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A Simplified Method for Determining Bile Salts in Bile.

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The metabolism of bile salts has received little attention due to the lack of a quantitative method which would give accurate results on small amounts of material. Five types of method have been tried: (1) using the Pettenkofer color reaction between cholic acid, furfural and concentrated sulfuric acid; (2) using separation of the bile acids and determination by weight; (3) stalagmometric methods depending on the property of bile salts to lower surface tension; (4) involving hydrolysis of the salts and subsequent determination of the amino nitrogen of the free taurine and glycocholic acid; (5) utilizing the fact that ferric salts of bile acids are insoluble.

Detailed criticisms of these methods by Hooper and Foster[1] and Schmidt and Dart[2] show that they are either too inaccurate or too laborious to be practical. One of the simplest recent methods is that of Szilard[3] who made use of a principle that has long been known, that bile salts are precipitated by ferric chloride,[4] and found that the iron in the precipitate is proportional to the bile salts present. In attempting to use this method and the modifications introduced later by Katayama,[5] it was found that the method was inaccurate and uncertain for small quantities of bile salts. Attempts to improve the technic have resulted in a new method which resembles that of Szilard only in its basic principle.

The new procedure is as follows: 5 cc. of bile are pipetted into a 100 cc. flask and diluted with 90 cc. of distilled water. Two cc. of 2 M (44%) calcium chloride, and 2 cc. of 4 M KOH are added and the whole is thoroughly mixed by shaking. Let stand for 10 minutes and then filter. The filtrate should be water clear and practically free from bilirubin, and if it is not, the bile is probably too concentrated and a greater dilution should have been made. Twen-

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[1] Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, **38**, 355.

[2] Schmidt, C. L. A., and Dart, A. E., *J. Biol. Chem.*, 1920, **45**, 415.

[3] Szilard, P., *Biochem. Z.*, 1926, **173**, 363.

[4] Hammarsten, O., Textbook of Physiological Chemistry, tr. by J. A. Mandel, New York, 5th ed., 1909, pp. 312-314.

[5] Katayama, I., *Arch. Int. Med.*, 1928, **42**, 916.

ty-five cc. of this filtrate are pipetted into a 50 cc. beaker and 1 M HCl is added drop by drop until it is just acid to litmus. One cc. of 1.0% ferric chloride is then added and, on mixing, the bile salts are seen to precipitate in flocculent masses. When the amount of bile salt present is small, precipitation takes place slowly and one hour should be allowed. The contents of the beaker are then transferred to a 50 cc. Jena sintered glass filter funnel, grade 4, the surface of which is covered with a thin layer of asbestos fibers. The presence of the asbestos is important because without it the filtration process is too slow to be practicable. The filtration is done under moderate suction and the precipitate washed 4 times with 1 cc. portions of distilled water to remove the excess ferric chloride, and is then washed through into a 30 cc. test tube with 4 cc. portions of 95% ethyl alcohol. The alcoholic filtrate is mixed and sufficient additional alcohol is added to completely dissolve any precipitate which may have formed due to the presence of a small amount of water. One cc. of 2 M ammonium hydroxide is then added, and the tube is allowed to stand until the ferric hydroxide has completely precipitated, which usually requires from 5 to 15 minutes. The precipitate is then filtered out on the same filter, which has been previously washed with 1 M HCl and distilled water, and washed with 2 portions of distilled water of 1 cc. each and then washed through into a test tube with 1 cc. of 1 M HCl and two 1 cc. portions of 0.1 M HCl followed by 1 cc. of distilled water. The contents of the tube are then transferred to a 25 cc. volumetric flask, and the iron is determined after the method of Lyons,[6] which is as follows: 1 drop of thioglycolic acid is mixed with the flask contents and 2 M ammonium hydroxide is added drop by drop until a permanent red color develops, which is compared with a standard prepared in the same way using 0.2 mg. of iron. The standard iron solution is 0.703 gm. ferrous ammonium sulfate dissolved in a liter of 0.1 M HCl, so that 1 cc. contains 0.1 mg. of iron. The amount of iron in the sample is determined by comparing the 2 solutions in a colorimeter, and the amount of bile acid present in the original sample of bile is obtained by multiplying by the conversion factor and correcting for dilution, as indicated in the equation:

