

A more sensitive and stable colorimetric determination of free fatty acids in blood

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Summary A sensitive method is proposed for the colorimetric determination of free fatty acids (FFA). Diphenylcarbazide containing diphenylcarbazone is used as the color developing reagent instead of diethyldithiocarbamate which was employed in the previous method (1965. *J. Lipid Res.* 6: 16–20). The present method was successfully applied to the determination of FFA present in as little as 40 μ l of whole blood.

Supplementary key words diphenylcarbazone

A number of methods have been proposed for the colorimetric determination of FFA in biological fluids (1–4). Owing to their simplicity in comparison with the titration techniques (5, 6), these colorimetric methods have been widely used for clinical and experimental purposes. Most of these colorimetric methods, however, are applicable to the determination of concentration of plasma FFA only if more than 0.1 ml of plasma is available. A more sensitive method is required for experiments in which repeated samplings of blood are undertaken to follow periodic changes in the plasma FFA level in small animals.

Turkington and Tracy (7) reported that the molar absorbancy of the complex of copper with diphenylcarbazide is about 12 times higher than that of the

complex with diethyldithiocarbamate (2). The currently available methods using diphenylcarbazide as a color developing reagent (8, 9) are of somewhat limited use for routine assay, because the color has to be measured within 5–15 min due to its instability.

The purpose of the present paper is to report a more sensitive color developing reagent as well as the conditions under which the color complex is stable.

Experimental

Withdrawal of blood specimen. Forty microliters of whole blood (withdrawn from the tail vein) was mixed with 0.7 ml of 0.9% NaCl in a centrifuge tube. The tube was centrifuged at 3000 rpm for 5–10 min to sediment blood cells and to obtain 0.5 ml of the clear supernatant as a test solution used in the standard procedure below.

Standard procedure. One-half ml of the test solution thus obtained was added to a glass-stoppered Pyrex test tube (16 \times 150 mm) containing 4 ml of chloroform and 0.5 ml of phosphate buffer (0.5 M, pH 6–7). The tube was then shaken for 80 sec. After 15 min or longer (30–60 min), the upper layer was carefully taken off by suction with a fine-tipped pipette, and to the residual chloroform layer was added 2 ml of a Cu–triethanolamine solution (Cu–TEA) (2). This solution consisted of 1 M triethanolamine–1 N acetic acid–6.45% $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ 9:1:10 (v/v/v). The tube was shaken 20 times, and after 15 min or longer (30–60 min), the upper layer was taken off by suction and the residual chloroform layer was filtered into another test tube as described previously (2). The color developed by the addition of 1.5 ml of a 0.5% solution of a mixture of diphenylcarbazone and diphenylcarbazide (5:95) in methanol was then determined in a spectrophotometer at 550 nm against a reagent blank.

Abbreviations: FFA, free fatty acids; Cu–TEA, copper–triethanolamine solution.

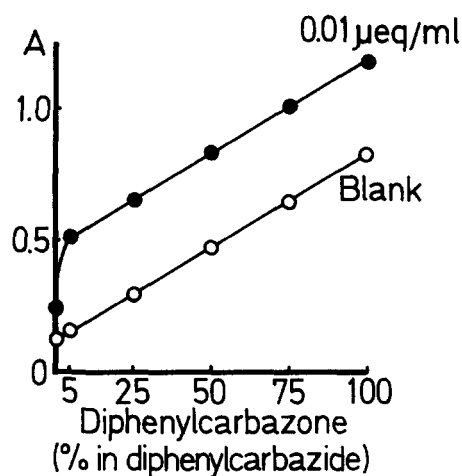


Fig. 1. Color intensity as a function of the ratio of diphenylcarbazone to diphenylcarbazide in a color-developing reagent. The mixtures of diphenylcarbazone and diphenylcarbazide were used at a concentration of 0.1% in methanol. The absorbances of sample (0.01 μeq FFA/ml) and of the blank were measured against distilled water at 550 nm.

