A RAPID AND SIMPLE METHOD FOR THE DETERMINATION OF ESTERIFIED FATTY ACIDS AND FOR TOTAL FATTY ACIDS IN BLOOD

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Carboxylic acid esters react with hydroxylamine in alkaline solution to form hydroxamic acids. These acids produce a red to violet reaction with ferric chloride which has been utilized by Feigl (1949) for the microchemical detection of carboxylic acid derivatives. Lipmann and Tuttle (1945) developed a method for the quantitative assay of acylphosphates by allowing hydroxylamine to react with these substances in aqueous solutions at pH 6.0. Hestrin (1949) adapted the reaction for the estimation of short-chain esters by employing alkaline aqueous reagents. Under these conditions esters of higher fatty acids did not react. In order to achieve a reaction of neutral fats and other natural lipids with these reagents, Bauer and Hirsch (1949) employed strictly anhydrous conditions and developed a method for the determination of the total esterified fatty acids in blood serum. Since almost all the fatty acids in blood serum are in esterified form, this method gives results identical with those testing total fatty acids in blood serum.

Although the method of Bauer and Hirsch is simpler than most other available methods for blood fat determination, it still involves several time-consuming steps. The blood extracts have to be evaporated to dryness at 60° C. and the evaporation has to be repeated, with the strict exclusion of water.

In connexion with work on the uptake of fat from blood serum by tissues *in vitro*, a convenient and fairly accurate method was required for blood fat estimation. It was learnt by personal information from Oren and Hestrin that higher fatty acid esters react at room temperature with hydroxylamine in alkaline solutions in aqueous alcohol. The presence of alcohol makes the reaction possible and also keeps the long-chain

hydroxamic acids in solution (Lipmann and Tuttle 1950). This reaction was therefore examined to establish optimal conditions for an analytical procedure. As a result of these experiments a method was evolved which is very simple to execute and which gives highly reproducible values, agreeing with those of well-established macro-methods. It does not require evaporation and no special precautions are needed for the exclusion of water.

Method

Reagents.—The following are required.

Alcohol-ether Mixture.—Three parts of alcohol and 1 part of ether (redistilled).

Hydroxylamine Hydrochloride.—An M-2 solution in water (kept in refrigerator).

Sodium Hydroxide.—Aqueous solution, 3.5N.

Hydrochloric Acid.—One part of the acid (sp. gr. 1.18) diluted with 2 volumes of water.

Ferric Chloride.—Ferric chloride, M-0.37, dissolved in 0.1N HCl.

Standard Stock Solution.—This is made up of 295 mg. triolein or 72.7 mg. triacetin (1 mEq.) in 25 ml. alcohol-ether (3:1) or alcohol respectively.

Procedure

Serum, 0.1–0.3 ml. containing 2–5 mEq. of fatty acid esters is delivered into a glass-stoppered measuring cylinder containing approximately 8 ml. of the alcohol-ether mixture. This mixture is brought to the boil, cooled, made up to 10 ml., and filtered and 3 ml. of the filtrate measured into a 16-mm. test tube. A blank containing 3 ml. of the alcohol-ether mixture is included in every run. Then 0.5 ml. of the hydroxylamine solution and 0.5 ml. sodium hydroxide solution are added and mixed, the tubes are stoppered and allowed to stand for 20 minutes at room temperature. After this period 0.6 ml. of hydrochloric acid solution is added, and, after mixing, 0.5 ml. of the ferric chloride solution is introduced. The tubes are mixed again and the colour

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developed is measured in a Fisher electrophotometer with an F. 525^B filter using micro test-tubes.

A calibration curve is obtained as follows: a standard solution containing 2 mEq. of ester in 1 ml. is prepared by diluting the stock solution 20 times with alcohol ether. For the test 0.5 to 2 ml. are used made up to 3 ml. with alcohol-ether and carried through the same procedure as the test filtrates. A typical standard curve is shown in Fig. 1A.

