Gradient-thickness thin-layer chromatography for the isolation and analysis of trace amounts of free fatty acids in large lipid samples

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ABSTRACT A thin-layer chromatographic method for quantitative isolation of free fatty acids is described. This method appears to be more satisfactory than existing methods in offering the combination of advantages of specificity, simplicity, rapidity, reproducibility, accuracy, high sensitivity, and applicability as a preparative technique.

The method involves chromatography on a thin-layer plate on which the layer of Silica Gel G decreases linearly in thickness from 1000 μ at the base to 125 μ at the upper end. This gradient-thickness design allows the separation and densitometric quantitation of very small traces of free fatty acids from relatively large and complex lipid samples in a single chromatographic step.

The method has been shown to be applicable directly to the crude total lipid extracts of several mammalian tissues. It appears to generate little if any artifactual free fatty acids from the breakdown of complex lipids, in contrast to the undesirable behavior of silicic acid columns in this respect.

Gradient-thickness thin-layer chromatography promises to be useful for the quantitative isolation of trace amounts not only of other types of lipids but also of classes of compounds other than lipids.

SUPPLEMENTARY KEY WORDS charring · quantitative densitometry · preparative chromatography · protection against peroxidation · artifactual free fatty acids · brain · liver · heart · blood plasma · coenzyme Q · triglycerides · hydrocarbons

A LARGE NUMBER of methods have been described for the quantitative isolation and(or) analysis of the FFA of biological lipid samples. These methods involve acid-base titration (1–3), colorimetric measurements (4–11), column chromatography (12–14), TLC (15, 16), GLC, or combinations of these techniques (17–22). Some of these methods have been incorporated into automated determinations for application to large numbers of samples (23–25).

Current work in our laboratory is concerned with the FFA of brain. Since FFA occur as trace components of lipids, a method of isolation and quantitation that offers the combined features of specificity, applicability as a preparative technique, capability of quantitation of trace amounts of FFA in relatively large lipid samples, simplicity, rapidity, reproducibility, and accuracy is required. Space does not permit a systematic and critical review of existing methods for FFA analysis, but the situation can be summarized with the statement that no existing technique offers a suitable combination of all of these features. A new technique was therefore devised in order to allow study of FFA in tissues such as brain. The method, which involves TLC on a layer of graded thickness, is described in the present paper.

A number of laboratories have published methods for the isolation of FFA by TLC. The FFA spot on the plate is subsequently quantitated by charring and densitometry (15) or by charring and measurement of spot area (16), or the FFA are eluted from the TLC adsorbent and

Abbreviations: FFA, free fatty acids; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; BHT, butylated hydroxytoluene.

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quantitated by colorimetry (20) or by GLC (21, 22). All the TLC methods now in the literature utilize adsorbent layers of constant thickness. By contrast, the gradient-thickness TLC method described in the present report offers the advantage of allowing the isolation, detection, and quantitation or elution and further characterization of exceedingly small traces of FFA in relatively large lipid samples. To the best of our knowledge, gradient-thickness TLC has not previously been described in the literature.

A preliminary report of portions of this work has appeared (26).

MATERIALS AND METHODS

A major feature of the present method is the use of a plate (Fig. 1) designed such that the thin layer of adsorbent decreases in thickness in a linear fashion from 1000 μm at the base to 125 μ at the top (Chromaflex T.L.C. Gradient Plate; Kontes Glass Co., Vineland, N.J.). This allows the use of relatively large lipid samples in order to permit detection and quantitation of trace components. Gradient-thickness TLC can be considered to be analogous to the recently described procedure of multibore column chromatography (27, 28) which has been shown to allow finer separations than are generally achieved with columns of uniform thickness.

