THE ESTIMATION OF DICOUMARIN IN BLOOD

BY

M. LUBRAN

From the West Middlesex Hospital, Isleworth, Middlesex

(RECEIVED FOR PUBLICATION AUGUST 18, 1950)

Dicoumarin (3,3'-methylene-bis (4-hydroxy-coumarin)) is widely used to prolong the blood clotting time in the treatment of thrombosis, dosage being controlled by repeated measurements of the prothrombin index. There are no published data concerning the blood levels of dicoumarin during therapy, nor their correlation with the prothrombin index. The method described in this paper has been devised in order to study this problem.

Dicoumarin is extracted from acidified plasma with ethylene dichloride. This, in turn, is extracted with alkali and the aqueous extract buffered to pH 7. It is then coupled with diazotized dianisidine to produce a stable red colour.

Procedure

The following reagents are required.

Ethylene Dichloride.—The technical grade material is washed with 1/5 its volume of normal sodium hydroxide, then with a similar volume of normal sulphuric acid, and finally three times with a similar volume of distilled water.

5N Hydrochloric Acid (Approx.).—Concentrated hydrochloric acid (AR), 50 ml., is diluted with distilled water to 100 ml.

M/15 Disodium Hydrogen Phosphate.—Anhydrous disodium hydrogen phosphate, 9.465 g., is dissolved in distilled water on a water bath, cooled, and made up to 1 litre.

2/15M Potassium Dihydrogen Phosphate in 0.1% Gum Ghatti.—A muslin bag containing 1 g. of gum ghatti "tears" is suspended just below the surface of 500 ml. of distilled water contained in a tall cylinder. After two to three days the gum solution is filtered on a water pump, and 18.145 g. anhydrous potassium dihydrogen phosphate is dissolved in 500 ml. water and made up to 1 litre with the gum solution. The solution is kept in the refrigerator.

Diazo-salt Solution.—Brentamine fast blue salt (Imperial Chemical Industries), 0.25 g., is dissolved in ice-cold distilled water to give 100 ml., and 1 ml. added of normal sulphuric acid. The reagent is mixed and filtered, and kept cold. Kept in the refrigerator, the solution is stable for two weeks.

Standard Dicoumarin Solutions : Stock Standard (10 mg./100 ml.).—Pure dicoumarin, 0.1000 g., is dissolved in M/15 disodium hydrogen phosphate to make 1 litre, and stored in a dark bottle in the refrigerator. The solution will keep for at least one month.

Working Standard (1 mg./100 ml.).—The stock is diluted ten times with M/15 disodium hydrogen phosphate. It should be made freshly each time.

Extraction.—A quantity of 5 ml. of plasma or serum (not whole blood) is pipetted into a 1 oz. screw-capped universal container, with a cellophane disk covering the rubber liner

of the cap. Then 1 ml. of 5N hydrochloric acid, and 10 ml. of ethylene dichloride are added and the solution shaken on a Kahn shaker for 30 minutes. It is centrifuged at maximum speed for 15 minutes. Three layers are obtained; an upper aqueous, a lower ethylene dichloride, and a solid middle layer. The upper layer is discarded and the lower layer removed with a teated pipette. About 7 ml. should be obtained. Should an emulsion form in the lower layer, it can be readily broken by brisk stirring with a thin glass rod and recentrifugation. Then 6 ml. of the extract is pipetted into another universal container, 4 ml. of disodium hydrogen phosphate added, and shaken mechanically for five minutes. The solution is centrifuged and the upper layer removed with a teated pipette.

Test.—A 3-ml. quantity of the disodium hydrogen phosphate is measured into a labelled tube. Into the blank tube 3 ml. of M/15 disodium hydrogen phosphate is measured, and into a third tube 3 ml. of working standard. To each is added 1 ml. of potassium dihydrogen phosphate solution, mixed well by tapping, and 0.1 ml. of diazo-reagent, freshly removed from the refrigerator, is added. Mixing is by inversion. The colour is read after ten minutes, preferably with a photo-electric absorptiometer and an Ilford spectrum filter No. 624 (yellow-green) zeroing on the blank. The calculation is made as follows:

$$\frac{4}{3} \times \frac{\text{reading of test}}{\text{reading of standard}} = \text{mg. dicoumarin/100 ml. plasma or serum.}$$

Results.—Normal serum or plasma gives a zero dicoumarin value, but with marked haemolysis; there are apparent values of the order of 0.2 mg. dicoumarin/100 ml. Recovery experiments gave over 95% recovery on every occasion. In patients on prolonged dicoumarin therapy, who had been given over 0.6 g. of dicoumarin, plasma dicoumarin values ranging from 0.9 to 2.8 mg./100 ml. were found, corresponding to prothrombin indices ranging from 50% to 12.5% of normal.

Discussion

Axelrod, Cooper, and Brodie (1949) described a method for estimating dicoumarin by measuring the optical density of an alkaline solution at 315 μ . n-Heptane was used to extract acidified plasma or urine. They gave no results for human bloods. Their method, although simple, requires quartz cuvettes and a Beckmann type instrument and is hardly suitable for the routine chemical laboratory. Clayton and Larmour (1935) described a coupling reaction for coumarin and melilotic acid, using diazotized p-nitroaniline. However, this reaction lacks specificity and sensitivity and proved unsuitable for dicoumarin. Gömöri (1949) suggested the use of stabilized diazonium salts, readily obtainable as dye-stuff intermediates, for the estimation of phenol. After examining nine such salts, one was found to be suitable at pH 7. At a more alkaline reaction a yellow colour was produced, but this was not as good as the red colour for estimation. Further, interference from phenols and salicylates occurred at an alkaline pH. There is also a colorimetric method for the estimation of dicoumarin described by Pulver and Kaulla (1948).

The new method will detect dicoumarin in a concentration of 1 μ g./ml. of final solution, equivalent to a plasma concentration of 0.13 mg./100 ml. A stabilizing colloid is essential, as the azo-compound responsible for the colour is poorly soluble in water at pH 7. Beer's law is obeyed up to a concentration of 15 μ g./ml. of final solution. For higher concentrations it is satisfactory to dilute with a pH 7 buffer solution.

The reaction seems to have a high degree of specificity. Phenol, tyrosine, and coumarin do not interfere. Salicylates give a similar colour only when their concentration exceeds 20 mg./100 ml.

Summary

Dicoumarin can be coupled with diazotized dianisidine to give a stable red colour.

This reaction can be used for the determination of plasma or serum dicoumarin, after extraction with ethylene dichloride.

Dicoumarin concentrations of 0.9 to 2.8 mg./100 ml. have been found in patients on prolonged dicoumarin therapy.

I wish to thank Dr. W. R. Horsfall and Dr. J. M. Johnstone for determining the prothrombin indices, Organon Laboratories, Ltd., for a supply of pure dicoumarin, and Imperial Chemical Industries (Dye-Stuffs Department) for supplying the stabilized diazonium salts.

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