A Method for Spectrophotometric Determination of Chenodesoxycholic Acid in Bile

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In 1924 Wieland and Revery¹ isolated a new bile acid from human bile, which they called antropodesoxycholic acid, but which proved to be identical with chenodesoxycholic acid, isolated at the same time from goose bile by Windaus, Bohne and Schwarzkopf². This acid (3,7-dihydroxycholanolic acid) could be prepared in relatively large amounts from human bile, but its physiological rôle does not seem to have been studied until the last few years. There has not even been a method for its quantitative determination in bile. Since 1951 there have been published some methods for separating the bile acids by columnar chromatography and determining the different fractions by means of titration (Bergström and Sjövall³, Sjövall⁴) or infrared spectrometry (Wootton⁵ Wootton and Wiggins⁶). It has been shown⁶ that chenodesoxycholic acid is to be found in human bile in the same proportion as cholic acid (3,7,12-trihydroxycholanolic acid)* while desoxycholic acid (3,12-dihydroxycholanolic acid) forms a minor fraction and lithocholic acid (3-hydroxycholanolic acid) is usually absent.

A perfect separation of the two dihydroxy acids is however not yet obtainable by chromatographic technique⁴⁻⁶ and infrared spectrometry is not an ordinary laboratory method. It therefore seemed to be of importance to have such a colorimetric method available as would make it possible to determine the chenodesoxycholic acid in the presence of other bile acids. This was not the case with Saba's⁷ different colorimetric methods — the only ones referred to in the literature.

Wieland and Revery¹ showed that chenodesoxycholic acid gave a positive Liebermann-Burchard reaction, and here one modification of this reaction is described, which may be used for quantitative determinations of chenodesoxycholic acid in an extract of hydrolysed bile and in the presence of the other common bile acids.

* According to Saba⁷, however, this was already reported in 1939 by Ohyama and Nagaki in a Japanese journal: *Nippon-Shokakibyogakkai-Zasshi*. 38 (1939) 245.

METHOD

Reagents: Sodium hydroxide, 1 *N*; hydrochloric acid, approx. 2.5 *N* (titrated against the *N* hydroxide); ethyl ether, redistilled, peroxide-free; silver nitrate, approx. 0.05 *N* in 3 *N* nitric acid; light petroleum, b.p. 40–70° C, redistilled or Ed. XI; ethanol, 70 % (v/v); ethanol, absolute; ethyl acetate, *p. a.* or Ed. XI. (The grade of the ethyl acetate is very important. I have used the grade Ed. XI, b. p. 74–77° C, free from acid when tested with litmus paper. Technical ethyl acetate must usually be purified, *cf.* Gilman *); sulphuric acid, conc. *p. a.*; acetic anhydride, *p. a.*; chenodesoxycholic acid standard *: 1.00 mg/ml absolute ethanol (macro method) or 0.10 mg/ml absolute ethanol (micro method).

A. Hydrolysis and extractions:

1. Hydrolyse a suitable amount (about 0.2–1 ml fresh or 25–100 mg lyophilized bile) with 30 ml *N* sodium hydroxide for 5 hours at 110°–120° C.
2. Neutralize with an equivalent amount of the 2.5 *N* hydrochloric acid and acidify with one additional ml.
3. Dilute to about 50 ml with water and extract with 3 portions of 50 ml ethyl ether. Evaporate the combined extracts to about 50 ml and wash with water until the water gives no opalescence with the silver nitrate solution.
4. Evaporate to dryness and subject the residue to a three-stage counter-current extraction between 70 % ethanol and light petroleum 50 : 50. Evaporate the three combined ethanol extracts to complete dryness, dissolve in absolute ethanol and make up to volume (10.00 ml).

