THE DETERMINATION OF POLDINE METHYL METHOSULPHATE IN BIOLOGICAL FLUIDS

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A spectrophotometric method is described for the quantitative determination of the quaternary ammonium compound, poldine methyl methosulphate in aqueous solution and in biological fluids. The method involves coupling with methyl orange with simultaneous extraction into a mixture of ethylene dichloride and isopentanol and the determination of the optical density of the resulting acidified solution. The effect of pH on the coupling and extraction and the specificity of the reaction towards related compounds are examined.

FOLLOWING successful clinical trials (Douthwaite, Hunt and Macdonald, 1957; Douthwaite and Hunt, 1958) as a gastric secretion inhibitor, the distribution, metabolism and rate of excretion of poldine methyl methosulphate (Nacton) were studied.

Published methods for the analysis of quaternary nitrogen compounds were all unsuitable when applied to the determination of this compound in biological fluids.

The bromophenol blue method of Levine and Clark (1955) was too insensitive and attempted coupling under acid and alkaline conditions with other sulphone-phthalein indicators followed by solvent extraction was not successful. The ammonium cobaltothiocyanate (Helgren, Theivagt and Campbell, 1957; Singleton and Wells, 1960), bromothymol blue (Helgren and others, 1957) and the bromocresol purple methods (Fogh, Rasmussen and Skadhaugl, 1954) were also investigated, but were again too insensitive. An examination of the chloranil reaction and the aconitic anhydride reaction of Sass, Kaufman, Cardenas and Martin (1958) revealed some interesting differences in the reactions of poldine and similar compounds, but no quantitative method of sufficient sensitivity resulted.

The method finally adopted for use in distribution and excretion studies was based on that suggested by Brodie and Udenfriend (1945) in which simultaneous coupling with methyl orange and extraction into an organic solvent is used, followed by spectrophotometric measurement after acidification.

EXPERIMENTAL

Reagents

Ethylene dichloride. Reagent grade ethylene dichloride was washed successively with N Analar NaOH (2 × ⅕ vol.), distilled water (2 × ⅕ vol.), N Analar HCl (2 × ⅕ vol.) and then 6 times with distilled water. It was then filtered through absorbent cotton wool to remove water. The ethylene dichloride was purified every 4 weeks.

Isopentanol. This was washed in the same manner as the ethylene dichloride and then fractionally distilled. After removal of the water, the clear distillate (b.p. 130–132°) was used.

Methyl orange reagent. Solid methyl orange was exhaustively extracted with ethylene dichloride in a Soxhlet extractor and then a 0·5 per cent w/v solution in water was made up. Immediately before use, equal volumes of this aqueous methyl orange solution and saturated aqueous boric acid were mixed. The mixed solution had a limited stability and was not used when turbidity had appeared.

Ethanolic sulphuric acid. Conc. H_2SO_4 (2 ml. S.G. 1·84) in ethanol (100 ml.) 5N-*Hydrochloric acid* (Analar grade).

Dipotassium hydrogen phosphate. Reagent grade (50 per cent) aqueous solution stored at +4°.

Determination of Poldine in Aqueous Solutions*

To 4 ml. of the test solution or of poldine standard solution normally containing 25–100 μg. were added ethylene dichloride (10 ml. containing 5 per cent v/v isopentanol, hereafter called EDC reagent), followed by methyl orange reagent (0·5 ml.). After manual shaking for 2 min. in a glass-stoppered tube, the mixture was centrifuged at about 700 *g*. The top aqueous layer was removed by aspiration. Any residual water droplets containing methyl orange were removed by filtration of the lower EDC layer into a dry test tube. 5 ml. of the filtrate was pipetted into another dry test tube, followed by ethanolic H_2SO_4 (1 ml.). After thorough mixing of the added acid, the resulting pink colour was read in a 2 cm. glass cell of the Unicam SP600 at 525 mμ against a blank, using distilled water initially in place of the test or poldine standard solution.

If the colour was too dense to read, the solution was suitably diluted with EDC reagent. Beer's law was then obeyed up to 1000 μg. poldine.

When analysing unknown solutions for poldine, standards were performed simultaneously since there was a small but appreciable variation in standard graphs prepared on different days.

Determination of Poldine in Urine

The pH of the urine was adjusted to 7·5 by the addition of 50 per cent K_2HPO_4. After measuring the volume, it was centrifuged at about 2000 *g* for 10 min. to remove any solid present. 4 ml. of supernatant was added to a glass-stoppered tube containing water or standard poldine solution (4 ml.), EDC reagent (10 ml.) and methyl orange reagent (0·5 ml.). The procedure was then as for the determination of poldine in aqueous solutions.

