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A DIFFERENTIAL DETERMINATION OF THE DIHYDROXY-BILE ACIDS IN BILE

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Abstract

A differential determination of deoxycholic and chenodeoxycholic acids in the dihydroxy-bile acid fraction, which were obtained from bile samples, human, bovine, dog and rat biles, was colorimetrically performed, using Kiliani's method. The colorimetric data proved to be well coincident with those one obtained by gas-liquid chromatography.

INTRODUCTION

A colorimetric determination of the individual dihydroxy-bile acids in bile has not yet been reported, though it has recently been performed by somewhat tedious technique of gas-liquid chromatography[1].

The present paper deals with a differential determination of deoxycholic and chenodeoxycholic acids using Kiliani's method[2] after the dihydroxy-bile acid fraction was prepared from some bile samples and its clinical applicability was suggested.

MATERIALS AND METHODS

1. *Bile Acids and Bile Samples*

Cholic acid (CA), deoxycholic acid (DC) and lithocholic acid (LC), stock preparations in the Department of Biochemistry, Tottori University School of Medicine, and chenodeoxycholic acid (CDC), prepared from cholic acid, all were purified by recrystallization to be used as standard samples. The following bile samples were examined: bladder biles of bovines and dogs; fistula biles of human being and rats.

2. *Ferric Chloride-sulfuric Acid (FCSA) Reagent*

Ten g. of ferric chloride (Wako Junyaku, Osaka) was dissolved in 100 ml of glacial acetic acid and reserved as stock solution. One ml of the stock solution was diluted with 100 ml of concentrated sulfuric acid (d: 1.82) before being used and protected from moisture.

3. Preparation of the Dihydroxy-bile Acid Fraction from the Bile Samples

An equal volume of ethanol was added to each bile sample and the precipitates were filtered off. After the solvent was removed in a stream of nitrogen, the residue was dissolved in 2N alcoholic potassium hydroxide solution and heated in an autoclave at 120°C for 6 hr. The hydrolyzate was acidified with 2N HCl and extracted with ethyl acetate. The extract was washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to dryness to obtain the bile acid mixture. From the mixture, the dihydroxy-bile acid fraction was prepared by the following procedures, a and b.

a) From bovine, dog and human-I bile samples

The dihdroxy-bile acid fraction was separated from each bile acid mixture by column chromatography using a silicic acid (Mallinckrodt Chemical Works, 100 mesh) and ethanol-chloroform as eluent[3]. Aliquotes of effluents were checked by thin layer chromatography (TLC) using Kieselgel H (E. Merck, A. G., Darmstadt) plates (0.25 mm thick) and a solvent system of isooctane : ethyl acetate : acetic acid = 20 : 40 : 1 (v/v). The portions eluted with 4% ethanol-chloroform were collected and proved to be the aimed fraction.

b) From human-II and rat bile samples

Each bile acid mixture was subjected to preparative TLC using the same supporter (0.5 mm thick) and solvent system as described above. The plates were marked with iodine vapor and the zone corresponding to the dihydroxy-bile acid fraction (chenodeoxycholic acid) was removed from the plates and extracted with methanol, and the solvent was evaporated.

4. Spectrophotometric Determination

a) Absorption curve

Each standard bile acid, 120 μg, was weighed in a test tube and dissolved in 3.0 ml of glacial acetic acid; 2.5 ml of the FCSA reagent was added and mixed well. After 15 min, each absorption spectrum in the visible region (380-600 nm) was automatically described against water, using a Hitachi spectrophotometer model 323 or a Shimadzu UV-200.

b) Calibration curves of deoxycholic and chenodeoxycholic acids

These acids, 0-150 μg, were treated as above and subjected to spectrophotometric determination at 438 and 562 nm, respectively, affording their calibration curves as shown in Figs. 2 and 3.

c) Quantitative analysis of the dihydroxy-bile acid fraction

The dihydroxy-bile acid fraction (ca. 100 μg) in 3 ml of glacial acetic acid was spectrophotometrically determined at 438 and 562 nm as described above and quantities of deoxycholic and chenodeoxycholic acids were calculated, using the calibration curves obtained from the above explanation.

5. Gas-liquid Chromatography (GLC)

The dihydroxy-bile acid fraction was methylated with diazomethane in ether, followed by acetylation with acetic anhydride as usual. Methanol was added to the mixture and evaporated under reduced pressure to dryness. GLC analysis of the acetyl methyl ester was performed with a Shimidzu gas chromatograph model GC-4BPTF (column : 0.75% SE-52, 4 mm×2 m ; temperature, 230°C). The identification of deoxycholic and chenodeoxycholic acids was carried out by comparing with acetyl methyl esters of the standard samples.