The plate was placed on a bench top on a small spot of water for the purpose of holding it by capillarity during the spreading step. The slurry, made by mixing 15 g of Silica Gel G with 30 ml of water for each plate, was immediately poured over the thick end of the plate and spread over the surface by an Applicator Rod (Kontes Glass Co.). The plate was allowed to dry on the bench top for at least 2 hr and was then activated at 110–120°C for 30 min. Alternatively, the plate can be left to dry on the bench top for only 10 min and then placed in a nearly vertical position in an air current in a fume hood for approximately 30 min prior to activation. The activated plate is then allowed to cool under a Plexiglas cover (29) in a stream of dry nitrogen.

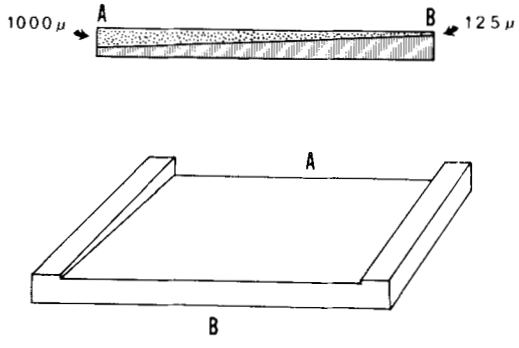

FIG. 1. Schematic diagram of a gradient-thickness TLC plate (lower drawing). Certain proportions of the plate are exaggerated in order to show clearly the raised borders and the varying depth of the wedge-shaped space that will contain the TLC adsorbent. The slurry is poured into this space and spread by means of a precisely straight glass rod until the surface is flat. The height of the raised glass borders thus determines the thickness of the layer at any given distance along the plate. The upper drawing is a diagram of a cross-section taken through the plate after the adsorbent layer has been spread. The cross-section is taken between points *A* and *B* of the lower drawing.

Lipids were extracted from tissues with chloroform–methanol 2:1 (30). It was found usually to be unnecessary to remove nonlipid contaminants from the extract. Aliquots of the crude unwashed lipid extract were evaporated under a stream of nitrogen and redissolved in a small volume of chloroform–methanol–water 15:5:0.8. This solution was used for spotting the thin-layer plates. The presence of water in the solvent mixture usually prevented the contraction and falling off of the spot that happened occasionally during evaporation of the solvent when there was more than 4 mg of lipid per spot. However, even using the above solvent, some trouble with retraction of spots was encountered in the case of total brain lipids unless the extract was freed of solvent under a stream of nitrogen at 50–55°C and redissolved in chloroform–methanol 2:1. Under these conditions most of the proteolipid protein remained insoluble. The proteolipid protein precipitate was separated by centrifugation, and the supernatant solution was transferred to another tube. The precipitate was washed with a small volume of chloroform–methanol 2:1, and the centrifugation was repeated. The supernatant solutions were combined and evaporated to dryness at 50–55°C under nitrogen. The lipid sample was then redissolved in chloroform–methanol–water 15:5:0.8 for spotting. At least 11 mg of brain lipid could then be applied as a single spot.

As an aid in the protection of the polyunsaturated components of the FFA against peroxidation, the antioxidant BHT may be added not only to the solvent used for spotting the lipids on the plate but also in a concentration of 0.05% to the developing solvent. BHT is superior to other antioxidants for use in the developing solvent insofar as it migrates on the plate faster than FFA, and thus the FFA are continuously in contact with the antioxidant during the entire developing step and following evaporation of the developing solvent.

FFA standards (usually palmitic acid) and unknown samples were spotted near the thick end of the layer while the plate was in a nitrogen atmosphere under the Plexiglas cover. The spot area for the standard at the origin was made to be of such a size that after development it yielded a spot area that approximated the area of the developed FFA spots from the unknowns. After evaporation of spotting solvent, the plate was placed in a filter paper-lined tank containing petroleum ether (bp 37–

51°C)–diethyl ether–glacial acetic acid 50:50:2 as the developing solvent. The solvent (approximately 200 ml) was allowed to wet the filter paper thoroughly and equilibrate for a period of at least 45 min before the plate was placed in a chromatography tank. Two plates could be run simultaneously in a single 30 × 10 × 28 cm tank. The plate was removed as soon as the solvent reached the entire length of its upper edge, and the solvent was then allowed to evaporate thoroughly before visualization of spots was attempted.