Determination of Poldine in Tissue

Preparation of tissue. After the removal of fat and all connective tissue, the organs were briefly rinsed in distilled water, blotted dry and

* In the text the approved name refers to the methyl methosulphate except where stated otherwise.

weighed. They were then homogenised in an M.S.E. homogeniser with an equal weight of water and transferred to a measuring cylinder. The homogeniser blades and tissue container were rinsed with water and the washings added to the homogenate. The homogenate was diluted to give a volume numerically three times the weight of the tissue and 3 ml. of homogenate (i.e. 1 g. tissue) was used for each determination. The consistency of this homogenate was such that it could be pipetted using a wide jet pipette. It was found that the alimentary tract was more difficult to homogenise but by grinding the frozen tissue after cutting into small pieces and then using the above procedure, a satisfactory homogenate could be obtained.

Method of deproteinising. 3·0 ml. homogenate prepared as above (i.e. 1·0 g. tissue) was heated in a boiling water-bath for 30 min. with 1·0 ml. 5N HCl and 5·0 ml. water or poldine standard solution. A glass bulb was used to prevent evaporation from the tube. The mixture was then rapidly cooled and transferred to a 15 ml. centrifuge tube and centrifuged for 10–15 min. at about 1000 g. An aliquot of the clear supernatant (5·0 ml.) was added to a glass-stoppered tube containing aqueous K_2HPO_4 solution (4 ml.), EDC reagent (10 ml.) and methyl orange reagent (0·5 ml.). These quantities gave a pH of 7·2. The extraction, coupling and reading procedures were as described above.

Determination of Poldine in Blood

The method described for tissues was equally suitable for the determination of poldine in blood. 1·0 ml. blood was used instead of 3·0 ml. homogenate and after removal of protein, 4·0 ml. of the supernatant was taken.

RESULTS

Stability of Methyl Orange Complex

Both the acidified and non-acidified forms of the methyl orange complex in EDC reagent are stable for at least 24 hr. at +4°. After a week the optical density of an acidified solution changed only from 0·87 to 0·91.

Reproducibility and Stability in Homogenates

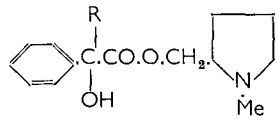
A standard poldine solution was added to a rat liver homogenate which was stored at −17°. Daily determinations over a period of 7 days showed that the recovery of poldine was 99·2 ± 1·8 per cent (mean ± s.e.m.).

Efficiency of Extraction

A comparison of the standard graph obtained from aqueous poldine solutions with a standard graph of poldine in EDC reagent showed that the efficiency of the extraction of the methyl orange-poldine complex was 89 per cent over the range 25–100 μg. poldine. Investigation of variation in the shaking time showed that extraction was completed within 1 min.

Specificity of Methyl Orange Coupling Method

The specificity of the methyl orange coupling reaction was investigated in a series of compounds based on poldine.



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Using the method described for aqueous solutions, it was found that the tertiary esters, (1-methyl-2-pyrrolidyl)methyl benzilate (poldine base) (I, R = Ph) and (1-methyl-2-pyrrolidyl)methyl α-cyclohexylmandelate (I, R = cyclohexyl), both couple satisfactorily with methyl orange. However, the resultant standard graphs of optical density against concentration show a slight deviation from Beer's law. The sensitivities of poldine base and methyl methosulphate and the methyl methosulphate of I (R = cyclohexyl) are very similar but that of the latter base is considerably less.

N-methyl prolinol is comparatively insensitive to methyl orange coupling and *N*-methylprolinol methyl methosulphate is completely insensitive.

The Effect of HCl Treatment on the Recovery of Poldine from Rat Liver Homogenate, Faeces and Urine

The HCl method was the standard procedure for the determination of poldine in tissue homogenates. The method without HCl treatment used 3·9M sodium chloride (1·0 ml.) in place of the HCl and the aliquot of the supernatant (5·0 ml.) was added to the phosphate buffer pH 7·4 (4 ml.; 50 per cent) in place of the aqueous K_2HPO_4 solution to retain the same molar salt concentration at the coupling stage. For an aqueous comparison, water (3·0 ml.) was substituted for the 3·0 ml. of homogenate.

With liver, faeces and urine the standard graphs showed that different amounts of complex were obtained (see Fig. 1). Taking the recovery of poldine in the absence of tissue under these conditions as 100 per cent, the recoveries from liver homogenate in the presence and absence of HCl were 89 and 68 per cent respectively; from faeces the recoveries were 91 and 80 per cent and from urine they were 95 and 88 per cent. These figures stress the importance of the HCl treatment for the determination of poldine in tissues.

Effect of pH on the Coupling Reaction

The pH range of coupling was investigated by the aqueous method modified so that the molar salt concentration was made the same as in the HCl deproteinising method.

Sodium dihydrogen phosphate and dipotassium hydrogen phosphate with varying volumes of hydrochloric acid and sodium hydroxide solution were used to obtain the different pH values at equal molarities.

From Fig. 2 it can be seen that the range of pH for efficient coupling was between pH 4·0 and 9·0. Above pH 9·0 and below pH 4·0 the colour yield fell off very sharply.

pH Stability

Under strongly acid or strongly alkaline conditions no extraction of poldine by EDC reagent occurred nor was it possible to couple and extract simultaneously under these conditions. Further qualitative experiments showed that on making a poldine solution strongly alkaline and then adjusting the pH to about 6·5 and attempting to couple, no coloration was produced. However, if the solution were made strongly acid and then the pH readjusted to about 6·5, coupling did occur.