B. Colour reaction (macro method):

5. Evaporate an aliquot amount of the final alcohol extract (plus some standard solutions containing 0.2–1.5 mg chenodesoxycholic acid) to dryness in test tubes on water bath, then put the tubes into an oven at 100° C for about 5 minutes.
6. Prepare the EtS-reagent by mixing ethyl acetate and concentrated sulphuric acid in the proportion 15 : 1; cool if necessary under tap water to room temperature.
7. When the test tubes have cooled down to room temperature add 3.00 ml of the EtS-reagent to a set of the tubes and mix thoroughly with glass-rods.
8. Add 2.00 ml acetic anhydride to the tubes at appropriate intervals. Mix thoroughly again with the glass-rods.
9. Determine the absorbancy in a spectrophotometer at 6150 Å in a 10 mm cuvette 12–15 minutes after the addition of the acetic anhydride to the tube.

Micromethod: Add to the samples and standards (containing 0.01–0.15 mg chenodesoxycholic acid) 1.00 ml EtS-reagent and at intervals 0.67 ml acetic anhydride. After 12–15 minutes read the absorbancy with a Pulfrich photometer, using 50 mm microcuvettes and filter S 61.

DISCUSSION OF THE METHOD

A. Hydrolysis and extractions. This part is in the main identical with the one ordinarily used for isolation of bile acids, see for instance Bergström and Sjövall⁹. The hydrolysis has proved to be complete — higher temperature, higher concentration of the sodium hydroxide or longer hydrolysing time has not given a higher yield. On the other hand, experiments with pure chenodesoxycholic acid show that nothing is destroyed during the process and at the same time that the extractions are quantitative. Only when working

* The author is indebted to Professor S. Bergström, Lund, for the samples of chenodesoxycholic acid and other bile acids tested in the experiments described in this paper.

Fig. 1. Absorption curves. $\times$ chenodesoxycholic acid (0.95 mg), $\bullet$ bile extract, prepared as described in the text. $\square$ cholic acid (1.0 mg), Δ desoxycholic acid (1.0 mg), $\circ$ lithocholic acid (1.0 mg). In this and following figures EtS = 15 : 1 means a reagent consisting of 15 parts ethyl acetate and 1 part sulphuric acid. EtS:Ac₂O = 3:2 means a mixture of 3.00 ml of the EtS-reagent in use and 2.00 ml acetic anhydride.

with small amounts (less than 5 mg of the acid) is the recovery less than 95–100 %.

For the colour reaction it is absolutely necessary that the final extract should be quite free from hydrochloric acid and cholesterol. (The reaction will however unmask the presence of minute amounts of these substances, see below). Acidification is therefore recommended with a small amount of dilute hydrochloric acid. The washings with water and the counter-current distribution must not be omitted.

B. Colour reaction. a) *The choice of reagents.* Chenodesoxycholic acid dissolved in acetic anhydride gives a positive Liebermann-Burchard reaction after the addition of a few drops of concentrated sulphuric acid. The colour is not stable and therefore not suitable for colorimetry. In order to stabilize the colour a number of solvents were tried. With glacial acetic acid a promising absorption curve developed with a maximum at 5100 Å, which in certain proportions of the reagents showed good colour-stability. However, both cholic acid and desoxycholic acid absorbed at the same wave-length to such an extent (10–20 % of chenodesoxycholic acid) that the method was unsatisfactory. This was also the case when chloroform was used as a solvent.

With ethyl acetate, used instead of glacial acetic acid, only a plateau was formed at 5100 Å, but in addition it developed a broad-based absorption peak with maximum at 6100–6200 Å (Fig. 1). The reagents mixed in certain proportions gave a sufficiently stable colour; the presence of other bile acids had no influence and the absorbancy obeyed Lambert-Beer's law (Fig. 2). Thus, ethyl acetate could be regarded as an ideal solvent.

