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Biochem. J. (1963) **88**, 7

The Colorimetric Micro-Determination of Long-Chain Fatty Acids

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The investigation reported here arose from the need for a simple micro-method for the determination of long-chain fatty acids eluted from paper chromatograms. When the acids were labelled with ^{14}C or ^{3}H, measurement of the radioactivity of a portion of each eluate would then permit the specific activities of the individual fatty acids to be calculated.

Titration of weak acids at the required level (a few millilitres of a 10 μM solution) is not simple, and interference might be caused by traces of acetic acid from the chromatographic solvent. A colorimetric method for long-chain fatty acids was devised by Ayers (1956), who measured the extinction of a chloroform solution of their copper soaps, prepared by shaking a solution of the potassium soaps with copper nitrate and chloroform. The useful range was 8–40 mM. In a modification of the method, Iwayama (1959) used a copper nitrate–triethanolamine reagent shaken with a chloroform solution of the free fatty acids, and claimed greater sensitivity. This was still not high enough for the required purpose, however, and some means of increasing it was sought. It seemed possible that this might be achieved by adding to Iwayama's copper-containing chloroform solution one of the reagents used for the colorimetric micro-determination of copper. Sodium diethyldithiocarbamate was chosen for investigation, since it is inexpensive and fairly sensitive, the reaction conditions are not critical and the coloured complex formed with copper is soluble in chloro-

form. The present paper describes a method, with this reagent, for the micro-determination of long-chain fatty acids in chloroform solution, a preliminary note on which has appeared (Duncombe, 1962). Barreto & Mano (1961) also used sodium diethyldithiocarbamate as a colour developer, but with an ammoniacal copper sulphate reagent, in a similar method of much lower sensitivity than that described here.

Hlynczak, Sysa, Toczyski & Horbacewicz (1961) have determined long-chain fatty acids on paper chromatograms by treating with copper acetate, eluting the copper with acid and determining with dithizone, but this is not applicable when the radioactivity of the fatty acids is to be measured.

EXPERIMENTAL

Reagents

Reagents. The copper reagent (Iwayama, 1959) consisted of 9 vol. of aq. 1 M-triethanolamine, 1 vol. of 1 N-acetic acid and 10 vol. of 6·45% (w/v) $Cu(NO_3)_2,3H_2O$ (AnalaR).

The diethyldithiocarbamate reagent was a 0·1% (w/v) solution of sodium diethyldithiocarbamate (AnalaR) in redistilled butan-2-ol.

Both the reagents were stored in the refrigerator and used within 7 days.

Standard solutions of fatty acids. These were made up in AnalaR chloroform in the range 10–100 μM. Myristic acid, palmitic acid and stearic acid (all of purity greater than 99%), linoleic acid (99% pure) and linolenic acid (98·9% pure) were from L. Light and Co. Ltd., Colnbrook, Bucks.; butyric acid, hexanoic acid, octanoic acid, decanoic acid, lauric acid and oleic acid were from British Drug Houses Ltd., Poole, Dorset; undecanoic acid was from Eastman Kodak, Rochester, N.Y., U.S.A.; palmitoleic acid ('purum') was from Fluka A.-G., Buchs, Switzerland.

Procedure

About 5 ml. of the chloroform solution of fatty acids in the concentration range stated above was placed in a 10–15 ml. stoppered centrifuge tube, together with about 2·5 ml. of the copper reagent (the ratio of these volumes is not critical; see the Results section). The tube was stoppered and shaken vigorously for at least 2 min. It was then centrifuged for a few minutes to separate the phases cleanly, and the supernatant aqueous phase removed by suction with a fine hypodermic needle. The surface of the chloroform phase can easily be left clean, with only traces of the aqueous phase adhering to the wall of the tube. A portion (3·0 ml.) of the chloroform solution was pipetted into a clean dry tube, care being taken that the pipette did not touch the inner wall of either tube as traces of copper-containing aqueous phase might be transferred. Then 0·5 ml. of the diethyldithiocarbamate reagent was added to the chloroform solution and, after the solutions had been mixed, the extinction was read at 440 mμ in a 1 cm. light-path cell against a blank solution that had been subjected to the same procedure. (The latter gave an extinction of 0·02–0·05 against a solvent blank of 3 ml. of chloroform plus 0·5 ml. of diethyldithiocarbamate reagent.)

RESULTS

Fig. 1 shows the relationship between the extinction and the concentration for a number of palmitic acid standards. Analysis of variance on 55 determinations showed that the data could be fitted by the regression line:

$$E^{1\,\text{cm.}}_{440\,\text{m}\mu} = 0{\cdot}0856[10^5 \times \text{concn. (M)}] - 0{\cdot}0203$$

which is shown in Fig. 1. The standard deviation of extinction within replicates was ±0·008, and single determinations had a standard deviation from the regression line of ±0·0107. This corresponded to about ±2·5% of the fatty acid concentration at 50 μM.

