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Note

Thin-layer chromatographic separation of free fatty acids

Analysis and purification of radioactively labelled fatty acids

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In a research project of this laboratory, binding of fatty acids to bovine serum albumin is used as a model system for studying drug–biomacromolecule interaction. Determination of the concentration of free and bound fatty acids in equilibrium-dialysis experiments can be accomplished by using ^{14}C-labelled fatty acids; although in the dialysis experiments, the ^{14}C-labelled fatty acids are diluted many times with the ^{12}C-isotope analogues, the binding of the labelled compounds is monitored. For this reason, the labelled fatty acids must be essentially free from contaminating radioactive compounds.

To check their purity, chromatography is chosen, as this technique can be applied for analysis as well as purification of the compounds. In order to limit the number of operations with radioactive material before and after the purification, there is a preference for analysis of the free fatty acids (FFA) rather than their derivatives. Moreover, as the relative change in physico-chemical properties (lipophilicity) is larger in a series of FFA than in their derivatives or salts, chromatographic separation of the FFA is preferable. Paper[1] and thin-layer[2] chromatographic (TLC) methods described for separating FFA as ammonium salts report R_F values for the C_7 to C_9 acids that are too close to allow easy purification.

The difficulty in obtaining reproducible R_F values in TLC with methyl acetate–2.5% aq. ammonia (95:5, v/v) as developing solvent was another factor that persuaded us to seek an alternative chromatographic method.

The excellent gas chromatographic[3–5] and electrophoretic[6] separation of FFA cannot easily be adapted to the preparative separation of radioactive FFA. This contrasts with TLC, which, in addition, is relatively inexpensive and requires only compact equipment[7].

As silanised silica gel has become commercially available for TLC, it is now possible to separate FFA by reversed-phase chromatography[7,8]. Thus, it appeared to us to be worthwhile to look for a simple solvent system for use with this lipophilic adsorbent. As will be shown, we have succeeded in obtaining good separations for the C_2 to C_9 FFA with a methanol–water system, and have devised a system satisfactory for the purification of ^{14}C-labelled C_5, C_6 and C_7 FFA.

MATERIALS

The propionic (C_3), butyric (C_4) and hexanoic (C_6) acids were obtained from Baker (Deventer, The Netherlands). The pentanoic (C_5) acid was from Koch-Light (Colnbrook, Great Britain) and the heptanoic (C_7) acid was from Fluka (Buchs, Switzerland). The purity of these acids was checked by gas chromatography at 200° on a column of Porapak Q and was found to be better than 99% by the internal-standard procedure.

The [1-^{14}C]-labelled acids, as sodium salts, were obtained from The Radiochemical Centre (Amersham, Great Britain) and had specific activities ranging from 10–20 mCi/mmole. These labelled salts were stored at −20° as stock solutions in methanol containing 50 μCi/ml.

The TLC plates (20 × 20 cm) were prepared with Desaga equipment (Desaga, Heidelberg, G.F.R.) according to the specifications for the silica gel (HF 254, silanised; E. Merck, Darmstadt, G.F.R.).

The scintillation liquid, Insta-gel, was from Packard (Brussels, Belgium) and counting was performed on a Packard Tricarb liquid scintillation spectrometer. The direct-contact X-ray film was Kodak RP 54.

All other materials used were from E. Merck.

METHODS AND RESULTS

The liquid non-labelled FFA were diluted five times with methanol before being applied to the plate. For the analytical separation, 10 μl of the stock solution of the radioactive acid (C_n, sodium salt) was placed in the tip of a conical tube, and 1 μl of undiluted FFA mixture (C_{n-1}:C_n:C_{n+1} = 1:1:1) was added. This mixed sample was sufficient for about five spots of diameter 3 mm. All processes involving FFA were carried out at 4°.

For the preparative separation of radioactive FFA, 250 μl of the radioactive stock solution was placed in the tip of a conical tube and the solvent was evaporated at 60° by aspirating off the vapour. The tip was then rinsed with 3 μl of 1 *N* hydrochloric acid in methanol, and the solution was applied as a "zone" of 4 cm in length. The initial spots were placed about 2 cm from the edge of the plate, and the solvent front was allowed to move 10 cm above the spots. All solutions were applied to the plate at 4°, and development[7] was carried out at the same temperature.

