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Modified colorimetric ultramicro method for estimating Nefa in serum

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SYNOPSIS A method is described for the estimation of serum free fatty acids based on the ultramicro method of Novák. The difficulty of obtaining an accurate standard graph has been overcome and the interfering effects of phospholipids have been reduced. The method is simple to perform and gives accurate, precise results in 50 μl samples; it is thus suitable for work on newborn babies and small laboratory animals.

The usual titrimetric methods (Dole, 1956; Gordon, 1957) for determining serum non-esterified fatty acids (Nefa) are difficult to perform when scaled down for use with micro quantities. If double extraction procedures are employed to overcome interference from lactic acid this involves the use of larger quantities of serum (Trout, Estes, and Friedberg, 1960). Methods based on colour reaction of *p*H indicators (Mosinger, 1965) are very difficult to use on a micro scale because of interference from carbon dioxide. Other methods based on the selective transfer of copper or cobalt soaps into chloroform (Ayers, 1956) are very sensitive when combined with triethanolamine buffer (Iwayama, 1959) and diethyldithiocarbamate for copper detection (Barreto and Mano, 1961; Duncombe, 1962, 1963), but it is difficult to separate the lower chloroform layer from the upper aqueous copper nitrate mixture without contamination of the former with copper, especially when the volume of the chloroform is reduced. Other coloured substances, such as bilirubin, may interfere.

These problems have been overcome by Novák (1965), who, by making the aqueous reagent denser than the chloroform phase, has made the removal of the latter more easy. He uses cobalt instead of copper nitrate with l-nitroso-2-naphthol as indicator. There is no interference from bilirubin at the wavelength at which the colour is read, or from lactic acid which does not form chloroform-soluble salts with copper or cobalt (Duncombe, 1963; Novák, 1965).

However Novák's method still suffers from some disadvantages. The standards (palmitic acid made up in Dole's mixture) do not give reproducible readings and their molar extinction differs from that of palmitic acid in serum. There is also interference from phospholipids (Duncombe, 1963). The volumes of reagents used are small and require special constriction pipettes for accurate measurement.

This paper describes a modification of Novák's method which overcomes some of these problems.

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IMPROVEMENT OF THE STANDARD GRAPH

If a small quantity of n-butyric acid (0·1 m-equiv/l.), is added to the heptane used in Novak's procedure, the optical density of the standards is increased and made more reproducible without changing serum readings (Fig. 1). Butyric acid appears to act as a catalyst either in the formation of cobalt soaps of long-chain fatty acids or in their transfer into chloroform. The fact that butyric acid has no effect when added to serum may be due to the presence of small amounts of short-chain fatty acids in the serum. The concentration of butyric acid is not critical.

INTERFERENCE FROM PHOSPHOLIPIDS

According to Duncombe (1963), lecithin, which has a molar extinction similar to that of Nefa, is the only other lipid which interferes with copper/cobalt methods. The effect of lecithin on Novák's method was therefore investigated by adding various quantities of L-α-lecithin to the 300 μl of heptane (Fig. 2). As the amount of lecithin was increased the reading of the standards without butyric acid

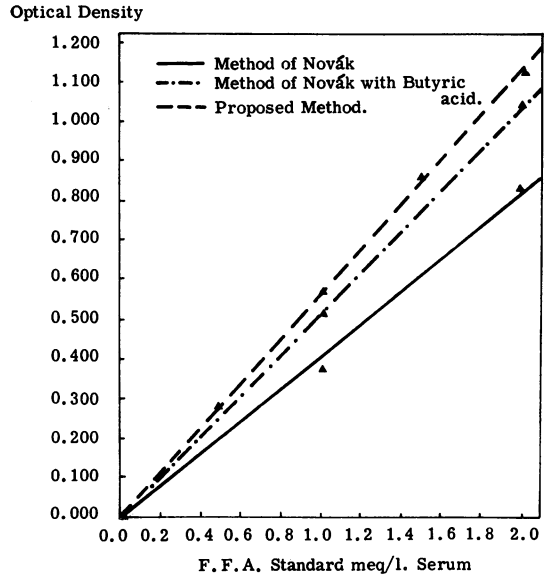

FIG. 1. *Effect of butyric acid on the method of Novák. Standards were made with palmitic acid and the butyric acid (0·13 m-equiv/l.) was added in the 300 µl of heptane.*