These results, derived from experiments carried out on a number of different occasions, showed that the regression line can be used as a permanent calibration for the determination of palmitic acid without the necessity of running standard solutions.

With C_{10}, C_{11}, C_{12}, C_{14} and C_{18} saturated acids (73 determinations), the regression:

$$E^{1\,\text{cm.}}_{440\,\text{m}\mu} = 0{\cdot}0776[10^5 \times \text{concn. (M)}] + 0{\cdot}0312$$

was obtained, with a standard deviation of ±0·0329 (±8·5% at 50 μM). For palmitoleic acid, oleic acid, linoleic acid and linolenic acid (57 determinations) the equation was:

$$E^{1\,\text{cm.}}_{440\,\text{m}\mu} = 0{\cdot}0764[10^5 \times \text{concn. (M)}] + 0{\cdot}0023$$

with a standard deviation of ±0·0204 (±5·4% at 50 μM).

A comparison of these results with those obtained for palmitic acid suggests that each acid has an individual response line but that these lie fairly close together. Acids of shorter chain length (butyric acid, hexanoic acid and octanoic acid)

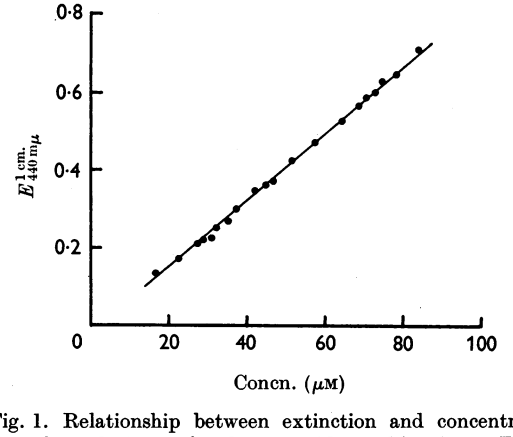

Fig. 1. Relationship between extinction and concentration for solutions of palmitic acid in chloroform. The standard procedure was carried out as described in the Experimental section. Each point is the mean of two to five replicates.

Table 1. *Behaviour of compounds other than fatty acids when subjected to the analytical procedure*

Lipids were dissolved in chloroform and thereafter treated like fatty acid solutions (see the Experimental section). Water-soluble compounds were added in 0·5 ml. of aqueous solution to exactly 5·0 ml. of chloroform, and the normal procedure was then carried out. The equivalent concentration of fatty acid was obtained by using the calibration on palmitic acid.

Compound	Concn. of solution tested	$E^{1\,cm.}_{440\,m\mu}$	Equivalent concn. of fatty acid
Cholesterol palmitate	0·32 mM	0·05	—
Cholesterol	0·52 mM	0·005	—
Tristearin	0·22 mM	0·014	—
Dipalmitoyl-lecithin* (synthetic; L. Light and Co.)	0·25 mM	1·28	—
Lactic acid	14 mM†	0·03	—
Sodium stearate	0·701 mM†	0·579	0·704 mM†
Human serum albumin (freeze-dried)	1% (w/v)†	0·61	0·09 mM†
Sodium stearate + human serum albumin	0·701 mM† 1% (w/v)†	0·602	0·732 mM

* Freed from non-esterified fatty acids by repeated extraction of a chloroform solution with aqueous bicarbonate.
† Concentration in aqueous solution.

were also examined, but each produced a very much smaller colour response.

The extinctions produced by a number of other compounds are shown in Table 1. Lactic acid was without effect and, of the lipids tested, interference was caused only by lecithin (freed from fatty acids by repeated extraction with bicarbonate). As might be expected, sodium stearate gave approximately the same molar extinction as the free acid, since it is the fatty acid anion that reacts with the copper reagent. Albumin gave a small positive reading, probably due to bound fatty acids; the value for the equivalent fatty acid content for sodium stearate and albumin together was 7·8% smaller than the value for the sum of the two measured separately.

The influence of a number of factors that might affect the final result was investigated. No difference was found when 5 ml. instead of 2·5 ml. of copper reagent was used per 5 ml. of fatty acid solution in chloroform. The smaller volume evidently provides sufficient excess of reagent and, as the ratio of volumes is not critical, accurate pipetting of the two solutions is not necessary. In the next stage of the procedure vigorous shaking by hand for 1 min. was as effective as shaking by machine for 10 min., though results were slightly lower after shaking by hand for only 30 sec. To ensure reproducibility, shaking by machine for at least 2 min. was normally employed.

Aging of the copper reagent gave low results. Aging of the diethyldithiocarbamate reagent caused little difference, but both reagents were normally made up weekly and kept in the refrigerator.

