Journal of Chromatography, 130 (1977) 139–143

CHROM. 9301

QUANTITATIVE RECOVERY OF FREE AND ESTERIFIED FATTY ACIDS FROM THIN-LAYER PLATES COATED WITH SILICA GEL

M. C. ELPHICK and J. P. LAWLOR

The Department of Child Health, University Hospital and Medical School, University of Nottingham, Nottingham NG7 2UH (Great Britain)

(Received May 3rd, 1976)

SUMMARY

A method is described for the recovery of fatty acids from silica gel on thin-layer plates. The method is simple and rapid and depends on the conversion of silica gel to potassium silicate, using potassium hydroxide, followed by acidification in such a way that the silicic acid is kept in solution. Fatty acids are then extracted by shaking with solvent. Fatty acid recovery from free fatty acid, phospholipid, glyceride and cholesterol ester zones is more than 90% efficient. There is no oxidation or isomerization of unsaturated fatty acids.

INTRODUCTION

In connection with work on fetal lipid metabolism, we required a simple method for the recovery of fatty acids from glyceride, phospholipid, cholesterol ester and free fatty acid zones on thin-layer chromatographic (TLC) plates. The procedure had to be efficient and suitable both for radioactivity determination and for total fatty acid measurement, as well as for subsequent analysis of the individual fatty acids by gas–liquid chromatography (GLC).

Most methods for recovering lipids from TLC plates depend on elution with solvents[1,2]. However, the more polar compounds are difficult to recover quantitatively and recovery varies with the amount of lipid present. Moreover, elution is time-consuming, the solvents have to be very pure, different zones need different solvent systems and subsequent evaporation is usually required before the lipids can be analysed further. Methods are available for liquid scintillation counting of zones scraped directly off TLC plates[3]. The efficiency of these methods is high, but they are limited by the phenomenon of self-absorption. Self-absorption can be overcome by initially dissolving the gel in hydrofluoric acid before scintillation counting[4], but the problem remains of further analysis of the lipids, for example for determination of specific activity.

In the present method this has been achieved by dissolving the gel in potassium hydroxide, then acidifying the extract under conditions in which the silica remains in solution. The free fatty acids and any unhydrolysed esters are extracted by shaking

with solvent. Saponification of esters not hydrolysed by this system (cholesterol esters) is continued on the dried extract using alcoholic potassium hydroxide and the fatty acids recovered after acidification.

EXPERIMENTAL

Reagents

Reagents used were: 2.62 *N* KOH, kept cold under nitrogen; 1 *M* polyethylene glycol, M.W. 200; 2.80 *N* HCl; *n*-pentane; 0.5 *N* KOH in 80% methanol; 1 *N* HCl. All aqueous reagents are made up in distilled water.

Procedure

Samples of up to 200 mg of silica gel are scraped from a TLC plate into polythene-stoppered glass test tubes (10 cm × 15 cm). 1 ml of 2.62 *N* KOH solution is added followed by immediate mixing. The tubes are gassed with nitrogen and incubated in boiling water. Free fatty acids require a 2-min incubation, glycerides and phospholipids 20 min and cholesterol esters 2 min, followed by a second hydrolysis step. After cooling on ice, 3 ml of polyethylene glycol is added followed by mixing and the addition of 1 ml 2.80 *N* HCl. The tubes are very quickly stoppered and their contents mixed. Redistilled *n*-pentane (4 ml) is added and the tubes shaken for 2 min. The organic layer is then decanted and the extraction repeated. Chloroform or a mixture of chloroform–*n*-heptane (70:30, v/v) can be used instead of pentane*. The extracts are combined and either evaporated to dryness under nitrogen followed by methylation for GLC analysis, or analysed for total free fatty acid content[5]. The extract can be divided and half used for scintillation spectrometry and half used for mass analysis. A blank extraction must always be done using the same quantity of gel as used with the test because the gel contains measurable quantities of fatty acids.

For cholesterol esters where hydrolysis is not complete, the extract is evaporated to dryness, in the same size of tube and 1 ml 0.5 *N* alcoholic KOH added followed by flushing with nitrogen, stoppering and incubating at 65°. After 30 min the tubes are cooled on ice and 0.75 ml 1 *N* HCl added followed by 5 ml of redistilled *n*-pentane or chloroform. The tubes are gassed again and shaken for 2 min. When the phases have separated the organic layer is removed and the extraction repeated.

