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STUDIES ON BILE

Part I. Quick Method of Cholic Acid Estimation in Bile/Bile Concentrate

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Abstract. For the determination of cholic acid Reinhold and Wilson's modification of the modified Gregory–Pascoe reaction was investigated. When applied on bile or bile concentrate the results obtained were definitely on the lower side in the presence of proteins and pigments. The presence of deoxycholic acid and cholesterol in bile seems to have no effect.

Natural bile is a rich source of bile salts and bile acids, which find varied applications in pharmaceutical industry. For reasons of making the transportation of this byeproduct of slaughter house, easier and cheaper, the bulk of the natural bile is reduced by removal of its water contents. The concentrated bile which may have solid residue from 50 to 75% is commerically known as 'bile concentrate'. Under the international specification for bile concentrate the cholic acid contents have also been fixed to a minimum of 45% in the fat, protein and pigments free dry residue.

Since the removal of fat, protein and pigments involve time consuming procedures, there was need for a method that could be applied directly on bile or bile concentrate, that is, without isolation, extraction or purification of bile acids from companion substances.

Doubilet used the method of Reinhold and Wilson's[1] modification of the modified Gregory–Pascoe[2] reaction for the estimation of cholic acid in duodenal drainage, but in this case also the pigments and proteins were removed prior to the estimation of cholic acid. We applied this reaction for the estimation of cholic acid directly in bile/bile concentrate without involving any step of purification.

Although the modified Gregory–Pascoe reaction is said to be specific for cholic acid with reference to other bile acids; its specificity with regard to other substances, as may be encountered in natural bile, has not been ascertained. Therefore, in order to have an idea of the extent to which, cholesterol, deoxycholic acid, proteins and pigments, will affect the estimation of cholic acid by the modified Gregory–Pascoe reaction, the present studies were undertaken.

Reagents and Apparatus. Sulphuric acid of analar grade was used.

Cholesterol[3] was prepared from the spinal cord of cattle, m.p. 147–148°; $[\alpha]_D^{26} = -39$ to -40.

Pure deoxycholic acid was obtained from laboratory grade deoxycholic acid (BDH) by crystallization from glacial acetic acid.

Cholic acid analytical grade was a gift from Dr. S. Sabir Ali, Biochemistry Department, University of Karachi. It was dried at 110°C for 24 hr before preparation of standard solution.

Standard solution of cholic acid was prepared by dissolving 200 mg dried cholic acid in 8.0 ml, NaOH (0.1N) and making up the volume to 100 ml.

Furfural solution was prepared by diluting 0.9 ml freshly distilled colourless furfural of laboratory grade (BDH) to 100 ml with distilled water. For the measurement of colour Unicam model SP-500 and Beckman DB spectrophotometers were used.

Experimental

Determination of Maxima. The colour was developed by heating together 0.1 ml standard cholic acid solution, 6 ml (16N) H_2SO_4 and 1 ml (0.9%) aqueous furfural for 8 min at 70°C in a water-bath. The spectrum shows two peaks exhibiting maxima at 607 and 380 nm (Fig. 1). For the estimation of cholic acid the measurement of colour was made at 610 nm.

Determination of Time Period for Maximum Colour Development and Colour-Decay with Time. The colour was developed in six separate glass-stoppered cylinders containing 0.2 mg cholic acid in each case. The set was designed in such a manner that at the end of 15 min each cylinder was heated for a period of 6, 7, 8, 9, 10 and 15 min. The reaction was arrested by chilling and O.Ds were measured. Maximum colour development occurs after 8 min of heating. It further shows no change in chromogenity of developed colour even after 15 min at 70°C. The blue colour starts developing after a lag period of about 4 min. Contrary to the observation made by Doubilet the colour remains fairly stable at room temperature even after 22 min (Table 1).

Effect of Cholesterol and Deoxycholic Acid. Figure 1 gives absorption curves due to cholesterol and deoxycholic acid. Cholesterol shows no absorption in

TABLE 1. STABILITY OF DEVELOPED COLOUR AGAINST TIME.

Time* (min)	O.D.	Time (min)	O.D.
7	0.320	16	0.320
9	0.320	19	0.319
10	0.319	22	0.320
13	0.320	—	—

*Time was recorded after development of maximum colour.

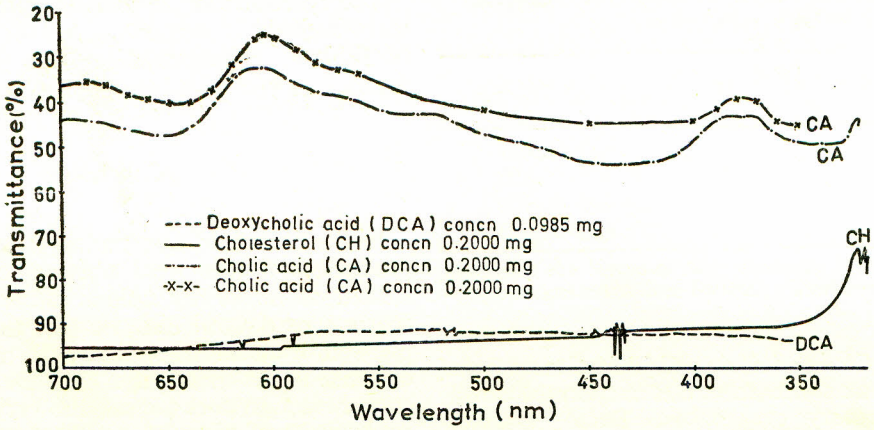

Fig. 1. Absorption spectra of cholic acid (CA), deoxycholic acid (DCA) and cholesterol (CH) after modified Gregory–Pascoe reaction.

the region around 610 nm. Deoxycholic acid shows negligible absorption, which could be due to the traces of cholic acid present as impurity. Assuming that the background absorption is due mainly to deoxycholic acid, nevertheless, it can safely be neglected, since the presence of deoxycholic acid as compared with cholic acid in bile of slaughtered animals is reported in the range of 10–15%. Since we are using dilute bile solutions for estimation in which the concentration of cholic acid is around 0.2 mg hence the amount of deoxycholic acid present in each estimation will be in the range of 0.02–0.03 mg. The absorption curve shown is due to 0.0985 mg deoxycholic acid, therefore, in actual estimation this background absorption will be further reduced by 1/4.9 to 1/3.3.