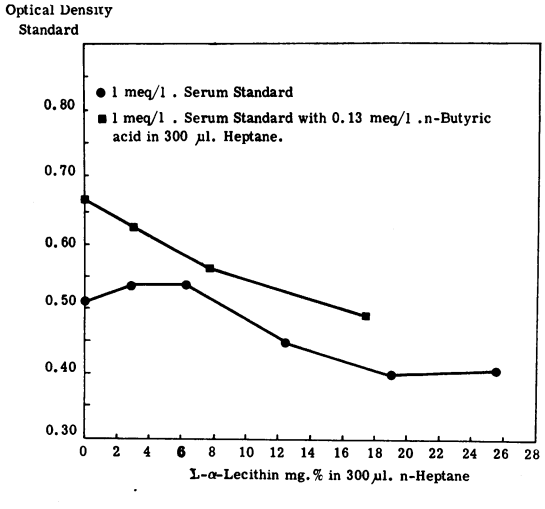

FIG. 2. *The effect of L-alpha-lecithin on the method of Novák. Standards were made up in Dole's mixture and the lecithin added in the 300 µl of heptane.*

at first rose but a greater concentration of lecithin produced a fall. When butyric acid was included the optical density was reduced even by low levels of phospholipid. The blanks (not shown) were also of greater optical density when lecithin was included. It was found that about 30% of the phospholipids that are extractable with warm ethanol/ether (3:1) were recovered by the modified Dole's extraction procedure employed by Novák, as estimated by the method of Bartlett (1959).

MODIFIED EXTRACTION TECHNIQUE

An extraction procedure for Nefa which overcomes interference from phospholipids has been described by Itaya and Ui (1965), whereby serum is diluted with buffer at *p*H 6·2 and shaken with chloroform for 90 seconds. This extracts as much Nefa as does Dole's procedure (1956), but only 1·3% of the total serum phospholipids.

The procedure has been modified by using only one sixth of the volumes so that 50 µl of serum can be extracted. In order to conserve all the chloroform, as much as possible of the buffer/serum layer is first aspirated. The last traces are removed by saturation with sodium sulphate and the addition of hexane, thus making the organic phase less dense than the aqueous phase which falls to the bottom of the tubes. The upper chloroform/hexane extract can then be easily removed. This procedure was found to extract less than 3% of the total phospholipids that were extractable with warm ethanol/ether (3:1).

Cobalt soaps are formed with the cobalt reagent as in Novák's method. By using larger volumes of organic solvents, however, and adding only a very small quantity of indicator to the final solution, the use of micro constriction pipettes has been avoided.

COBALT INDICATOR

2-Nitroso-l-naphthol has been chosen as indicator for cobalt estimation (Almond, 1953) instead of the 1-nitroso-2-naphthol used in Novák's method, because it gives better sensitivity at 530 mµ, at which wavelength interference from bilirubin is lessened (Fig. 3).

MODIFIED METHOD

APPARATUS Test tubes should have an internal diameter of about 10 mm and a capacity of 5 ml, and be fitted with airtight polythene stoppers. Pasteur capillary pipettes must be made with their ends drawn out to a fine point. A mechanical shaker, and a Sanz pipette (Beckman-Spinco) calibrated to 50 µl are also required.

REAGENTS Chloroform should be redistilled and 0·02 ml of n-butyric acid is added to 100 ml redistilled hexane.

STANDARD Only one standard is required. Dissolve 25·6 mg purified palmitic acid in chloroform and make

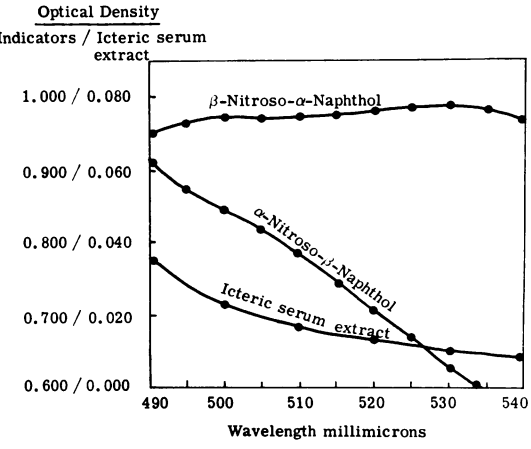

FIG. 3. *Absorption spectra of cobalt complexes with 1-nitroso-2-naphthol and 2-nitroso-1-naphthol. (Cobalt nitrate dissolved in ethanol and mixed with equal volume of 0·03% of either reagent in 96% ethanol, and read against corresponding blank.) Also shown is an absorption spectrum of a Dole's heptane extract of a highly icteric sample of serum.*

up to 100 ml; this gives a solution containing 1 m-equiv/l. Dilute this by a factor of 10 with chloroform for the working standard solution (0·10 m-equiv/l.) which is equivalent to serum containing 2·0 m-equiv/l. of Nefa.