Results and discussion

Effect on color intensity of the addition of diphenylcarbazone to diphenylcarbazide. When diphenylcarbazide alone was used as a color developing reagent, the maximum color intensity was obtained within a few minutes, but thereafter a rapid decline in intensity occurred (see Fig. 2, \circ, \bullet). Since it was found that diphenylcarbazone produced a more stable color complex with copper than did diphenylcarbazide, the effect of adding diphenylcarbazone to diphenylcarbazide on the color development was studied with 0.01 $\mu\text{eq/ml}$ of palmitic acid as a standard (Fig. 1). In

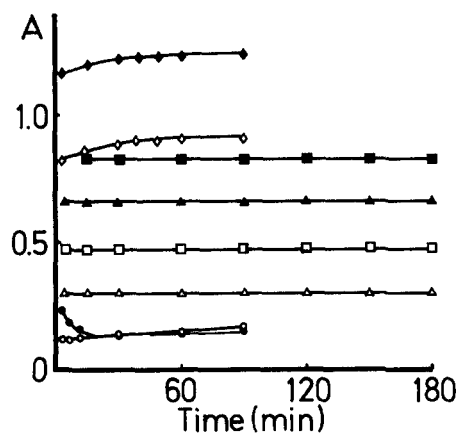


Fig. 2. Stability of color complex of diphenylcarbazone, diphenylcarbazide, and mixtures of both. The color was developed according to the standard procedure (see text). Reagents used are as follows: \circ and \bullet , diphenylcarbazide only; \triangle and \blacktriangle , diphenylcarbazone:diphenylcarbazide (1:3); \square and \blacksquare , diphenylcarbazone:diphenylcarbazide (1:1); \diamond and \blacklozenge , diphenylcarbazone only. The open symbols represent the reagent blanks and the solid symbols represent samples containing 0.01 $\mu\text{eq/ml}$ of palmitic acid.

this experiment, the color developed with or without (the reagent blank) palmitate was measured against distilled water. As is shown in Fig. 1, the addition of diphenylcarbazone to diphenylcarbazide in the ratio of 5:95 caused an increase in absorbance in the presence of FFA without a significant increase in the absorbance of the reagent blank. Further addition of diphenylcarbazone gave rise to further increases in absorbances of both tubes (with and without FFA) in a parallel fashion. Thus, 5% diphenylcarbazone in diphenylcarbazide could be used as a color developing reagent to afford a more sensitive colorimetric method than with diphenylcarbazide alone.

Effect of diphenylcarbazone on stability of color. The intensity of color plotted as a function of the time after adding different color developing agents to chloroform is shown in Fig. 2. There was a marked fading of color in a few minutes in the case of diphenylcarbazide (\circ, \bullet). With diphenylcarbazone alone, there was a slight increase in color intensity in 30 min, regardless of whether the chloroform contained palmitic acid (0.01 $\mu\text{eq/ml}$) or not. The color developed with the reagent containing 3–80% of diphenylcarbazone was stable for 3 hr.

The stability of the color complex obtained with pure diphenylcarbazide seemed to be dependent on the quality (grade and/or Lot No.) of other reagents, such as chloroform, present in the tube. The addition of diphenylcarbazone to diphenylcarbazide successfully stabilized the color in any lot of chloroform employed. Though it is not known why diphenylcarbazone stabilizes the color, the use of this reagent made it possible to determine the FFA content routinely in as little as 0.04 ml of blood. The mixture of diphenylcarbazone and diphenylcarbazide can be used as the color reagent for FFA not only in chloro-

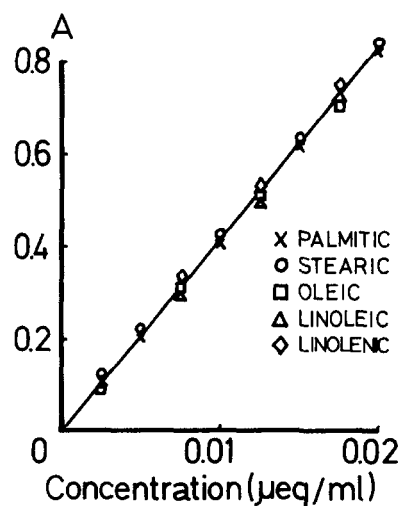


Fig. 3. Standard curves for different fatty acids obtained by the present method. One ml of the color-developing reagent was added to 2 ml of chloroform containing the copper soap.

