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A Colorimetric Method for the Determination of Cinchona Alkaloids

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During determinations of the concentrations of cinchona alkaloids in the blood of chicks, difficulty was experienced in finding a sufficiently sensitive method for the non-fluorescent alkaloids, cinchonine and cinchonidine. Young chicks up to 4 weeks old were used, so that samples of not more than 1 ml. of blood and plasma were obtainable. The oral doses of alkaloids were only 50 mg./kg. body weight. It was therefore necessary to find a method of determination sensitive to at least 1 μg. of alkaloid.

The silico-tungstate nephelometric method of Kyker, Webb & Andrews (1941) was tried, but even after modification to suit our conditions, it was not possible to detect less than 10 μg. of alkaloid. Previously our attention had been attracted to the method of Prudhomme (1940), in which quinine combines with eosin to form a coloured compound extractable with chloroform. This method was rejected mainly because the eosin colours were too weak, and the coloured compound in chloroform solution was readily adsorbed on to the walls of the tube.

Our interest in this reaction was revived when it was discovered by one of us (E. W. R.) that bromothymol blue formed a similar compound with quinine. If to a solution of quinine bromothymol blue indicator was added and the mixture allowed to stand, a yellow colour could be extracted by shaking with chloroform. The depth of colour extracted was proportional to the concentration of quinine. This reaction was given by the four principal alkaloids of cinchona bark, quinine, quinidine, cinchonine and cinchonidine, by certain derivatives of these alkaloids, and by some other nitrogen-containing compounds (see later). It was decided to investigate the possibilities of this reaction as a quantitative method for determining cinchona alkaloids in biological material.

EXPERIMENTAL

Factors influencing colour production

Optimum time for bromothymol blue alkaloid reaction. Mixtures of quinine and bromothymol blue solutions were allowed to stand for varying intervals of time before extraction. The amount of colour extracted reached a constant level in 60 min.

Choice of solvent for colour extraction. Although chloroform was used by Prudhomme (1940) to extract the eosin-quinine compound, its use had certain objections. Being heavier than water, it was difficult to pipette off the lower chloroform layer into colorimeter cells without contamination with the aqueous layer. Chloroform readily formed emulsions on shaking with aqueous solutions, which were difficult to break down even by centrifuging. Other organic solvents

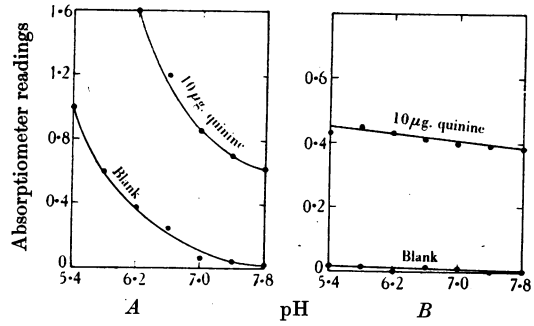

Fig. 1. The influence of pH on the extraction of bromothymol blue cinchona compound by (*A*) chloroform, and (*B*) benzene.

were therefore tried, including light petroleum, carbon disulphide, carbon tetrachloride, *n*-butyl and amyl alcohols, benzene, toluene and xylene. Petroleum failed to extract any colour, while *n*-butyl and amyl alcohols extracted bromothymol blue itself. The other solvents were about equally efficient, and benzene was finally chosen.

Effect of pH *on extraction.* Decrease of pH was found to increase the amount of colour extracted by chloroform.

Investigations over the range of Sorensen's phosphate buffers (pH 5·4–8·0) showed that the colour extracted from a 'blank' bromothymol blue solution without any alkaloid also increased rapidly as the pH fell. On the other hand, pH variations had little effect on the depth of colour extracted by benzene (Fig. 1).

Optimum time for extraction of colour. Benzene was added to bromothymol blue-alkaloid solutions which had stood for 60 min. in narrow bore tubes. The tubes were tightly closed with corks covered with cellophan and were shaken mechanically along the line of the long axis of the tube, at 200 complete oscillations/min. Under these conditions, the amount of colour extracted reached a constant level after 30 min.