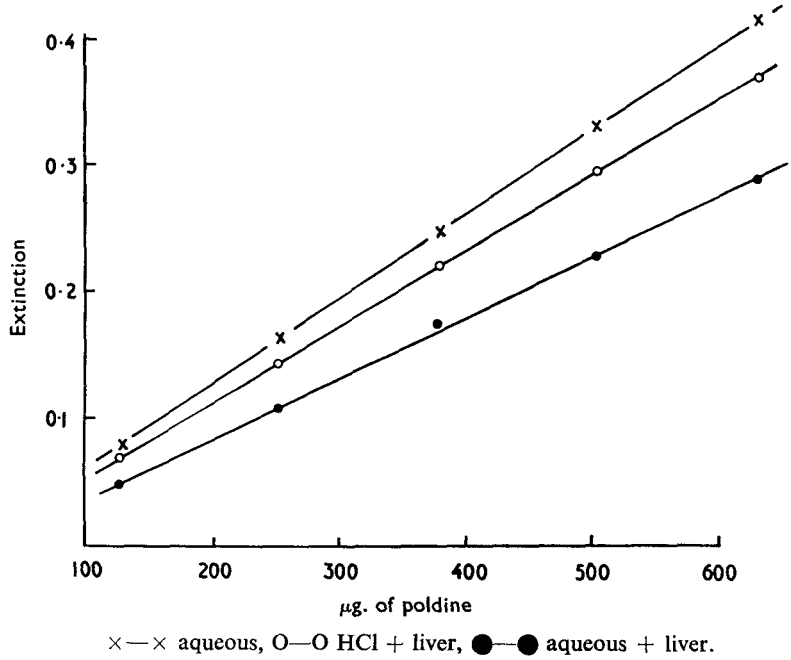

FIG. 1. The effect of HCl treatment on the recovery of poldine from liver homogenates.

DISCUSSION

As poldine is decomposed at a high pH and is insoluble in light petroleum and only sparingly soluble in ethylene dichloride, chloroform, carbon tetrachloride or benzene, the method of Brodie, Udenfriend and Baer (1947) required modification. The complex formed between methyl orange and poldine is, however, soluble in chloroform and ethylene dichloride and the latter was found to be slightly better for extraction of the complex.

The experiments on the recovery of poldine from rat liver homogenate, faeces and urine showed that a proportion of the poldine was not being determined by the ordinary aqueous method in the presence of tissue or faecal homogenate, or urine. The analyses with HCl present resulted in increased coupling but still not to the extent of that obtained in aqueous solution. It is thus apparent that the tissue is either destroying the poldine, or is affecting the extraction. As standards are performed with all tests, the quantitative determinations should not be affected.

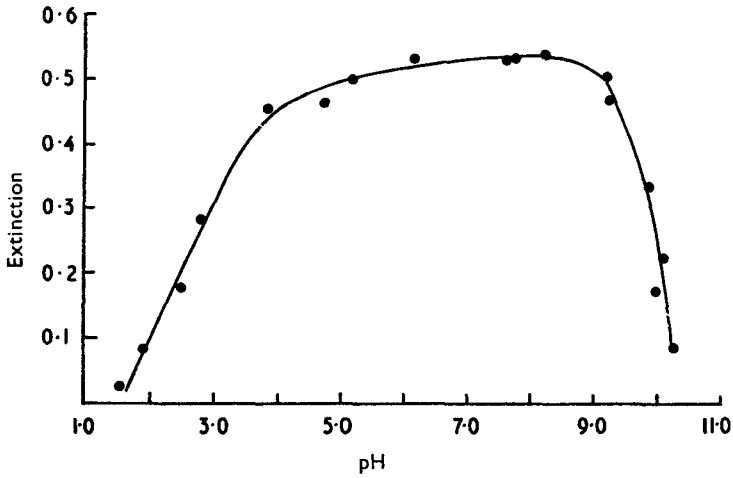

FIG. 2. The effect of pH on the coupling of poldine with methyl orange and on the simultaneous extraction with EDC reagent.

In view of the fact that poldine base and methyl methosulphate and *N*-methyl prolinol all couple to some extent with methyl orange and the coupling product can be simultaneously extracted into the EDC reagent, it is not possible by this method to establish whether the compound assayed in the tissue distribution and excretion studies is in fact the unchanged quaternary compound. Although the base has a similar sensitivity towards coupling as the methyl methosulphate, a metabolite may differ in its sensitivity.

The fact that poldine couples well with methyl orange whereas *N*-methyl prolinol methyl methosulphate does not couple at all, shows that the benzilic acid moiety has some influence on the coupling reaction. This can be compared with the work of Singleton and Wells (1960), who found that whereas poldine couples with ammonium cobaltothiocyanate, *N*-methyl prolinol methyl methosulphate does not react with this reagent.

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