Journal of Chromatography, 80 (1973) 133–136

CHROM. 6664

Note

Separation and quantitative recovery of lipid classes; a convenient thin-layer chromatographic method

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(Received November 20th, 1972; revised manuscript received February 22nd, 1973)

The introduction of thin-layer chromatography (TLC) somewhat more than a decade ago has made separation of lipids into compound classes a practical matter. Separation of lipid classes on a quantitative basis, however, has remained a problem. None of the current methods for class separation were satisfactory in giving clear separation of amounts of lipid needed by our laboratory. Neither were they conveniently adapted to readily available laboratory equipment. This report shows how bands of cholesterol esters, triglycerides, free fatty acids, cholesterol, and phospholipids are separated from total lipid extracts for quantitative recovery on 20 × 20 cm commercially prepared sheets of silica gel by a two-step development procedure.

Because of the polarity extremes among lipid classes, use of a single-step solvent system has not proven feasible. The difficulties inherent in trying to chromatograph a mixture of non-polar and more polar lipids by using a one-step developer have been discussed by Freeman and West[1]. In 1969 Manners *et al.*[2] reported that no one-step development was available although earlier Storry and Tuckley[3] had used special equipment to accomplish such resolution. Some of the difficulties in achieving lipid class separation are solved by using a step-wise procedure of development that utilizes a polar solvent system preceding, or following a non-polar system. The rationale for using a step-wise approach for lipid class separation instead of a single-step system has been reviewed by Browning[4].

METHODS AND MATERIALS

Total lipids were extracted from rat and porcine livers by the method of Folch *et al*[5]. The extract was evaporated to a known volume and an aliquot dried for weighing with a Cahn electro-balance. From the determined concentration of lipid an aliquot representing 20 mg of lipid was concentrated to 200 μl and applied with a Hamilton syringe to a silica gel plate at a height of 20 mm. The plates used were the commercially available Eastman Chromagram Sheets No. 6061 Silica Gel. Lipid standards were obtained from Sigma Chemical Co., St. Louis, Mo. A standard mixture of cholesteryl oleate, cholesteryl stearate, trilinolein, oleic acid, stearic acid, cholesterol, sphingomyelin, and lecithin was applied to one side of the plate. Individual standards and standard mixtures were chromatographed to determine their positions relative

to the origin. Development of the chromatogram was carried out at room temperature in covered glass tanks lined with filter-paper to saturate the environment. The developer was added to a tank-depth of 10 mm.

Developer I was a system of benzene–hexane (15:85). Developer II was composed of acetic acid–ethyl ether–hexane (2:29:69). Acetic acid has been recommended as preventing streak and tail formation[6]. The sheets were developed in each system to 19.5 cm requiring 40 to 45 min for each plate. Until the bands were recovered by elution, the sheets were stored in a nitrogen atmosphere to prevent alteration of any lipids by oxidation.

In our laboratory, the general lipid classes and accompanying standard spots were located by an exposure of 30 sec at room temperature to iodine vapors. The brownish bands were encircled with a No. 2 soft lead pencil and labeled as to class because the iodine fades during storage or during elution and drying. Iodination causes some loss of polyunsaturated fatty acids. If fatty acids are to be recovered unaltered, other spray reagents can be used to detect the general or specific lipid classes.

The penciled areas were cut from the sheet and divided into small strips which were collected by funneling into labeled flasks for recovery by elution. All classes except phospholipids were removed by alternating two elutions of ethyl ether with two elutions of chloroform–methanol (4:1) (ref. 7). Since phospholipids are somewhat difficult to remove from silica gel, a separate elution procedure was necessary. For phospholipid bands, two elutions of chloroform–methanol (2:1) were alternated with two elutions of chloroform–methanol–water (3:5:2) (ref. 8). Agitation of 2 min by shaking or vibration were used during each wash. The washes from each eluted band were pooled into centrifuge tubes and evaporated to dryness by a nitrogen stream. 4 ml of chloroform–methanol (2:1) was added to redissolve the lipid fraction. These 4-ml solutions contained suspended particles of silica gel and were cleared by centrifugation at approximately $1000 \times g$ for 10 min. If necessary, any traces of suspended silica gel can be removed by vacuum filtration through a sintered-glass funnel, correcting for changes in volume. A 0.5-ml aliquot of the cleared or filtered solution was removed, dried, and weighed.

To determine whether this development system and the silica gel sheet actually permitted separation and recovery in quantitative amounts, known amounts of standards were applied to sheets for development. A known amount of mixed standards (10 to 15 mg) was applied to each sheet. After development the bands were cut out and eluted as above. The eluate was dried, then taken up to 4 ml in chloroform–methanol (2:1), centrifuged and filtered. A 0.5-ml aliquot of the clear, 4-ml volume

TABLE I

RECOVERIES OF STANDARDS FROM BANDS ON CHROMAGRAM SHEETS

Lipid	*Recovery (%)*
Cholesteryl stearate	95.6
Trilinolein	99.3
Stearic acid	101.8
Cholesterol	98.9
Lecithin	97.0

was placed in an aluminum boat, dried, and weighed on the electro-balance to determine the percent recoveries listed in Table I. Duplicate trials were made, using 10- to 15-mg samples per trial.