The system was developed and tested using [1-^{14}C]palmitic acid and glycerol tri-[1-^{14}C]palmitate; both were obtained from the Radiochemical Centre (Amersham, Great Britain). Radioactivity was determined in a toluene-based scintillation fluid using a Walac LKB liquid scintillation spectrometer. An external standard was used to correct for quenching. The extraction of arachidonic acid, glycerol triheptadecanoate, glycerol trilinolenate, DL-α-phosphatidylcholine (dipalmitoyl) and cholesterol oleate was examined by GLC analysis of silica gel extracts. Fatty acids and fatty acid esters, including carrier palmitic acid and glycerol tripalmitin were obtained from Sigma (St. Louis, Mo., U.S.A.) (approx. 99% pure). Methyl esters were prepared with boron trifluoride–methanol complex (Fisons, Loughborough, Great Britain). After hydrolysis of excess methylating reagent and extraction into *n*-pentane the methyl

* When using chloroform replace polyethylene glycol with 2.17 *M* glycerol.

esters were injected into glass columns (2 m × 2 mm I.D.) packed with 20% diethylene glycol succinate on Chromosorb W HMDS, 100–120 mesh. The chromatograph was a Perkin-Elmer Model F17 fitted with flame ionization detectors. Separation was done isothermally at 180° and peak quantitation was performed with a digital integrator (Pye Unicam SP 80). Nitrogen carrier gas was used at a flow-rate of 25 ml/min. TLC was performed on glass plates coated with a 0.4 mm thickness of silica gel H (Merck) previously activated at 110° for 30 min. Samples were either deposited on the plate in a narrow band (3 cm wide) and run in equilibrated tanks with a solvent system consisting of petroleum ether (b.p. 60°–80°)–diethyl ether–glacial acetic acid (60:40:1), or by evenly spreading the samples on areas 2 × 4 cm marked out on the plate. Zones were made visible with iodine vapour.

RESULTS

Unesterified fatty acids were extracted by dissolving the gel in KOH with a 2-min incubation at 100°. Recovery of labelled palmitic acid in 5 tests averaged 93% (Table I). Recovery of 1.6 mg labelled tripalmitin using the same method in 4 tests averaged 94% (range 92–96%; streaked on TLC plates and developed in solvent). A short incubation of the gel with KOH in boiling water resulted in only partial hydrolysis of trilinolenin and triheptadecanoin, but complete hydrolysis was achieved by incubating the tubes for 10–20 min (Fig. 1). There was no loss of linolenic acid. Arachadonic acid subjected to a similar procedure had 94% of the mass and had the same retention time on GLC analysis as an equivalent amount of original solution. Significant oxidation of both linolenic and arachidonic acids was found to occur, however, when the plates were exposed to air for some time. This can be avoided by using 1 mg DL-α-tocopherol (Sigma) for every 100 mg fatty acid[6], without giving spurious peaks on GLC analysis. Dipalmitoyl lecithin on silica gel was completely hydrolysed after just a short incubation at 100° with KOH. Cholesterol esters were not fully hydrolysed, however, even after 30 min incubation. Therefore, the alternative method of quickly dissolving the gel, acidifying, extracting with pentane and saponifying with alcoholic KOH was employed.

Compared with heptadecanoic acid standard, the oleic acid of cholesterol oleate was recovered in 5 tests with an efficiency of 97% with a range of 95–106%.

TABLE I

RECOVERY OF PALMITIC ACID

Recovery of 75,000 dpm [1-^{14}C]palmitic acid added to 100 mg lots of silica gel on an area of plate about 1 × 4 cm (0.4 mm thick). Various amounts of carrier palmitic acid were added along with the label and the gel extracted into 1 ml KOH followed by a 2-min incubation at 100° (see text) followed by extraction into pentane.

