conveniently carried out by cellulose acetate electrophoresis which unfortunately does not always give a clear separation of fibrinogen and β-globulin. For this reason a comparison of plasma and serum lipids was carried out. As is illustrated in Table IV there is some alteration of the lipid pattern during the clotting process which makes analysis of serum less meaningful than that of plasma.

The parameters for the liquid chromatograph cited in the experimental section were found to be satisfactory and were used throughout in the experiments discussed in this paper. The gas flow rates were those recommended by W. G. Pye & Co. Ltd. The other parameters were selected bearing the following points in mind. It is desir-

able to keep the evaporator temperature as low as possible in order to reduce the loss of solute, but at the same time it is important to remove all the solvent. Solvent selection must therefore allow the greatest possible difference between solvent and solute boiling point. The detector temperature must be sufficiently high to allow pyrolysation of the solute but should be at least 200° below that of the cleaner oven so that contaminants, if any, that remain on the wire will not be pyrolysed. Increased sensitivity may be achieved by increasing the wire speed and decreasing the column flow rate. However, neither of these procedures was found to be necessary.

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REFERENCES

1 J. J. WREN, *J. Chromatog.*, 4 (1960) 173.
2 J. HIRSCH AND E. H. AHRENS, JR., *J. Biol. Chem.*, 233 (1958) 311.
3 D. L. FILLERUP AND J. F. MEAD, *Proc. Soc. Exptl. Biol.*, 83 (1953) 574.
4 B. T. COLE, *Proc. Soc. Exptl. Biol.*, 114 (1964) 731.
5 W. R. BLOOR, *J. Biol. Chem.*, 24 (1916) 227.
6 A. ZLATKIS, B. ZAK AND A. J. BOYLE, *J. Lab. Clin. Med.*, 41 (1953) 486.
7 M. J. ALBRINK, *J. Lipid Res.*, 1 (1959) 53.
8 W. G. DUNCOMBE, *Biochem. J.*, 88 (1963) 7.
9 V. P. DOLE, *J. Clin. Invest.*, 35 (1956) 150.
10 C. P. STEWART AND E. B. HENDRY, *Biochem. J.*, 29 (1935) 1683.
11 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
12 M. LAMBERT AND A. C. NEISH, *Can. J. Res.*, 28 (1950) 83.
13 F. SNYDER AND N. STEPHENS, *Biochim. Biophys. Acta*, 34 (1959) 244.
14 J. FOLCH, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
15 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
16 H. WAGENER, *Nature*, 205 (1965) 386.
17 J. A. LOVERN, *Biochem. J.*, 63 (1956) 373.
18 M. W. BATES, *J. Am. Physiol.*, 194 (1958) 446.