RESULTS AND DISCUSSION

Fig. 1 shows the separation and location of lipid bands from porcine liver and a mixture of standards. Developer I moved the cholesterol esters away from the origin where all other classes remained. Developer II moved cholesterol esters, triglycerides, fatty acids, and cholesterol varying distances from the origin, while the phospholipids remained at the origin. The phospholipid band may possibly contain some glycolipids, but this was not explored by making a phosphorous determination. Developer II alone resulted in some separation, but the cholesterol ester and triglyceride bands were superimposed. It was found that a development system benzene–hexane (15:85) separated cholesterol esters and triglycerides. Discrete bands from lipid extracts were formed on the Chromagram sheets when amounts up to 20 mg of lipid were applied in a line at the origin. Single lipid standards would not form a discrete band if the amount applied was over 15 mg.

Early procedures for TLC of lipid compounds used a combination of ab-

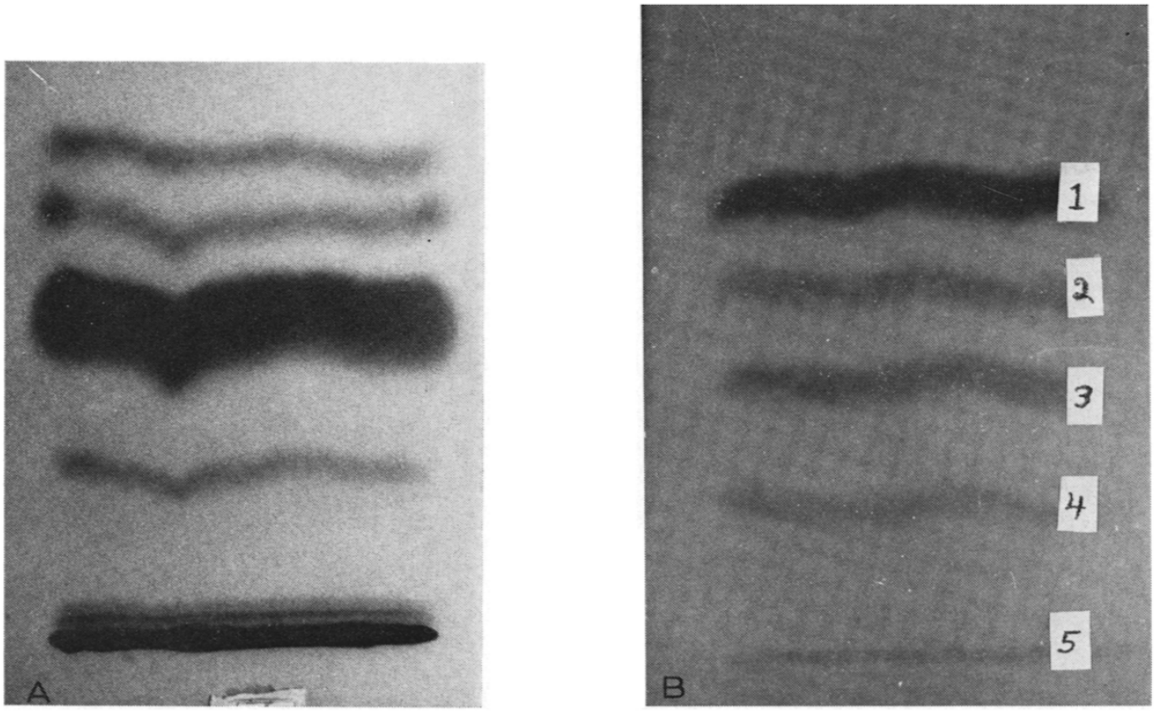

Fig. 1. These chromatograms show the developed bands of lipids as they appear after exposure to iodine vapor. (A) Chromatogram from a lipid extract prepared from a porcine liver homogenate. (B) Separation of the eight standards into bands numbered 1 to 5. 1 = Cholesterol esters (cholesteryl oleate, cholesteryl stearate); 2 = triglyceride (trilinolein); 3 = free fatty acids (oleic acid, stearic acid); 4 = cholesterol; 5 = phospholipids (lecithin, sphingomyelin).

sorbents, single solvent systems and techniques such as reversed-phase TLC[6]. In 1966, reports began to appear on the separation of lipid classes by TLC using multi-solvent systems[1-3,6,7,9-12]. Most methods just referenced were concerned with spot identification on specially prepared glass plates. The Freeman and West[1] method required hand-made gel plates of non-standard size and did not resolve bands for large recoveries. Katyal *et al.*[9] reported that they isolated neutral lipid bands after separating out polar lipids with column chromatography. The method of Skipski *et al.*[7] was followed to separate classes in a total lipid sample. We found the separation of lipid classes into discrete bands poor. Compounding this disadvantage in the methodology of Skipski is the requirement of a trial-and-error process for selection of the second solvent system. The Skipski method requires that the second solvent system be altered to achieve clear-cut separation of bands when the lipid extract contains a large proportion of one lipid class. Limitations in sample size and laboratory time preclude use of a trial-and-error process to find a workable solvent system. Finally, better separation of lipid classes is desirable.

This paper reports the development of a system of solvents that gives clearer band separations regardless of the ratio of lipid classes in the extract. The method evolved makes use of standard, commercially produced TLC plates which avoids the labor, special equipment, and variation of hand-made plates. Using this TLC method, isolated quantities of separated lipid classes can be recovered for quantitative comparison by radio-counting, gravimetric determination, or they can be subjected to further fractionation.

ACKNOWLEDGEMENT

This work was supported by United States Public Health Grant HD-01784.

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