The plate was sprayed with a charring reagent that consisted of a solution of 0.6 g potassium dichromate in a mixture of 55 ml of concentrated sulfuric acid and 45 ml of water. The spraying flask (Fisher Scientific Company, Pittsburgh, Pa.) was held 50 cm from the plate and was operated at a high rate of gas flow in order to create a fine mist. The spraying was judged to be optimal when the thin end of the silica gel layer (always at the top during spraying) had just begun to take on a light yellow appearance. The plate was charred in an oven at 180°C until fumes were no longer evolved from the plate (approximately 1 hr). The charred plate had a lighter background if the oven was well ventilated during the charring process.

After remaining charring reagent was removed from the edges and back of the plate, the amount of FFA in each spot was determined by densitometry. A Photovolt Photometer Model 520-A with Transmission Density Unit Model 52-C was used, and the density was recorded by a Varicord Model 42-B (Photovolt Corp., New York). The slit length was kept the same for all spots on any given plate and was slightly longer than the width of the widest spot on the plate. The response control knob was kept at a setting of 7. The scanning was begun near the thin end of the plate. The base line increased in a linear fashion due to the increasing thickness of the adsorbent layer (Fig. 2). Therefore the lamp brightness adjustment was set for each spot such that the minimum in the optical density tracing that occurs at the termination of the FFA peak was at the same per cent of full scale for all the FFA spots on the plate. For most plates this minimum was set at 10% of full scale. The area under each peak was determined by a planimeter (Keuffel and Esser Co., Hoboken, N.J.). If densitometric equipment is not available, it is possible to elute the isolated FFA from the adsorbent and apply a suitable colorimetric determination (e.g. references 4–11).

RESULTS AND DISCUSSION

Application of Method to Mammalian Tissues

The method was applied to total lipid extracts of several rat tissues, including plasma, liver, heart, and brain. The results of a typical TLC run are shown in Fig. 3.

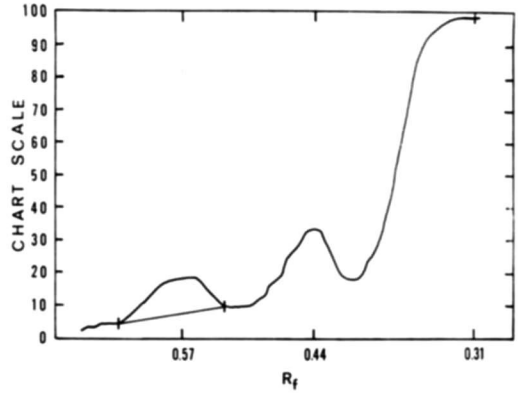

FIG. 2. Densitometer tracing obtained from scanning the lane containing the liver lipid sample in the TLC plate illustrated in Fig. 3. The base line increases with decreasing R_f because of the linear increase in thickness of the adsorbent layer. The usual manner of drawing the base line under a peak is shown. The enclosed area is measured by means of a planimeter.

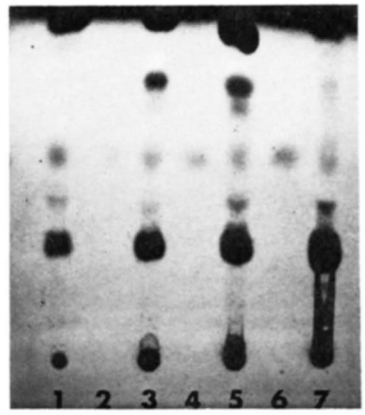

FIG. 3. Gradient-thickness TLC on Silica Gel G. The developing solvent is petroleum ether(bp 37–51°C)–diethyl ether–glacial acetic acid 50:50:2. Lane *1* is the total lipids from 60 μl of plasma; line *3* is 2.1 mg of heart total lipids; lane *5* is 4.2 mg of liver total lipids; lane *7* is 11.5 mg of brain total lipids; lanes *2*, *4*, and *6* are 1, 5, and 10 μg of palmitic acid standards, respectively. The pronounced streaking at the origin in the case of the brain sample is due to the large sample size but has no adverse effect on the FFA spot. The darkness of the background increases progressively in the direction of the origin, due to the linear increase in thickness of the plate.