Extraction of alkaloid from biological material

The method used by Prudhomme (1940) for blood involved the precipitation of proteins by H_2SO_4 and large quantities of Na_2SO_4, which caused considerable inconvenience by crystallizing out in the filtrate. The extraction procedure used by Kyker *et al.* (1941) consisted of a long continuous extraction with ether in a special apparatus and was applicable only to fluid material. A more satisfactory method for our purpose was the extraction process described by Kelsey & Geiling (1942), and a modified form of this method was finally adopted.

METHOD

Lake 1 ml. of oxalated blood in 29 ml. of water; add 5 ml. N-NaOH. Heat for 30 min. on a steam-bath. Cool and transfer to a separating funnel. Add 2 ml. acetone and 50 ml. ether, shake thoroughly, allow to stand for a few minutes and shake again. Allow to stand till both layers are quite clear; discard the aqueous layer. Shake the ether with 50 ml. 0·5N-KOH; allow to stand till both layers are clear; discard the KOH. Shake the ether with 50 ml. of water; allow to separate, and discard the water. To the washed ether add 2·5 ml. 0·05N-HCl; shake thoroughly, allow to separate and run the acid into a 25 ml. beaker. Extract the ether with a second 2·5 ml. of acid and mix the acid extracts. Warm on a sand-bath to drive off dissolved ether.

To the acid extract add 1 ml. 0·04 % (w/v) bromothymol blue solution and neutralize to approximately pH 7·0 (green colour) with N-NaOH (use 0·1N-NaOH towards the end-point). Transfer the neutralized solution to a narrow bore tube. Add 1 ml. Sorensen's buffer of pH 7·0, mix and allow to stand for 60 min. Add exactly 1 ml. benzene, close the tube with a tight-fitting cork covered with cellophan, and place on the shaker for 40 min. Centrifuge at moderate speed for 10 min. Pipette the separated benzene layer into a 0·5 ml. colorimeter cell.

The depth of colour was measured in our determinations in a Spekker Photoelectric Absorptiometer, using Ilford Spectrum filters no. 601 (violet). Standard curves were prepared by adding known amounts of alkaloid to volumes of blood equal to those used in actual determinations. Where larger volumes of blood are available, 5 or 10 ml. samples may be used. The ether extract should then be extracted with two 5 ml. portions of HCl and 5 ml. each of bromothymol blue solution and buffer added. The final colour may be extracted with 5–15 ml. of benzene, which enables visual colorimeters of the Klett type to be used.

The extraction procedure described above can be used for material other than blood. Fluid materials should be treated in the same way as blood; tissues should be dropped into 2 % NaOH and heated until completely disintegrated before extraction.

RESULTS

Fig. 2 shows standard curves prepared using (*A*) 1 ml. of chick blood and extracting finally with 1 ml. of benzene, and (*B*) 5 ml. of chick blood and extracting with 7 ml. of benzene. Curves obtained

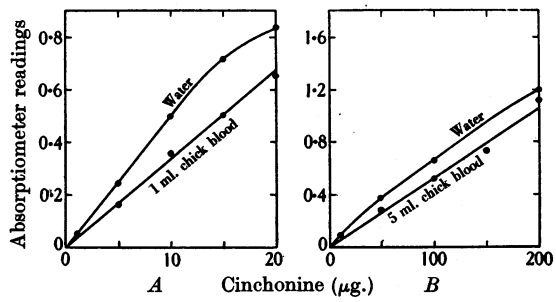

Fig. 2. Standard curves, using Spekker Photoelectric Absorptiometer with Ilford Spectrum filters no. 601. *A*, extracted with 1 ml. benzene; *B*, extracted with 7 ml. benzene.

by determination of the alkaloid in simple aqueous solution are also shown. There was considerable discrepancy between the 'water' and 'blood' standard curves, but this apparent 'loss' of alkaloid when extracted from biological material appeared to be constant. The error can therefore be eliminated by using standard curves prepared by extracting graded amounts of alkaloid from biological material.