In human sera, especially with high cholesterol contents, a turbidity forms, disturbing the photometric reading. This can be overcome by the addition of 1 ml. of ether at the end of the reaction. The addition of ether does not change the colour intensity beyond the dilution factor, but the results have to be multiplied by the factor 1.2 or read from a standard

Fig. 1.—Standard curve: A, obtained with triolein by the method described; B, obtained with triolein plus 1 ml. of ether added at the end of the reaction. Optical densities are given after deduction of the blank values.

curve made with the addition of ether (Fig. 1B). The choice of this procedure is based on the following experiments:

Since the long-chain fatty acid esters do not react with hydroxylamine in aqueous solutions, and since, on the other hand, the use of anhydrous reagents requires evaporations and strict exclusion of traces of water, tests were made to determine at what alcohol concentration in water the reaction becomes optimal and is least susceptible to changes in the concentration of the reagents. All the tests were conducted at room temperature without evaporation. Fig. 2 gives the result of these experiments.

It is evident from Fig. 2 that very low colour intensities were obtained with reagents made up in 95% alcohol when developed at room temperature (Curve A). This is due partly to the low concentration of

Fig. 2.—Effect of alcohol and reagent concentrations on the colour intensities. A: With 0.21% hydroxylamine (M-0.03) in the reaction mixture; 1.5 ml. of an alcohol-ether solution containing 2 μ Eq. of triacetin+0.3 ml. of 3.5% hydroxylamine (M-6.5) and 0.3 ml. 3.5% NaOH (M-0.5) both in 95% alcohol made up to 5 ml. with alcohol and water to bring to the final alcohol concentrations given in the figure. Hydrochloric acid and ferric chloride solution were added as described in the method. B: With 0.70% hydroxylamine (M-0.1) in the reaction mixture. As in curve A but with 1 ml. of the hydroxylamine and NaOH solutions. C: With 1.75% hydroxylamine (M-0.25) in the reaction mixture. As in curve A but with 0.5 ml. of 14% hydroxylamine hydrochloride (M-2) and 0.5 ml. 14% NaOH (3.5N) in aqueous solutions.

the reagents (hydroxylamine and sodium hydroxide). Increasing this concentration is not feasible in non-aqueous solvents due to the low solubility. In addition, the presence of water actually increases the rate of the reaction at room temperature (Curve B). At the concentration in the reaction mixture of hydroxylamine (M-0.25) and alcohol chosen by us (70%) optimal conditions are obtained, and small changes in these concentrations have no effect on the final result (Curve C).

It can be seen from the standard curve (Fig. 1) that under these conditions the colour density is proportional to the concentration of the ester and that the addition of ether at the end of the experiment to avoid turbidities does not change this behaviour.

The proportion between concentration and colour intensity is also maintained when increasing aliquots of the serum filtrates are used for the test.

A comparison of the colour density obtained with various glycerol esters (Table I) shows that equivalent amounts of esters of highly different chain length give nearly the same results so that the method actually measures the total esterified fatty acids.

TABLE I
COMPARISON BETWEEN VARIOUS ESTERS

	mEq.	Photometer Reading	Reading—Blank
Blank ..	—	6.0	—
Triacetin ..	2	41.0	35.0
Tributyrin ..	2	38.5	32.5
Tricaprylin ..	2	38.5	32.5
Triolein ..	2	41.0	35.0

TABLE II
COMPARISON BETWEEN HYDROXAMIC AND
GRAVIMETRIC METHODS

No.	Gravimetric (mg.%)	Hydroxamic (mg.%)	Difference (%)
1	630	660	4
2	347	386	10
3	670	638	5
4	341	350	2
5	223	186	16.5
6	231	218	5.5
7	164	186	13

The hydroxamic method gives results comparable with those of a gravimetric macro-method, in which the fatty acids are extracted from a large amount, 3 ml., of serum and are weighed after saponification and acidification.

The method gives a high reproducibility with a standard deviation of $\pm 4\%$. Normal values in human blood serum were found to be around 350 mg. %

Summary

A greatly simplified adaptation of the hydroxamic acid method for the determination of long-chain fatty acid esters is described and its utilization for the determination of total fatty acids in blood is found highly satisfactory.

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