All the samples were obtained from male rats (Charles River Breeding Laboratories, North Wilmington, Mass; C-D strain, weighing 180–240 g) fed ad lib. The rats were sacrificed by decapitation, and the organs were excised. Heart and liver were quickly washed in saline, blotted, weighed, and minced, and the homogenization in chloroform–methanol 2:1 in a Virtis apparatus was begun within 2 min after decapitation. When the heart was excised special care was taken to avoid contamination by adipose tissue from the basal region. The sample was entirely heart ventricles. The brain samples also were washed and blotted, but the homogenization was done by means of a glass homogenizer with a motor driven Teflon pestle within 35 sec after decapitation.

The FFA spot in all instances is small and does not show appreciable tailing. In the case of each of the four tissues, the FFA spot appears to be well separated from other components of the total lipid extract.

The purity of the FFA spot from each of the four tissues illustrated in Fig. 3 was determined by the following experiment which was performed in duplicate. The same amount of lipid was applied to each of two TLC plates. Both plates were developed as described above. One plate was charred while the other plate was sprayed with 2,7-dichlorofluorescein (0.2% in ethanol), and the spots were visualized and outlined under ultraviolet light. This latter plate was then sprayed lightly with water, and the FFA spots were scraped off into centrifuge tubes containing 2 or 3 ml of 0.22% formic acid in chloroform (31). The contents of the tube were mixed well, and the silica gel was sedimented by centrifugation. The supernatant solution was transferred to another tube, and the silica was washed with 2 ml of the same solvent. After centrifugation, the two supernatant solutions were combined and their volume was reduced by a stream of nitrogen. The FFA content was then determined by the colorimetric method of Novak (10). For each of the four tissues examined, the direct densitometric measurement of the spot on the plate gave a value that was 97–99% of the amount of FFA determined by the colorimetric measurement of the isolated FFA, indicating that in each case the spot was essentially pure FFA. The high specificity of this TLC separation indicates that it can be used as a preparative technique for the isolation of trace amounts of FFA from relatively large biological lipid samples for the purpose of further characterization of the FFA fraction.

Table 1 shows the amounts of FFA in rat brain, liver, plasma, and heart as determined by charring and densitometry from a TLC plate such as that illustrated in Fig. 3. The tissues had been removed from the rats and homogenized in chloroform–methanol as rapidly as possible, since it is known that FFA can be produced very rapidly in tissues, especially brain, after decapitation of the animals (26, 32). The FFA content for rat liver shown in Table 1 is lower than previously published values (33–35). These differences may be due to differences in nutritional states of the animals, differences in the time intervals between killing of the animals and homogenization of the tissues, artifactual generation of FFA from complex lipids on silicic acid columns, or perhaps other factors. The value for brain FFA in Table 1 is much lower than published data for the rat (36) and human (18) and slightly lower than that for the mouse (37). The plasma FFA content in Table 1 is similar to previously reported data obtained by other techniques (38, 39).

TABLE 1 CONTENT OF FFA IN RAT TISSUES

Tissue*	No. of Samples	μg/g of tissue (wet weight) mean ± SEM
Plasma	7	96 ± 3.3
Heart	7	98 ± 4.4
Liver	6	73 ± 5.0
Brain	7	37 ± 1.4

* The samples were obtained as described in the l gend of Fig. 3. Each sample was from a different rat.

Recovery of FFA

As one check of the absolute accuracy of the method, a brain was homogenized in chloroform–methanol very soon (40 sec) after decapitation of the rat, and the homogenate was divided into two portions. To one portion palmitic acid was added in the amount of 50–100 μg/100 mg total lipid. The analysis for FFA was then carried out as described above, and it was found that the difference between the FFA contents of the two portions of the homogenate amounted to a recovery of 96–98% of the quantity of added palmitic acid.

