notes on methodology

Rapid assay of labeled free fatty acids in mixtures of labeled lipids

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SUMMARY The fatty acids in lipid mixtures are adsorbed on dehydrated hydroxy-charged ion exchange resin, the other lipids are removed by washing with solvent, and the adsorbed fatty acids are released with quaternary ammonium base for counting. All manipulations are carried out directly in scintillation vials.

SUPPLEMENTARY KEY WORDS liquid scintillation counting . lipases

THE USE OF triglycerides labeled in the fatty acid moiety as substrates for the study of lipolytic enzymes provides vastly increased sensitivity over microtitration procedures (1). With the specific activities of commercially available ^{14}C-triglycerides, the sensitivity of lipase assays can be increased by a factor of 10,000. However, before assay of the liberated fatty acids is possible, they must be separated from the other labeled lipids. This is usually done by various chromatographic methods, each of which has inherent disadvantages with respect to time, counting efficiency, or both.

A procedure is described here by which rapid assay of large numbers of samples is possible. Handling errors are minimized, and excellent duplicate values and high counting efficiency are achieved.

Materials. Ion exchange resin: any strongly cationic resin, such as Amberlite IRA 400 (Rohm & Haas Co., Philadelphia, Pa.) that is not attacked and does not yield colored substances when treated with the reagents employed is acceptable. The 20–50 mesh resin has sufficient adsorbing capacity and settles rapidly enough to be convenient. The resin is prepared (2) by hydroxylation with 100 volumes of 5% NaOH, washing with distilled water until neutral, washing with isopropyl alcohol, and finally washing with hexane.

Radioisotopes were purchased from New England Nuclear Corp., Boston, Mass., and checked for purity by counting thin-layer chromatography plate scrapings suspended in 4% Cab-O-Sil in toluene scintillation fluid.

Procedure. A large number of samples can be handled simultaneously. Approximately 0.5 g of the treated ion exchange resin is transferred to each of the required number of 20-ml glass scintillation vials. The lipid extract of each sample, containing not more than 5 mg of fatty acid in 1–4 ml of extracting solvent, is added. After the contents of the vials have been thoroughly mixed by swirling, the excess solvent is removed by aspiration through a Pasteur pipette with suction provided by a water pump. The resin is then washed four times with 5-ml portions of hexane, added with a syringe pipette. The solvent is removed by aspiration after each washing. These washings can easily be trapped either individually or collectively when desired. 1 ml of "NCS" solubilizer (a mixture of toluene-soluble quaternary ammonium bases, Nuclear-Chicago Corporation, Chicago, Ill.) (3) is added to each vial by syringe pipette. This displaces the fatty acids from the resin. 15 ml of toluene scintillation fluid (4% Liquifluor [Pilot Chemicals Inc., Watertown, Mass.] in toluene) is added and the samples are counted in a Nuclear-Chicago model 720 refrigerated liquid scintillation counter. Counting efficiencies were determined in the preliminary experiments after the addition of an internal standard of ^{14}C-toluene.

Results. The specificity and accuracy of this method were tested as follows. The binding capacity of the resin was measured by adding 2, 5, 10, 20, or 40 mg of palmitic acid to 0.5 g samples of the resin before adding ^{14}C-palmitic acid. Although in these experiments up to 40 mg of unlabeled fatty acid could be added before any radioactivity could be detected in the washings, the total lipid per vial in the final procedure was always kept to less than 5 mg. The affinity of the resin for fatty acids of various chain lengths was measured with ^{14}C-labeled lauric, myristic, palmitic, oleic, and stearic acids. Less than 0.05% of the added radioactivity of any of these compounds could be detected in the washings.

The efficacy of the washing procedure in removing the glycerides was also tested. ^{14}C-Tripalmitin with both low (about 5000 dpm) and high (about 200,000 dpm) activity was added to resin samples in vials. Triplicate sets of these resins were then washed 1–8 times with hexane after which the resins were examined for radioactivity. In all cases less than 0.09% of the applied radioactivity remained after three washings.

Finally, the method was tested with typical lipase hydrolysates prepared as follows. 0.1 mc of ^{14}C-tripalmitin-dissolved in ethanol was emulsified in water with the aid of lecithin and Tween 20 (polyoxyethylene sorbitan monolaurate). Ediol was added to the solution to yield a final lipid concentration of 5%. Samples of this labeled test emulsion were hydrolyzed with lipoprotein lipase from mouse heart powder and with pancreatic lipase. These preparations yielded hydrolysates with the label

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TABLE 1 RECOVERY AND COUNTING EFFICIENCY OF LABELED FATTY ACIDS OF VARIOUS CHAIN LENGTHS

Fatty Acid Tested	Activity Added*	Activity Measured*	Average Counting Efficiency	Average "Recovery"
	dpm	*cpm*		%
Lauric	3500 ± 64	2250 ± 65	67.2	95.6
Myristic	4040 ± 32	2580 ± 70	66.9	95.4
Palmitic	22,500 ± 255	14,140 ± 107	67.2	93.5
Stearic	4820 ± 106	3080 ± 76	67.6	94.5
Oleic	4780 ± 52	2970 ± 160	66.2	93.8

* Mean ± SD, n = 5.

distributed in triglyceride, diglyceride, monoglyceride, and fatty acid in the proportions, for lipoprotein lipase, 60:5:2:15 and for pancreatic lipase, 10:6:18:75. Samples of these mixtures were treated as described above and the washings were pooled and concentrated. Portions of these washings were then subjected to thin-layer chromatography (4); the appropriate spots were scraped into scintillation vials, suspended in 4% Cab-O-Sil in toluene scintillation fluid, and counted. All of the triglyceride and the partial glycerides were effectively eluted with the four hexane washes. No significant radioactivity was found in the area on the plate where fatty acid would appear.

Table 1 shows the reproducibility and the counting efficiency of this technique. The slight decrease in counting efficiency was due to the presence of the thin layer of resin beads at the bottom of the vials as well as of the solubilizing reagent. The counting efficiency was, however, extremely reproducible; it could be monitored by the channels ratio method with a quench correction curve established from standards.

The method was developed to measure lipoprotein lipase activity in eluates from micro starch-block electrophoresis. For these and other experiments hexane extracts or the hexane layer from the extraction mixture of Dole (5) were used as the source of the lipid. The 3:1 alcohol–ether mixture of Bloor could also be used, while chloroform–methanol extraction mixtures could not, because of their high density, which causes the resin to float.

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