Effect of Pigments and Proteins. The effect of pigments and proteins was studied by indirect method. Proteins and pigments were removed by the procedure of Harwood[4] by means of zinc hydroxide. The clear aqueous extracts of bile salts from treated bile were used for cholic acid estimation, and results compared with that obtained by direct estimation from bile solution (Table 3). Slightly higher percentage in case of purified bile may be attributed to the increased sensitivity of this colour reaction due to the removal of proteins and pigments.

Similar observation has been recorded by Reinhold and Wilson.

Removal of Pigment and Protein. In the original procedure of Harwood 3 ml (2N) KOH has been used. When we used the same volume recovery of cholic acid exceeded 100% (Table 2).

Recoveries above 100% are due to volume displacement effect caused by excess formation of $ZnCO_3$ which is formed because unreacted $ZnSO_4$ remains in the solution, if lesser volume of KOH solution is used. Hence instead of 3 ml (2N) KOH as given in the original method, we used 4.5 ml (2N) KOH solution.

Following is the scheme given for the estimation of cholic acid in deproteinized and depigmented bile.

10 ml dil bile (7 ml bile+3 ml water)

Untreated bile	*Treated bile*
4 ml diluted to 100 ml	4 ml+20 ml water+4·5 ml KOH (2N) +3 ml $ZnSO_4$ (40%), centrifuged and worked up according to the method given by Doubilet.[5] Extract made up to 100 ml.
Used 0.025 ml for estimation	Used 0.025 ml for estimation.

TABLE 2. EFFECT OF KOH VOLUME VARIATION ON CHOLIC ACID RECOVERY FROM ZINC HYDROXIDE GEL-PROTEIN AND PIGMENT REMOVAL METHOD.

Cholic acid/exp. (mg)	Vol of 2N KOH (ml)	Vol. of 40% $ZnSO_4$ (ml)	Cholic acid recovery (%)
0.2	3.0	3.0	122.5
0.2	3.5	3.0	109.3
0.2	3.9	3.0	108.3
0.2	4.2	3.0	106.0
0.2	4.5	3.0	96.0
0.2	5.0	3.0	96.0

TABLE 3. VALUES OF CHOLIC ACID IN THE DEPROTEINIZED AND DEPIGMENTED BILE AND UNTREATED BILE.

Animal	Cholic acid in treated bile (%)	Cholic acid in untreated bile(%)
Cow	4.51	4.44
Buffalo	3.58	3.37
Sheep	4.70	4.49
Calf*	1.37	1.29
Goat†	6.87	6.90
Fat-tailed sheep†	4.41	4.41

*Calf bile was estimated with 0·05 ml instead of 0·025 ml.
†The pigment was not completely removed.

TABLE 4. QUANTITATIVE RANGE FOR CHOLIC ACID DETERMINATION.

Amount of cholic acid/exp (mg)	O.D.* at 610 nm
0.05	0.125–0.155
0.10	0.245–0.310
0.20	0.480–0.610
0.40	0.850–1.100

*The range in the values of O.D. indicated the maximum and minimum colour intensities obtained in different sets of experiment.

Estimation of Cholic Acid in Bile/Bile Concentrate. Filtered bile or bile concentrate was evaporated down to dryness in rotary evaporator. Dried samples (0.1–0.2 g) were then taken and transferred in a glass-stoppered test-tube. These were further dried under high vacuum for 2 hr at 70°C. The weight of the samples was then determined by difference, and dissolved in water (25 ml), so that 0.05–0.1 ml of the test solution contains approximately 0.2 mg cholic acid. Suitable aliquots of the test solutions were then taken in test-tubes for estimation; in any case the volume of test solutions never exceeded 0.1 ml. When less than 0.1 ml volumes were employed, the initial volume of the test solution was then made up to a final volume of 0.1 ml. To this solution (0.1 ml) was then added 1.0 ml (0.9%) furfural solution followed by 6.0 ml (16N) H_2SO_4 and heated for 8 min at 70°C in a water-bath. At the end of this period the tubes were cooled quickly to room temperature in an ice-bath. Two standards, which were routinely prepared with the reading of each batch of test solutions contained 0.1 and 0.2 mg cholic acid. The O.Ds of the resultant blue colour were measured at 610 nm and cholic acid per cent was calculated according to the following formula;

$$\frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times \frac{\text{Concn of standard} \times 100}{\text{Amount of unknown}}$$

The reaction can be used to determine cholic acid quantitatively in amount of 0.1–0.3 mg as indicated in Table 4. The deviation from Beer's law is quite marked as one goes into higher concentrations.

Conclusion

In the course of our studies it was found that reproducible results were obtained when estimations were done in the dry residue rather than in bile concentrate or natural bile. Further the presence of the pigment even in trace amounts may also cause a decrease in the sensitivity. Thus the sensitivity of this colour reaction does not seem to vary with the increase or decrease of pigment quantity, but rather depends on its total absence or presence in any amounts. Considering these results it can be safely derived that for routine, analysis, this method is both quick and reliable. The results are liable to be on the lower side by 2–6% in presence of protein and pigments, while deoxycholic acid and cholesterol seem to have no effect.

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