The temperature at which the fatty acid solution was shaken with the copper reagent affected the observed extinction slightly (Table 2). The effect amounted to −0·55% of concentration/degree rise.

Table 2. *Effect of temperature of shaking on colour development*

Determinations were made on a palmitic acid solution by the standard method (see the Experimental section), solutions being brought to the temperature stated and maintained there during shaking.

Temp.	$E^{1\,cm.}_{440\,m\mu}$
4°	0·618
23	0·563
37	0·514

Experiments on paper chromatography. Long-chain fatty acids were separated on Whatman no. 2 paper impregnated with liquid paraffin [dipped in 10% (v/v) paraffin in ether and air-dried], the developing solvent being 80% (v/v) acetic acid saturated with paraffin. [*carboxy*-^{14}C]Palmitic acid was run in this system, the radioactive spot was cut out and extracted into chloroform, and the fatty acid determination was carried out on a known volume of extract. A blank area of paper from the same chromatogram at the level of palmitic acid was similarly treated to give a blank solution for the spectrophotometric reading. Another portion of the radioactive extract was evaporated to dryness and counted in a liquid-scintillation counter, with correction for internal quenching. In eight such experiments the specific activity calculated from the results ranged from 47 to 143% of the true value for the palmitic acid applied. These variations seemed to be largely due to the variable and often high extinction given by the paper blank, which might not be the true blank value for that area containing the palmitic acid spot. The

measured blanks ranged from 0·06 to 0·20 (against a solvent blank), and the cause of the variability has not yet been identified. It was apparently not due to acetic acid or paraffin in the quantities likely to occur on the paper, and pretreatment of the paper by chromatographic washing with ether before paraffin impregnation gave no significant improvement.

DISCUSSION

The results obtained with palmitic acid show that for this acid the micro-determination described gives an acceptable degree of accuracy and reproducibility for most biochemical purposes. Since the calibration lines of all acids are slightly different, for the best accuracy when dealing with any single acid a calibration should be made with suitable standards. When a mixture of long-chain acids is being investigated, it is usually necessary to refer readings to a calibration line made from standards of some typical acid, and some loss of accuracy will ensue. With these limitations the method can be used for saturated acids of chain length from C_{10} to C_{18}, and for palmitoleic acid, oleic acid, linoleic acid and linolenic acid, either singly or in mixtures. It can also be applied to the sodium soaps.

The useful range is 0·05–0·5 μmole of acid (in 5 ml.), which can be extended upwards by using cells of shorter light-path or by dilution, with appropriate calibration curves.

The chief advantages of the method are simplicity and sensitivity. Only two accurate pipettings are necessary (of the copper-containing chloroform solution and of the diethyldithiocarbamate reagent), and even these are not very critical since a 10% change in the usual ratio of the volumes produces only a 1% change in extinction of the final solution. The number of determinations is kept to a minimum since it is not necessary to run standards at the same time, though the calibration should be checked from time to time. Gross changes in laboratory temperature would appear to be the most likely sources of variation.

The sensitivity compares very favourably with other available methods. An extinction of 0·4 results from a sample containing about 60 μg. of palmitic acid. The methods of Barreto & Mano (1961) and of Iwayama (1959) require about 1·5 mg. and 25·6 mg. respectively to produce the same extinction. Coleman & Middlebrook (1957) have developed a method of equal sensitivity based on the enrichment of methylene blue at a water–heptane interface, but since the system is not in equilibrium when colour readings are made it is necessary to run standards concurrently. Further, there was little effect with linolenic acid and with myristic acid and shorter-chain saturated acids, and uniform standardization of the procedure and the maintenance of a constant ambient temperature were very critical factors.

Of the lipids and other compounds tested, only lecithin causes appreciable interference, an effect evidently not due to contaminating free fatty acids, but albumin appears to bind part of the acids added as sodium soaps. Cholesterol, cholesterol esters and triglycerides do not interfere except in comparatively high concentration, so that the possibility of the selective determination of non-esterified fatty acids in their presence is dependent on relative concentrations. It might not be applicable, for instance, to extracts of adipose tissue, with their high ratio of triglyceride to non-esterified fatty acid.

It is essential that the solvent used in this method should be chloroform. A number of other solvents have been tested but none gives an adequate sensitivity.

SUMMARY

1. A colorimetric procedure is described for the determination of long-chain fatty acids in chloroform solution in the range 0·05–0·5 μmole in 5 ml.

2. The method can be applied to C_{10}–C_{18} saturated acids and to palmitoleic acid, oleic acid, linoleic acid and linolenic acid.

3. For a single acid an accuracy of about $\pm$ 2·5% (S.D.) of the concentration at mid-range can be obtained with one determination. For mixtures of acids there is some loss of accuracy.

4. The method can be used for fatty acids in the presence of cholesterol, cholesterol esters and triglycerides.

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