As a second check on the accuracy, three samples of palmitic acid, four of arachidonic acid, and four of docosahexaenoic acid were applied to gradient plates. Each spot contained 50–100 μg of FFA. The acids migrated up the plate in the solvent system containing BHT as described above. The spots were visualized with 2,7-dichlorofluorescein under ultraviolet light and were scraped off. The FFA were converted to the methyl esters in BF_3-methanol. Aliquots of the original FFA solutions were also converted to methyl esters with BF_3-methanol. The over-all recovery was then determined by comparison of the original FFA samples with those subjected to TLC, using GLC for the quantification. The over-all recovery was found to be 98% for palmitic acid and 91% for both arachidonic and docosahexaenoic acids. Therefore the method is suitable for the nearly quantitative isolation of trace amounts of FFA, including polyunsaturated fatty acids, with only slight losses due to autoxidation or other causes.

Relative Extent of Charring of Different FFA

It has been reported that the extent of charring of certain lipids on a TLC plate can under some conditions be affected by the degree of unsaturation (40, 41). To test this for FFA under the conditions described in the present paper, duplicate 10-μg samples of palmitic, stearic, linolenic, and arachidonic acids were spotted on a gradient plate, and the chromatogram was developed and charred as above. Three such chromatographic separations were carried out for a total of six spots for each fatty acid. The area under the peak for the densitometry tracing for palmitic acid was taken as 100. The areas for stearic acid, linolenic acid, and arachidonic acid were 101 ± 5 (SD), 80 ± 4, and 68 ± 2, respectively. Since the experiment described in the previous paragraph shows that there is less than a 10% loss of

even the most highly unsaturated fatty acids during a chromatographic analysis, it is clear that the lower densitometric values for linolenic and arachidonic acids are due to a smaller extent of charring for these fatty acids and are not due to actual losses of FFA. Our results on the effects of the degree of unsaturation upon the extent of charring differ somewhat from previously published data (40, 41). For example, whereas we find that the extent of charring of FFA decreases with increasing unsaturation, the earlier workers reported that certain unsaturated lipids charred to a greater extent than the corresponding saturated lipids. The differences in results may be due to the fact that the published data do not include FFA or possibly to differences in experimental conditions such as composition of the charring reagent.

It is thus evident that when the method is used for quantitative analysis by densitometry, some systematic absolute error will be present when the sample contains polyunsaturated FFA. However, this error will not be serious unless the proportion of polyunsaturated FFA in the sample is unusually large. Since the above data show that the extent of charring of individual FFA is very reproducible, this does not present a problem for the usual type of comparative studies in which the various samples do not differ radically in their extent of unsaturation.

Sensitivity and Linear Range

Fig. 4 illustrates the sensitivity and linear range of the method. It can be seen that the linear range can be extended upward by use of a relatively longer slit or downward by use of a shorter slit. Use of the gradient-thickness plate allows detection of less than 1 μg of FFA originating from a single spot containing at least 11 mg of total lipid (Fig. 3). This high sensitivity permits the use of very small tissue samples. For example, only 50 μl or less of plasma is required for the accurate determination of normal FFA levels.

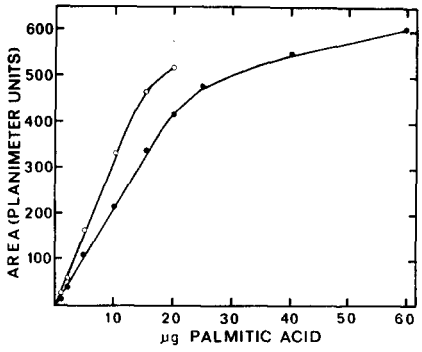

FIG. 4. Typical standard curves. The slit located between the TLC plate and the photocell of the densitometer was 13 mm long in the case of the data represented by the solid circles and 6 mm long in the case of the open circles. The slits were purchased from Photovolt Corp., New York. Both curves were obtained by scanning the same plate. The curve represented by open circles was not extended above 20 μg because the width of the spot containing such large amounts of FFA exceeded the 6 mm length of the slit.