Recovery experiments. Known amounts of cinchonidine were added to 1 ml. quantities of blood and the alkaloid content determined. Table 1 shows the amounts added and recovered, and the percentage recovery. The determinations on rabbit plasma and human urine were read from a standard curve prepared by using chick blood. Since good recoveries were obtained, it appears that the apparent 'loss' of alkaloid when extracted from biological material is constant whatever the nature of the material. If, however, any doubts are entertained as to the accuracy of a series of results, it is ad-

visable to prepare specific standard curves using the same material as in the actual determinations.

Table 1. *'Recoveries' of cinchonidine added to biological material*

Material	Cinchonidine added (μg.)	Cinchonidine recovered (μg.)	Recovery (%)
Rabbit blood	0·25	0·5	200
	1·5	1·5	100
	7·0	7·8	111
	13·0	13·1	101
	18·0	18·5	103
Rabbit plasma	13·0	13·4	103
Human urine	13·0	13·75	106

As a further check of the accuracy of the method, comparisons were made with the silico-tungstic acid method of Kyker *et al.* (1941). 15 μg. of cinchonine added to blood were determined by both methods. The recoveries of alkaloid were by this method, 15·4 μg., by the silico-tungstic acid method, 16·0 μg.

DISCUSSION

It was shown by Prudhomme (1940) that eosin would react with quinine to form a compound extractable with chloroform. In view of the similarity of chemical constitution between eosin and bromothymol blue, it is not surprising that the latter behaves similarly towards cinchona alkaloids. The reaction is not specific for this group of organic bases. Prudhomme reported that ephedrine, eserine, pilocarpine and atropine combined with eosin, but that caffeine, aspirin, sulphonamides, veronal, gardenal, urea and amino-acids did not. Auerbach (1943) reported that bromothymol blue or bromophenol blue would combine with quaternary ammonium salts in alkaline solution. We found that bromothymol blue combined with ephedrine, atropine, eserine and strychnine, but the colours extracted were less intense than those produced by equal amounts of cinchona alkaloids. Cysteine, *p*-aminobenzoic acid, histamine, urea, adrenalin, caffeine, morphine, nicotine, sulphanilamide, aspirin and glucose produced no extractable colour. This negative list includes most of the substances which might naturally occur in blood samples, and also some of the medicaments which might be administered together with cinchona alkaloids.

Using this reaction as a quantitative measure for cinchona alkaloids, we have shown that amounts of alkaloid as low as 0·25 μg. can be detected. The method is therefore almost as sensitive as a fluorimetric method, and should consequently be of value in determining the non-fluorescent alkaloids. The use of bromothymol blue eliminates the practical difficulties attending the eosin method already mentioned. Also, Prudhomme's method requires 10 ml. blood samples, and attempts to work with smaller volumes produced difficulties in filtration because of the large amount of sodium sulphate present.

The modifications of Kelsey & Geiling's (1942) extraction procedure were introduced chiefly to reduce the time factor. A thorough shaking with one portion of ether was found to be sufficient to extract practically 100% of the alkaloid, thus enabling the whole procedure to be carried out in one separating funnel. The 2 ml. of acetone were used instead of 20 ml. of ethanol in preventing emulsion formation to reduce the possibility of loss of alkaloid by reduction of the partition coefficient between the phases. A single washing with stronger potassium hydroxide was introduced, followed by a single washing with water to remove residual KOH. An important point to observe is that after all shakings the funnel should be allowed to stand until separation is absolutely complete, as indicated by the absence of cloudiness in either layer. This modified method of extraction has been used continuously in this laboratory for fluorimetric, nephelometric and colorimetric determinations of cinchona alkaloids.

SUMMARY

1. A colorimetric method for determining cinchona alkaloids, based on the formation of a coloured compound with bromothymol blue, is described.

2. Using small capacity cells in a photoelectric photometer, the method is sensitive to 0·25 μg. of alkaloid.

3. The specificity of the reaction has been investigated, and lists of substances which do or do not combine with bromothymol blue are included.

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