Palmitic acid (μg)	*Recovery (%)*
0.25	93
0.25	92
500	94
500	95
1000	93

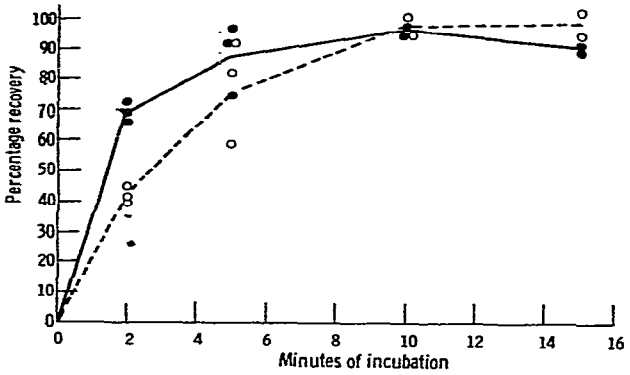

Fig. 1. Fatty acid recovery from 200-μg samples of glycerol triheptadecanoin and glycerol trilinolenin added to 200-mg areas of silica gel. The gel was incubated for various times at 100° with 1 ml KOH, acidified and extracted for fatty acids using pentane as described in the text. The fatty acids were estimated by GLC. Each point represents one separate determination. —○—, Heptadecanoic acid; —●—, linolenic acid.

Palmitic acid in dipalmitoyl lecithin was recovered in another 6 tests at an efficiency of 94% (88–100%). Linolenic acid from glycerol trilinolenin was extracted with an efficiency of 94% (90–98%) recovery (6 tests). Iodine used to stain the TLC plates was not found to cause significant quenching during scintillation counting.

DISCUSSION

TLC is a very widely used technique for separating lipids in extracts of biological material. The amount of lipid separated is small and quantitation is often done by charring or staining followed by densitometric measurement[7,8]. The method described here was designed to enable quantitation of lipid zones on TLC plates using precise analytical techniques but avoiding the lengthy extraction procedures currently available. The technique is over 90% efficient and is simple to perform. The efficiency of extraction is independent of the amount of lipid present.

If chloroform is used to replace the pentane, Duncombe's method[5] can be applied directly to the measurement of fatty acid concentrations. When the amounts are very small (< 0.5 μmole), a modification of Duncombe's method[9] can be used with chloroform–heptane (70:30) extracts. Glycerides (mono-, di- and triglycerides), and phospholipids can be completely hydrolysed during the digestion of the gel, but cholesterol esters require a further saponification stage with alcoholic KOH. An obvious disadvantage of the method is that it cannot be applied to the extraction of intact esters or any other lipid that is likely to be affected by strong alkali. The procedure does not, however, cause isomerization of linolenic acid which has been reported to occur with the use of prolonged saponification times with concentrated alkalies[10], nor does it result in oxidation of highly unsaturated fatty acids. Moreover, it is possible to analyse the acidified aqueous phase for glycerol and other water-soluble products. Lipids not containing fatty acids such as free cholesterol can also be extracted. Another advantage is that radioactivity, due to [^{14}C]- or [^{3}H]-labelled fatty acids, can be measured with minimal quenching and self-absorption problems. The

method can be applied to the determination of specific activities by measuring radioactivity in the same extract on which mass analysis is performed. Although oxidation of unsaturated fatty acid was not seen to occur during the procedure, precautions must be taken during the performance of the TLC by using antioxidants and exclusion of air. This is especially important when levels are low. The method is not suitable for use with silica gel containing binding agents.

REFERENCES

1 R. Bickerstaff and E. F. Annison, *Biochem. J.*, 118 (1970) 433.
2 B. Goldrick and J. Hirsch, *J. Lipid Res.*, 4 (1963) 482.
3 F. Snyder and N. Stephens, *Anal. Biochem.*, 4 (1962) 128.
4 W. A. Shaw, W. R. Harlan and A. Bennett, *Anal. Biochem.*, 43 (1971) 119.
5 W. G. Duncombe, *Biochem. J.*, 88 (1963) 7.
6 B. Century, B. C. Lloyd, L. A. Whitting, C. C. Harvey and M. K. Horwitt, *J. Nutrition*, 75 (1961) 341.
7 J. Clausen, H. O. Christensen-Lou and H. Andersen, *J. Neurochem.*, 12 (1965) 599.
8 A. N. Siakotos and G. Rouser, *Anal. Biochem.*, 14 (1966) 162.
9 M. C. Elphick, *J. Lipid Res.*, 16 (1975) 406.
10 H. J. Ast, *Anal. Chem.*, 35 (1963) 1539.