A MICROMETHOD FOR THE DETERMINATION OF CAROTENE AND VITAMIN A IN WHOLE BLOOD

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Numerous methods have been proposed for the determination of blood carotene and vitamin A. Calorimetric methods [7, 9] which have been widely used are cumbersome because of the requirement to determine the two vitamins separately; also no less than 3 ml of blood are required for the determination. The micromethod proposed by Bessey et al., [3] enables the spectra of carotene and vitamin A to be determined from a single sample of 0.06 ml of plasma. Despite the shortcomings of this method [4-6, 8] it has been widely used abroad and in this country.

In our previous paper [1] we concluded that Bessey's method could not be used for determination of carotene and vitamin A in the blood because ultraviolet rays do not exert a specific destructive action on vitamin A.

A general drawback of the methods used is the necessity to obtain plasma, which not only complicates the procedure but leads to a loss of vitamin A as a result of its destruction during manipulation. This line of thought had led to the development of a method for the determination of carotene and vitamin A in whole blood; the theoretical basis for this method has been described previously [1].

The method is based on obtaining a petrol-ether extract of vitamin A and carotene from whole blood, and unmixed with unsaponified impurities. Because sterols belong to this class of substance they are converted into a digitonin complex insoluble in water and in fatty solvents. The optimum formation of complex occurs at pH 7.4. In the first form of the method 1 ml of blood was required, and there were complications due to several laborious procedures. Therefore the further development has been directed towards simplification and towards performing the analysis of microquantities.

Here we describe the micromodification which enables the concentration of carotene and vitamin A to be determined in 0.1 ml of whole blood.

Reagents: 1) A 2 N alcoholic solution of NaOH is prepared from one part of 11 N aqueous NaOH and 4.5 parts of ethanol in which is dissolved an anti-oxidant; 2) a 0.25% alcoholic solution of the anti-oxidant butyloxytoluene; 3) a buffer solution at pH 7.4 prepared from 18.17 parts of 0.5 M K_2HPO_4, and 1.83 parts of 0.5 M citric acid; 4) 1 N HCl in ethanol; 5) 1% digitonin in 80% ethanol; 6) the petrol-ether fraction distilling at 70-80°; 7) liquid nitrogen.

Apparatus: SF-4 spectrophotometer.

Course of the analysis. First 0.1 ml of 2 N alcoholic NaOH was placed in bacteriological (Widal) tubes, to which was added 0,1 ml of whole blood taken from a finger or the ear. The vessels were than immersed in liquid nitrogen in a Dewar flask. When all the blood samples had been taken the tubes were picked up in turn by forceps from the liquid nitrogen, and at the moment when all the liquid nitrogen had changed into the gaseous form the tube was corked. By this means oxygen was excluded from the tube. Next the tubes were placed in a water bath for 15 min at 70°. The saponified samples were rapidly transferred into iced water, and the following reagents were added to the cooled samples, which were shaken: 0.2 ml ethyl alcohol with anti-oxidant, 0.15 ml of alcoholic HCl and 9.4 ml buffer at pH 7.4. After 1-2 min 0.025 ml of 1% alcoholic digitonin was added. Then after 1-2 min

0.4 ml of petrol ether was poured in, and the tubes were tightly corked. Extraction was continued for 30 min with continuous agitation in a mechanical shaker. After 2-3 min of centrifugation at 15,000 revs/min the spectrum was recorded in a spectrophotometer. The optical density was measured in a plane-parallel quartz vessel having a distance between the working surfaces of 8 mm, and between the lateral surfaces of 2.5 mm. The light flux at the output passed through a diaphragm. The aperture in the center had a diameter of 1 mm. Some padding was placed at the base of the holder to raise the vessel so that the light passed almost through the bottom of it. The position and centering of the container in the holder was first checked by eye when the photocells were removed, by use of a wavelength of 520-540 mμ and a slit width of 2 mm. In this case the green ray should pass through the solution without touching the bottom or sides of the container [2]. The arrangement of the spectrophotometer in the light beam was made in a blank experiment against petrol ether (water was used instead of blood). The optical density for vitamin A was measured at a wavelength of 328 mμ, and for carotin at a wavelength of 460 mμ. The concentrations were calculated from a calibrated graph drawn up by use of vitamin A-acetate and β-carotene.

To construct the calibration curve, 3.44 mg of vitamin A-acetate were dissolved in 100 ml of petrol ether (initial solution). The amount of vitamin A-acetate in 0.1ml of this solution is 0.00344 mg (3.44 μg). Dilution in arithmetical progression gave a reduction of 0.00344 mg, or 10 IU.

To construct the calibration graph for carotene 10 mg of β-carotene were dissolved in 100 ml of petrol-ether (initial solution). The amount of β-carotene in 1 ml of the initial solution was 100 μg. Dilution in arithmetical progression, as for vitamin A, gives a reduction of concentration of 100 μg.

A check of the method with the preparation of vitamin A-acetate showed losses which did not exceed 5-8%. The difference between parallel tests did not exceed the error of the instrument.

A comparison of the method described here with that of Kimbl' and Sobel' [1] showed that in whole blood there is 3-4 times as much vitamin A as carotene.

Because when Bessey's method is used irradiation of the solutions with ultraveiolet light causes destruction not only of vitamin A, but also of sterols, the extinctions observed do not correspond to the vitamin A content.

The advantage of the method we have described is that it enables an analysis of 0.1 ml of whole blood to be made.

SUMMARY

A microspectrophotometric method is proposed for determination of vitamin A and carotene in 0.1 ml of whole blood. The principle of the method is to obtain petrol-ether extracts from the nonsaponifying blood fraction, free from impurities which absorb within the 300-400 mm range. Direct spectrophotometry of the vitamin A and carotene in the extract obtained is then possible. The loss of vitamin A does not exceed 5-8%. The difference between parallel tests did not exceed the error of the instrument.

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