Detection of the non-radioactive FFA was by spraying with a saturated solution of methyl red in 0.2% methanolic sodium hydroxide. Detection of the radioactive FFA was by autoradiography or by zone analysis. With autoradiographic detection, the plates were first sprayed with the indicator solution, carefully dried and then sprayed with a solution of a fluid adhesive (Lero) in chloroform (this increased the resistance of the adsorbent to mechanical damage). Next, an X-ray film was placed in direct contact with the adsorbent, the contact time being 24 h for 0.05 μCi per spot. The zone analysis was performed by scraping the adsorbent from the wet plate in 3-mm zones at 4° (see ref. 9). With an analytical separation, the adsorbent of each zone was transferred to a counting phial containing 4 ml of water and 10 ml of Insta-gel, and the radioactivity in each phial was counted. For preparative separation, the

adsorbent from each zone was transferred to a conical centrifuge tube containing 1 ml of methanol, and the mixture was stirred.

To determine the position of the main peak, 1-μl aliquots of the supernatant solution in each tube were transferred to a counting phial containing 10 ml of Insta-gel, and the radioactivity in each phial was counted. The fractions containing the peak concentration of the acid were centrifuged at 3000 *g*, and the supernatant solution containing the purified acid was removed with a pasteur pipette. To check that the lower FFA had not volatilised during the processes preceding autoradiography, recovery experiments with [1-^{14}C]propionic acid were carried out; recovery of radioactivity was 97 $\pm$ 2%.

Development of a suitable solvent system

The solvent system dioxan–water–formic acid (60:35:5), although having a high resolving power for the higher FFA[8], is less suitable for separation of the lower FFA; this is mainly due to interference by this solvent system with the detection method used.

This disadvantage does not occur with the solvent system methanol–water, which gives excellent separation of the C_3 to C_9 FFA on silanised silica gel (see Fig. 1a). For optimal resolution, the methanol–water ratio depends on the length of the carbon chain of the acid being separated. The dependence of the R_F value on the methanol–water ratio is shown in Fig. 2 for the eight FFA investigated; from the curves in Fig. 2, an optimal ratio can be selected for a given mixture of FFA.

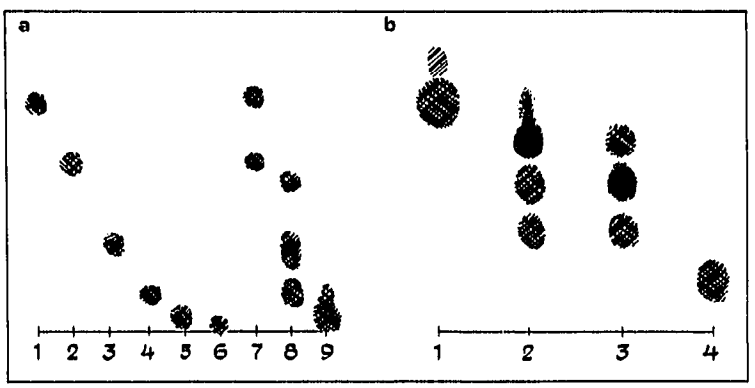

Fig. 1. Chromatogram of FFA on silanised silica gel: development time, 1 h at 4°; spray reagent, saturated methyl red solution in 0.2% methanolic sodium hydroxide. (a) Non-radioactive FFA; solvent: methanol–water (35:65). 1 = propionic acid; 2 = butyric acid; 3 = pentanoic acid; 4 = hexanoic acid; 5 = heptanoic acid; 6 = octanoic acid; 7 = mixture of propionic and butyric acids; 8 = mixture of butyric, pentanoic and hexanoic acids; 9 = mixture of hexanoic, heptanoic and octanoic acids. (b) Autoradiogram of ^{14}C-labelled fatty acids: solvent: methanol–water (40:60). 1 = propionic acid; 2 = mixture of butyric, pentanoic and hexanoic acids to which pure [^{14}C]butyric acid is added; 3 = mixture of butyric, pentanoic and hexanoic acids to which pure [^{14}C]pentanoic acid is added; 4 = heptanoic acid. The hatching indicates the area in which a red colour is visible after spraying. The blackness corresponds to that on the original autoradiogram and indicates the position of radioactive material. For further details, see text.