Fig. 2. Absorbancy of chenodesoxycholic acid. a) Micro method: Pulfrich-Photometer, Zeiss, 50 mm microcuvettes, filter S 61. EtS = 15:1; EtS:Ac₂O = 1.00 : 0.67. b) Macro method: Unicam SP 600, 10 mm cuvettes, 6 150 Å. EtS = 15 : 1; EtS:Ac₂O = 3 : 2. This means that the absorbancy for a given amount in the micro method will be the same as for an amount 15 times greater in the macro method.

The amount of sulphuric acid used in the colour reaction must necessarily be comparatively low. Before adding it, it therefore seemed better to mix it with either ethyl acetate or acetic anhydride. The last mentioned mixture is known from Schoenheimer-Sperry's method for determination of cholesterol¹⁰ to be unstable even when kept in an ice-bath. The mixture with ethyl acetate proved to be more stable and was therefore chosen.

b) *The amounts of the different reagents.* For practical reasons the total volume of the reaction mixture was fixed at 5.00 ml for the determination in a 10 mm cuvette of the Beckman model (which needs 3—3.5 ml).

The composition of the reaction mixture was investigated in order to achieve a) a sufficiently high molar absorbancy and b) a stable absorbancy during a sufficiently long time. From Figs. 3 and 4 it will be seen that a) is mostly under the control of sulphuric acid and b) controlled by the ratio of ethyl acetate: acetic anhydride.

In Fig. 3 the amount of acetic anhydride is fixed at 2.00 ml, the ethyl acetate is nearly constant (2.73—2.86 ml) and the sulphuric acid varies between 0.27 and 0.14 ml. This is achieved by dissolving the chenodesoxycholic acid in 3.00 ml of different EtS-reagents (*i.e.* mixtures of ethyl acetate and sulphuric acids in proportions stated in the figure). Decreasing amounts of sulphuric acid give decreasing absorbancy, but on the other hand increasing amounts give decreasing stability. The best combination of high and stable absorbancy is achieved with the EtS-reagent 15:1.

In Fig. 4 the amount of sulphuric acid is constant and equal to that in 3.00 ml of EtS-reagent 15:1, *i.e.* 0.19 ml. The ethyl acetate (and hence the acetic anhydride too) is however varied as stated in the figure. It is seen that the amount (included the sulphuric acid) must be at least 3.0 ml. On the other hand increasing amounts of ethyl acetate give an inconsiderably increased stability, appearing at about the same absorbancy level and much later. The best reaction mixture will therefore be composed of 3.00 ml EtS-reagent 15:1 and 2.00 ml acetic anhydride.

Fig. 3. The effect of the sulphuric acid on the absorbancy level (and stability). EtS: Ac₂O = 3 : 2. EtS-reagent: Δ 10 : 1, ◻ 12.5 : 1, × 15 : 1, ○ 17.5 : 1, ● 20 : 1.

c) *The influence of external factors.* The colour reaction may be performed in darkness or in light, but it is influenced by the external temperature. Fig. 5 shows that it is necessary to develop and read the colour at the same temperature. From the practical point of view it is difficult to use thermo-regulated cuvettes, and as far as I can see there is nothing to prevent work at room temperature. The temperature in the cuvette house of an Unicam spectrophotometer does not increase, not even after being in use for several hours.

d) *The course of the reaction.* When the reaction is performed in the way mentioned above, a series of different colours is developed. If the substrate is pure chenodesoxycholic acid a faint pink colour appears in the first minute, and in the second to third minutes it changes into dirty brown, soon shifting to dirty green. In the 5th–7th minutes the colour becomes more and more olive, which then remains but increases in intensity. The colour is maximal and stable from the 10th–12th until the 18th–20th minutes, then its intensity decreases slowly and after several hours it turns yellow.

When the substrate is a bile extract or chenodesoxycholic acid subjected to the whole procedure the maximum is reached in the 10th minute after the addition of acetic anhydride. It is stable no longer than till the 15th or 16th minute, and the yellow colour appears quicker, usually after 2–3 hours. The reason for this is possibly the presence of small amounts of hydrochloric acid (see below).