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[6] Lyons, E., *J. Am. Chem. Soc.*, 1927, **49**, 1916.

$$\frac{Rs}{Ru} \times S_f \times C \times \frac{Vt}{Va \times B} = \text{mg. bile acid in 1 cc. bile}$$

where Rs = reading of the standard
Ru = reading of the unknown
S_f = mg. iron in the standard
C = conversion factor (mg. bile acid per 0.1 mg. iron)
Vt = Volume of volumetric flask used
Va = Volume of aliquot of filtrate analyzed
B = Volume of bile taken

The amount of bile acid which corresponds to a given amount of iron can be calculated from the molecular weights of the acids, assuming that they combine with ferric iron stoichiometrically. In order to check this assumption, and at the same test the accuracy of the analytical method, samples of pure glycocholic acid and sodium taurocholate (generously supplied by Prof. C. L. A. Schmidt) were analyzed, with results shown in Table I. The findings justify the use of the conversion factor calculated from stoichiometric relations. The probable error of the method is about 1% when pure solutions of the salts in distilled water are analyzed.

TABLE I.

Material	Sample mg.	Iron Found mg.	Mg. Bile Acid per 0.1 mg. Iron	Calculated mg. Bile Acid per 0.1 mg. Fe
Glycocholic Acid (Pure)	5.0	0.198	2.52	2.51
" " "	3.6	0.145	2.48	2.51
Sodium Taurocholate (Pure)	6.3	0.206	3.06	2.98
" " "	8.6	0.275	3.12	2.98
" " (Practical)	9.4	0.226	3.28	2.98
" " "	7.8	0.235	3.32	2.98

In order to test the accuracy of the method when applied to bile, the recovery of added sodium taurocholate from dog bile was investigated with results indicated in Table II. These show an average recovery of added bile salt of 95%. This value has also been obtained in similar experiments with glycocholic acid.

TABLE II.

Bile cc.	Sodium Taurocholate mg. added	Iron Found mg.	Total Sodium Taurocholate mg.	Recovered Sodium Taurocholate mg.
1	0	0.412	12.3	—
1	0	0.396	11.8	—
1	10	0.725	21.6	9.6
1	10	0.715	21.3	9.3

The substances which ordinarily complicate bile salt determinations, such as cholesterol, amino acids, bile pigments, lecithin and lipoids, do not enter into consideration here since they do not form insoluble compounds with ferric chloride. Carbonates and phosphates which do form such compounds are removed by calcium chlorid and potassium hydroxide and the small amount which may remain is insoluble in alcohol and hence does not contribute to the error of the experiment. Precipitation of the pigments with calcium chlorid has been known for a long time but has not been used by other workers on this problem who instead employ charcoal in some form to adsorb the pigments. Kusui[7] has shown that such procedures also absorb an appreciable amount of the bile salts also.

Before this method can be used on bile from different animals, the relative proportions of the glycocholic and taurocholic must be known since the conversion factor of iron to bile acid depends on which acid is present. In the dog where only taurocholic acid is produced the problem is simple but in man where about equal amounts of both acids are found, an average value between the 2.9 of taurocholic acid and 2.5 of glycocholic acid, or 2.7, must be used.

The method was developed to study the bile acid excretion in patients with post-operative bile fistulas and particularly to study the effect of administering decholin (sodium dehydrocholate). It was found that decholin by mouth increased the amount of bile acids produced per hour in the fistula bile, reaching a high level in 3 to 5 hours after administration and then gradually falling off. The specific gravity and volume of the bile showed some increase.

Other samples of bile from various sources analyzed by this method show a great variation in concentration of bile acids. The highest concentration of bile salts was found in gall bladder bile from an ox which was 4.5%, and the least in fistula bile of a human which was from 0.1% to 0.4%.

This method can also be applied with no modification to the duodenal fluid obtained during a Lyon's drainage. Work is now in progress to adapt this method to the analysis of blood and urine.

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[7] Kusui, K., *J. Biochem.*, 1932, **15**, 399.