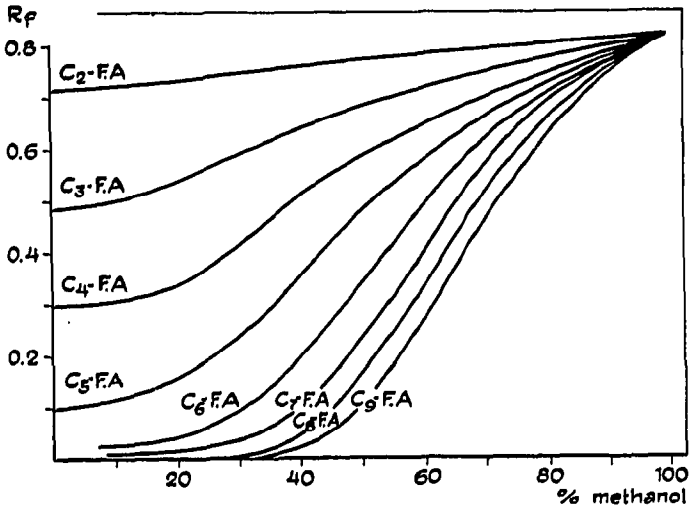

Fig. 2. Dependence of the R_F values of FFA on the methanol–water ratio. The curves permit selection of an optimal resolving ratio for a given mixture of FFA.

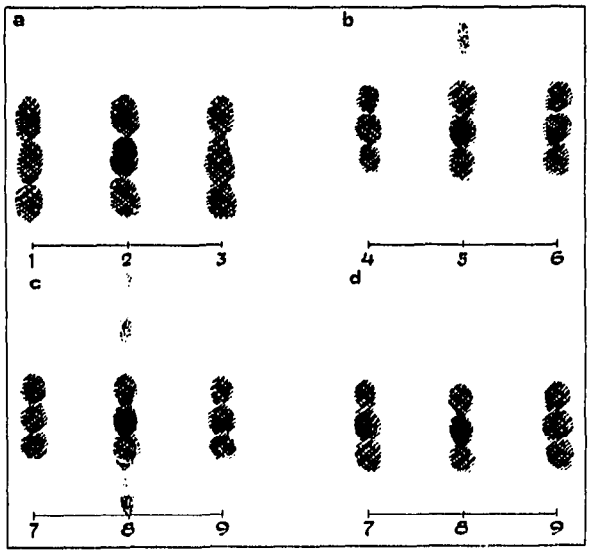

Fig. 3. Autoradiograms of ^{14}C-labelled FFA. (a): 1, 3 = mixtures of propionic, butyric and pentanoic acids; 2 = the same, but with the labelled butyric acid being analyzed added to the mixture. Solvent: methanol–water (20:80). (b): 4, 6 = mixtures of pentanoic, hexanoic and heptanoic acids; 5 = the same, but with the labelled hexanoic acid being analyzed added to the mixture. Solvent: methanol–water (50:50). (c): 7, 9 = mixtures of hexanoic, heptanoic and octanoic acids; 8 = the same, but with the labelled heptanoic acid being analyzed added to the mixture. Solvent: methanol–water (50:50). (d): 7, 9 = the same as in (c); 8 = the same, but with the purified [^{14}C]heptanoic acid added to the mixture. For further details, see legend to Fig. 1.

Analysis of labelled free fatty acids

The radioactive FFA were applied to the plate in the way described for the analytical separation; spots of non-radioactive mixtures were applied on either side of the radioactive spots to serve as reference spots. The detection of the labelled FFA was by autoradiography (see Fig. 1b), and depending on the results, one of the following procedures is carried out.

(a) If the autoradiogram indicates an impurity content much less than 1 %, as shown in Fig. 3a, there is no need for further chromatography. The result is confirmed by dilution analysis as the *p*-bromophenacyl ester.