If the EtS-reagent is more than 2 hours old, the course of the reaction is altered. The maximum is reached later, and is stable for a shorter time. It is therefore recommended that a new reagent will be prepared after 2 hours; as seen below the reproducibility is sufficiently good to allow this. The course of the reaction is also altered if the EtS-reagent is added to the substrate more than 30 minutes before the acetic anhydride. In a typical series where

Fig. 4. The effect of the ratio of ethyl acetate to acetic anhydride on the stability (and absorbance level). The EtS-reagent contains in every case 0.19 ml sulphuric acid (i.e. the same as in 3.00 ml EtS = 15 : 1).

- Δ 4.0 ml EtS + 1.0 ml Ac_2O , $\square$ 3.5 ml EtS + 1.5 ml Ac_2O ,
 $\times$ 3.0 ml EtS + 2.0 ml Ac_2O , $\circ$ 2.5 ml EtS + 2.5 ml Ac_2O ,
 $\bullet$ 2.0 ml EtS + 3.0 ml Ac_2O , $\ominus$ 1.5 ml EtS + 3.5 ml Ac_2O .

the EtS-reagent was added 0, 30, 60 and 120 minutes before the addition of acetic anhydride the absorbance was 0.400, 0.397, 0.388 and 0.373, respectively, for the same amount of chenodesoxycholic acid.

e) Specificity. Most bile compounds except the bile acids will have been eliminated before the colour reaction is performed and therefore without influence. The same is true of the added compounds. As mentioned above minor amounts of cholesterol or hydrochloric acid will influence the reaction. Cholesterol disturbs the stability, and the colour will deepen even after the 20th minute. Hydrochloric acid gives a quickly developing colour which reaches a maximum far below the expected and starts to drop in the 7th—8th minute and turns yellow before the 20th minute.

The final extract sometimes contains a faint amount of pigments; the main part has been destroyed during the hydrolysis. These small amounts in the extract may well be tolerated. It is not necessary and is theoretically wrong, to correct for the absorbance of the pigments in the EtS-reagent, as its absorption curve changes after the addition of acetic anhydride. In larger amounts, however, the pigments will disturb the reaction, making impossible the simple method of performing the colour reaction on an unhydrolysed bile extract, even if this extract has been freed from cholesterol by the procedure described by Isaksson ¹¹.

The common natural bile acids and some synthetic ones were tested, and from the results, listed in Table 1, it is seen that none of them showed a coloured product except chenodesoxycholic acid. This indicates the importance of the $\text{C}_7\text{—OH}$ group for the reaction, which is in accordance with the results of

Fig. 5. The effect of temperature. The colours were first developed for 15 minutes at the given temperatures, then read at room temperature (20° C). EtS = 15 : 1, EtS:Ac₂O = 3 : 2.

Idler and Bauman¹². They tested the Schoenheimer-Sperry modification¹⁰ of the Liebermann-Burchard reagent and pointed out the effect of the position 7 on the reactivity of cholesterol derivatives and certain other sterols. In addition the present results show the inhibiting power of the C₁₂-substituent, either in the form of a hydroxyl or of a keto group.

Thus, the given method is specific for chenodesoxycholic acid among the common natural bile acids. The specificity is also seen in Fig. 1, where the absorption curve for an ordinary prepared extract from human bladder bile is given. In the upper part of the spectrum the course of the curve is nearly identical with that of pure chenodesoxycholic acid; below 5 500 Å the slopes differ owing to the presence of the other bile acids in the extract.

Table I. The specificity of the colour reaction.

The determinations were made according to the macro method at 6 150 Å with an Unicam S. P. 600 spectrophotometer, using 10 mm cuvettes.