PHOSPHATE BUFFER (*p*H 6·2) Dissolve 31·2 g of sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$) in 800 ml of water, adjust to *p*H 6·2 with 10 N sodium hydroxide, and make up to 1,000 ml with water.

SATURATED SODIUM SULPHATE Saturated and stored at 40°C in contact with excess crystals.

SATURATED POTASSIUM SULPHATE Saturated and stored at 40°C in contact with excess crystals.

COBALT NITRATE SOLUTION Cobalt nitrate ($Co(NO_3)_2 \cdot 6H_2O$), 6 g, is dissolved in 0·8 ml of glacial acetic acid and sufficient saturated potassium sulphate to make the volume up to 100 ml. This solution must be kept at 40°C in a tightly stoppered bottle.

PREPARATION OF COBALT REAGENT Triethanolamine, 2·7 ml, is made up to 20 ml with the cobalt nitrate solution. This is then added to 14 ml of the saturated sodium sulphate. This reagent is very unstable and must be made up freshly for each series of analyses.

Indicator solution 2-Nitroso-1-naphthol, 450 mg, is made up in absolute ethanol to 100 ml.

PROCEDURE For the extraction of a single sample of serum, six test tubes are required: two for the sample, two for the blank, and two for the standard. Chloroform, 1 ml, is added to the sample and blank tubes and 1·0 ml of the 0·1 m-equiv/l. palmitic acid solution to the standard tubes. Then 0·3 ml of the phosphate buffer is pipetted into all tubes, followed by 50 μl of the serum under test to the sample tubes and 50 μl of water to the blanks and standards. The tubes are tightly stoppered, shaken mechanically for 90 seconds, and centrifuged until the phases have separated.

Using one of the Pasteur pipettes attached to a suction pump, most of the upper aqueous layer is removed without disturbing the chloroform phase. Thereafter 1·0 ml of hexane containing butyric acid is added and the tubes are gently mixed until the chloroform and hexane phases are homogenous. Then 0·3 ml of saturated sodium sulphate is added and the tubes are well mixed and centrifuged until the phases have separated.

The upper chloroform/hexane layer is then transferred using another fine pointed pipette fitted with a rubber teat. As much as possible of this phase should be removed, but none of the aqueous layer should be carried over with it.

Now 1·0 ml of the cobalt reagent is added to the chloroform/hexane extract and, after making sure that all the tubes are air tight, they are cooled under cold water and shaken vigorously by hand for three minutes. They are then centrifuged for at least 15 minutes at 2,500 rpm (centrifuge head 30 cm diameter).

The upper layer is then transferred with another Pasteur pipette. Very great care must be taken not to remove any of the aqueous cobalt reagent. Of the cobalt indicator, 0·05 ml is added to the extract; the colour is developed for 30 min and read in 1·5 ml cuvettes of 1 cm optical depth at a wavelength of 530 mμ.

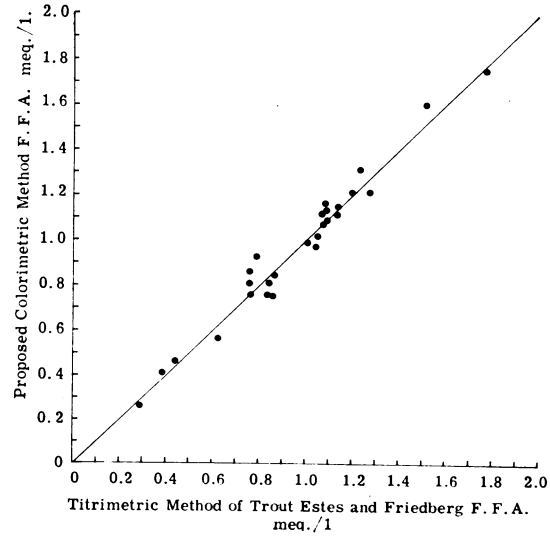

FIG. 4. *Comparison between titrimetric and colorimetric methods for analysis of serum Nefa.*

RESULTS

Twenty-seven samples of human serum were analysed both by this method and by the titrimetric method of Trout *et al* (1960). The results (Fig. 4) show good agreement.

A typical standard curve is shown in Fig. 1; all the points fall in a straight line up to 2 m-equiv/l. Thirty replicate determinations on one test serum gave a mean Nefa concentration of 0·50 m-equiv/l. with a standard deviation of ± 0·02. The method was also tested using n-heptane instead of hexane; the two gave similar results.

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