(b) If the autoradiogram indicates an impurity content of about 1 % (as shown in Fig. 3b for hexanoic acid), the FFA has now to be analyzed quantitatively by zone

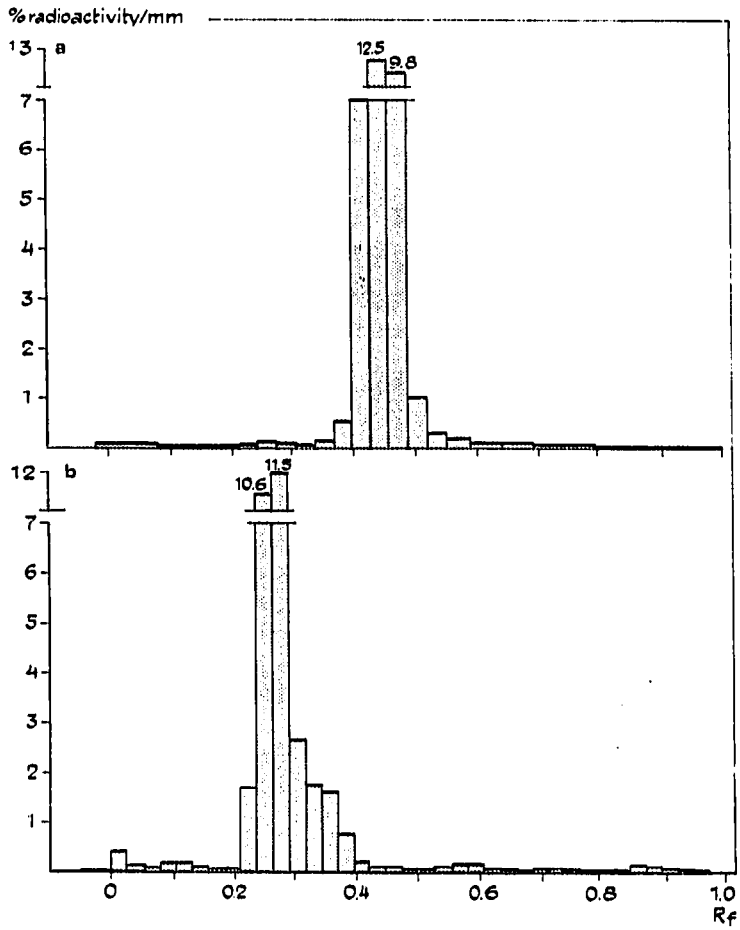

Fig. 4. Zone analysis and preparative separation of ^{14}C-labelled FFA on silanised silica gel. The ordinate gives the percentage of radioactivity recovered per mm of scraped zone; the abscissa gives the R_F value of the zone. (a) Zone analysis of [^{14}C]hexanoic acid; solvent: methanol–water (50:50). The calculated purity is 99 ± 0.2%. (b) Preparative separation of [^{14}C]heptanoic acid; solvent: methanol–water (50:50). Fractions 11 and 12, containing most of the pure heptanoic acid, were stored for further use.

elution. A fresh chromatogram is made by applying the FFA to the plate in the way described for the analytical separation, and detection is by the analytical zone-analysis method (see Fig. 4a). The zone analysis indicates, for hexanoic acid, a total amount of contaminating radioactivity of $1 \pm 0.2\%$, which is within limits considered as acceptable.

(c) If the autoradiogram indicates an impurity content greater than 1% (as for heptanoic acid in Fig. 3c), then, if the impurity has been clearly diagnosed, this FFA is directly purified according to the procedure described below.

Purification of labelled FFA

The radioactive FFA is applied to the plate as described for preparative separation, and detection is carried out as for preparative zone analysis; this is demonstrated for heptanoic acid in Fig. 4b. The fractions 11 and 12, containing pure heptanoic acid, are stored at −20°; the purity of these fractions is confirmed by autoradiography (see Fig. 3d).

DISCUSSION

The methanol–water solvent system in proportions ranging from 10:90 for C_2 to C_4 FFA up to 60:40 for C_7 to C_9 FFA gives excellent and reproducible resolution of the individual FFA on silanised silica gel. The "comets" of the FFA spots, which can impede interpretation of the purity of the labelled FFA (see Fig. 1b), can be avoided by applying mixed spots. When mixed spots are applied, the labelled C_n FFA is forced to distribute itself between the C_{n-1}, C_n and C_{n+1} FFA spots; this reduces "comet" formation dramatically (compare Figs. 1b and 3a).

The analytical TLC procedure described has been applied successfully to ^{14}C-labelled C_3 to C_7 FFA. It is noteworthy that, in general, the C_n FFA appears not to be contaminated with C_{n+1} or C_{n-1} FFA, but with some C_{n+2} and C_{n-2} FFA.

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