Common name	Structure	Absorbancy per mg substance			
		15 min.	20 min.	25 min.	90 min.
after the addition of Ac ₂ O					
Natural bile acids:					
Lithocholic acid	3-monohydroxy	0.010 *	—	0.010	—
Hyodesoxycholic acid	3,6-dihydroxy	0.001	—	0.002	0.006
Chenodesoxycholic acid	3,7-dihydroxy	0.420	0.418	0.395	—
Desoxycholic acid	3,12-dihydroxy	0.000	—	0.001	0.005
Cholic acid	3,7,12-trihydroxy	0.006	—	0.006	0.005
Synthetic bile acids:					
—	3,7-dihydroxy-12-keto	0.009 *	—	0.008	0.006
Dehydrocholic acid	3,7,12-triketo	0.000	—	0.000	0.001
Apocholeic acid	3,12-dihydroxy-Δ ^{8:14}	0.000	—	0.000	0.002
Sterol:					
Cholesterol	Δ ⁵ -cholestenol	0.435	0.550	0.600	—

* The alcohol extract of the acid was faintly discoloured, owing to small quantities of impurities.

Table 2. Reproducibility of the whole method, when starting with the same or different amounts of lyophilized human bladder bile.

mg dry substance	25	50	100	150	200	Mean
No.	chenodesoxycholic acid as a percentage of dry substance					
66	—	15.0; 16.1; 16.2	—	—	—	15.8
68	—	12.3	14.7	—	—	13.5
72	—	—	12.9; 13.6	—	—	13.2
85	10.9	12.1; 12.1; 13.0	12.4; 12.6	—	—	12.2
97	—	16.1	16.6	—	—	16.4
112	19.2	18.9	20.2	19.1	—	19.4
117	16.1	14.4	15.2	—	14.8	15.1
125	—	—	19.3; 19.5	—	—	19.4
126	—	—	22.0; 23.6	—	—	22.8
127	—	—	17.5; 17.3	—	—	17.4

REPRODUCIBILITY

Colour reaction. Determinations according to the macro method in an Unicam SP 600 on 20 samples of the same amount (0.95 mg) of pure chenodesoxycholic acid gave absorbancy values within the range 0.388—0.403, mean value 0.395 with a standard error of ± 1.3 %. The determinations were performed during several days and in all 12 different preparations of EtS-reagent were used.

Determinations in duplicate on 26 different extracts gave a standard error of ± 2.4 % in the macro method, and 10 duplicate determinations according to the micro method gave a standard error of ± 4.9 %.

In 11 recovery experiments the recovery percentage ranged from 97 to 104 % of the amounts added. Here the pure chenodesoxycholic acid added varied between 10 and 200 % of the original amounts (0.4—0.9 mg) in the bile extract.

The whole method. In all, 29 extracts prepared from different amounts of dry substance from 10 human bladder biles were examined. The standard error was ± 4.8 %. The results are listed in Table 2. Four recovery experiments showed a recovery of 98 to 103 % (to one of the duplicates was added 5.0 mg chenodesoxycholic acid, giving the following results: 1) 22.3 and 28.2 mg, 2) 12.9 and 18.3 mg, 3) 19.3 and 24.3 mg, and 4) 17.5 and 22.0 mg).

This communication is part of an investigation on the differences in the chemical composition of normal and pathological bile, which will be reported elsewhere¹³. However, from the results listed in Table 2 it is seen that the amount of chenodesoxycholic acid is of the same magnitude as is known for cholic acid. This is in accordance with the findings of Wootton and Wiggins⁶.

SUMMARY

1. A colorimetric method for the determination of chenodesoxycholic acid in bile is described.

2. The method is a modification of the Liebermann-Burchard reaction and is specific for chenodesoxycholic acid among the common bile acids.

3. The reaction may be performed on an extract of bile acids from hydrolysed bile with satisfactory accuracy.

This is part of an investigation partly supported by a grant from the *Swedish State Fund for the Promotion of Medical Research*. Thanks are due to Miss Ulla-Britt Josefsson for valuable assistance.

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Received March 22, 1954.