Reproducibility

The reproducibility of the method was checked by extraction of the total lipid from 1 ml of rat plasma with chloroform–methanol, and division of the crude lipid extract into five equal aliquots. Each aliquot was analyzed on a separate TLC plate and compared with its own palmitic acid standard. The mean FFA content ±standard deviation was 99.8 ± 5.3 μg/ml of plasma. The variability in the intensity of charring from one plate to another is greater than the variability within a single plate, and therefore at least one standard spot should be applied to each plate.

Artifactual Production of FFA on Silicic Acid Columns

Prior to development of the method described above, an attempt was made to use silicic acid columns either treated (14) or not treated (12, 13) with KOH in order to isolate a FFA-rich fraction from a total lipid extract of brain prior to subsequent purification by TLC. It was discovered, however, that there was a time-dependent artifactual generation of FFA on either type of silicic acid column. Even with relatively rapid use of the column, FFA were produced in an amount sufficient to give an apparent FFA content that was at least double the true value as obtained by the direct gradient-thickness TLC method described above. Since the FFA content of brain is approximately 0.04% of the total lipids, the amount of FFA artifactually generated on the silicic acid columns represents the destruction of only a tiny fraction of any of the major lipid species present and might remain undetectable except by direct measurement of the amount of FFA produced. It should be emphasized that the column methods are still applicable when the FFA represent a major component of the lipid mixture, even though the columns give erroneous results when the FFA are only a trace component such as in the brain.

Advantages of the Gradient-Thickness TLC Method

It is clear from the experiments described above that the gradient-thickness TLC method for FFA analysis offers a unique combination of advantages. Its high sensitivity and specificity allows the quantitative isolation of FFA, free of contaminating lipids, in a single chromatographic step even when the FFA occur only in trace amounts in a complex sample such as the total lipids isolated from brain. The method may be used for either preparative or analytical purposes. It is rapid and simple, and in most cases aliquots of a crude total lipid extract can be spotted

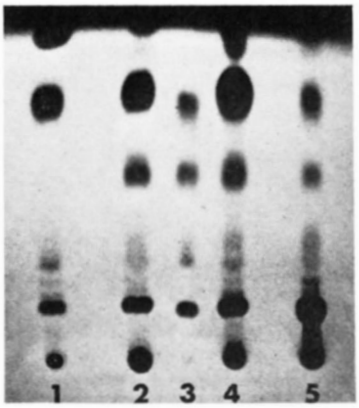

FIG. 5. Gradient-thickness TLC on Silical Gel G. The developing solvent is petroleum ether (bp 37–51°C)–diethyl ether–glacial acetic acid 70:30:1. Lane *1* is the total lipids from 46 μl of plasma; lane *2* is 1.6 mg of heart total lipids; lane *4* is 2.7 mg of liver total lipids; have *5* is 11 mg of brain total lipids; and lane *3* is a standard mixture composed from bottom to top of cholesterol, palmitic acid, coenzyme Q, and tripalmitin. Other conditions were similar to those described for Fig. 3.

directly on the plate after evaporation of solvent and redissolving in an appropriate small volume. The TLC method appears to generate little if any artifactual FFA from the breakdown of complex lipids, in contrast to the undesirable behavior of silicic acid columns in this respect.

From the chromatograms shown in Fig. 5, it can be seen that gradient-thickness TLC is also useful for the separation of trace quantities of other lipids from the crude total lipid extracts of tissues. For example, using petroleum ether(bp 37–51°C)–diethyl ether–glacial acetic acid 70:30:1, a complete separation of coenzyme Q (R_f = 0.5), triglycerides (R_f = 0.7), and hydrocarbon components (solvent front) of crude total lipid extracts from several rat tissues was obtained. By suitable manipulation of the solvent system and stationary phase, it should be easily possible to use this type of TLC plate for isolation of trace amounts of individual phospholipids and other relatively polar lipids as well as compounds other than lipids.

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