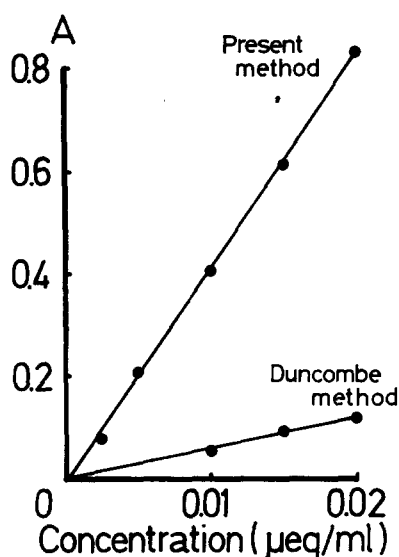


Fig. 4. Standard curves for palmitic acid obtained by two methods. Two ml of chloroform containing the copper soap of palmitate was treated with 1 ml of the color reagent by the present or the Duncombe method (10).

form but also in a mixture of chloroform and heptane (5).

Colorimetry of different fatty acids. **Fig. 3** shows the standard curves obtained by the present method with five different fatty acids including mono- and polyunsaturated acids. The chloroform solution containing the fatty acid was treated with Cu-TEA as described above. A 2-ml aliquot of the chloroform layer was transferred to another test tube and color was developed with 1 ml of the color-developing reagent. Unlike Duncombe's method (10), which results in slightly different standard curves with different fatty acids, the present method gave essentially the same standard curve for five different fatty acids.

Comparison with Duncombe's method. Comparison of the present method with the procedure of Duncombe (10) was performed on a standard chloroform solution of palmitic acid as well as on biological samples. **Fig. 4** shows that the color intensity obtained by the present method is about 6–7 times higher than that obtained by the procedure of Duncombe (10). Serum samples obtained from 12 rats were analyzed for FFA both by the present method and by Duncombe's method. The correlation of FFA values found by the present method with values by Duncombe's method is shown in **Fig. 5** together with statistical parameters.

The standard deviation was 15.6 for a mean value of 674 µeq FFA per liter of blood (five determinations) with diphenylcarbazone only, and 6.8 for a mean value of 643 µeq FFA per liter of blood (five determinations) with the mixture of diphenylcarbazone and di-

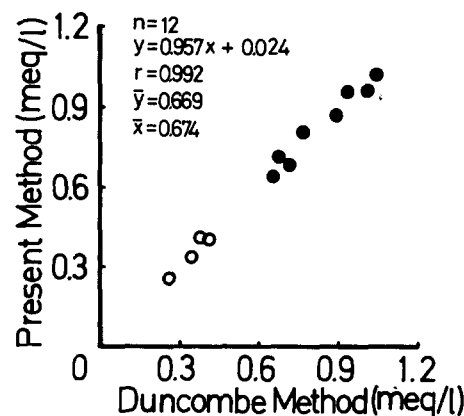


Fig. 5. Comparison of the present method with the procedure of Duncombe (10) for the determination of FFA content in serum. The open circles represent serum samples from fed rats. The solid circles represent serum from fasted (24–48 hr) rats. Application of the least squares method gave statistical parameters shown in this figure.

phenylcarbazide (5:95). Thus, the present method shows a good reproducibility. ■■

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REFERENCES

- Duncombe, W. G. 1964. The colorimetric micro-determination of non-esterified fatty acids in plasma. *Clin. Chim. Acta.* **9**: 122–125.
- Itaya, K., and M. Ui. 1965. Colorimetric determination of free fatty acids in biological fluids. *J. Lipid Res.* **6**: 16–20.
- Novak, M. 1965. Colorimetric ultramicro method for the determination of free fatty acids. *J. Lipid Res.* **6**: 431–433.
- Itaya, K., and T. Kadowaki. 1969. An improved method for the colorimetric determination of free fatty acids. *Clin. Chim. Acta.* **26**: 401–404.
- Dole, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **35**: 150–154.
- Gordon, R. S., Jr. 1957. Unesterified fatty acid in human blood plasma. II. The transport function of unesterified fatty acid. *J. Clin. Invest.* **36**: 810–815.
- Turkington, R. W., and F. Tracy. 1958. Spectrophotometric determination of ultramicro amounts of copper with 1,5-diphenylcarbohydrazide. *Anal. Chem.* **30**: 1699–1701.
- Laurell, S., and G. Tibbling. 1967. Colorimetric micro-determination of free fatty acids in plasma. *Clin. Chim. Acta.* **16**: 57–62.
- Mahadevan, S., C. J. Dillard, and A. L. Tappel. 1969. A modified colorimetric micro method for long-chain fatty acids and its application for assay of lipolytic enzymes. *Anal. Biochem.* **27**: 389–396.
- Duncombe, W. G. 1963. The colorimetric micro-determination of long-chain fatty acids. *Biochem. J.* **88**: 7–10.