RESULTS AND DISCUSSION

The visible absorption spectra of the standard samples are depicted n Fig. 1, and their colors developed and absorption maxima (λ_{max}) are listed in Table 1. As shown in Fig. 1, the absorption spectra of the individual bile acids, especially those of deoxycholic and chenodeoxycholic acids were so characteristic in shape that it was suggested that these dihydroxy-bile acids, even in a mixture, were differentially deter-

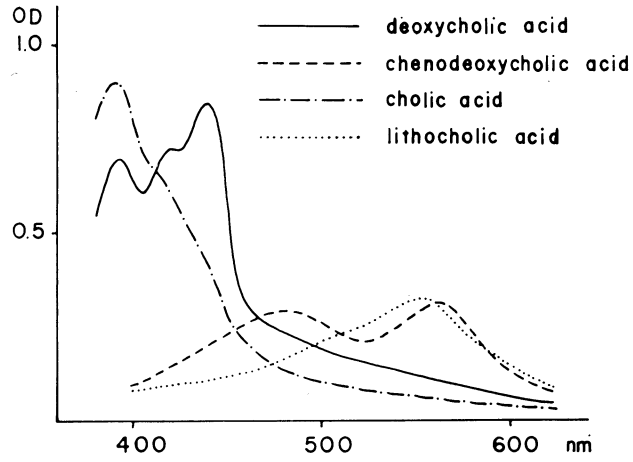

Fig.1. Absorption spectra of the bile acids in the FCSA reagent.

TABLE 1.

Colors developed in the FCSA reagent and their absorption maxima

Bile acids	Color developed	λ_{max} (nm)
Cholic	yellow	388
Deoxycholic	yellow	438
Chenodeoxycholic	rose	562
Lithocholic	rose	553

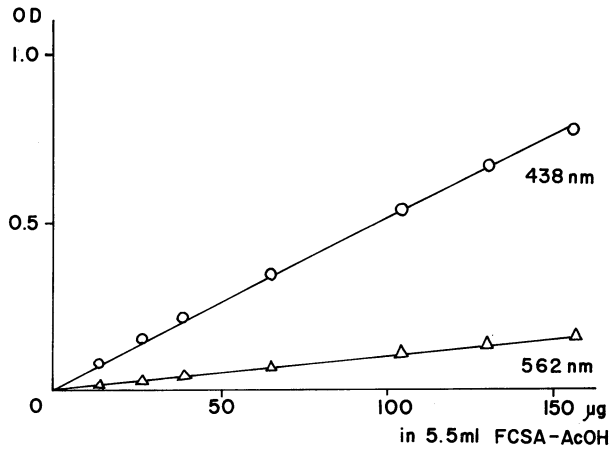

Fig.2. Calibration curves for deoxycholic acid.

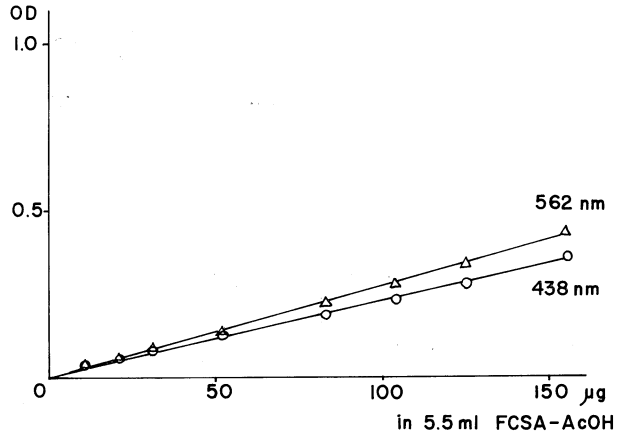

Fig.3. Calibration curves for chenodeoxycholic acid.

mined. Each calibration curve obtained for deoxycholic and chenodeoxycholic acids at 438 and 562 nm gave an approximately linear relationship as shown in Figs. 2 and 3 and reproducibility of their color development was satisfactory, and the colors developed being stable within 20 min. Then the contents of deoxycholic and chenodeoxycholic acids in a mixture of them can be calculated from the following equations:

$$Cda = \frac{\alpha c_2 A_1 - \alpha c_1 A_2}{\alpha d_1 \alpha c_2 - \alpha d_2 \alpha c_1}$$

$$Cca = \frac{\alpha d_1 A_2 - \alpha d_2 A_1}{\alpha d_1 \alpha c_2 - \alpha d_2 \alpha c_1}$$

Where Cda and Cca are the respective contents (μg) of deoxycholic and chenodeoxycholic acids; αd_1 and αd_2 are the tangent values obtained from the slopes of the calibration curves at 438 and 562 nm for deoxycholic acid; αc_1 and αc_2 are the corresponding values for chenodeoxycholic acid; A_1 and A_2 are the respective absorbancies (OD) of the mixture of the dihydroxy-bile acids at 438 and 562 nm. The values of Cda and Cca obtained from the individual bile samples are listed in Table 2,

TABLE 2.

Contents (μg) of deoxycholic (DC) and chenodeoxycholic (CDC) acids in the dihydroxy-bile acid fractions prepared from the respective bile samples

Bile sample	DC (μg)	CDC (μg)	Cda/Cca	GLC
Bovine	124.0	40.0	3.1	3.0
Dog	54.2	17.4	3.1	3.0
Rat	0	75.0	—	—
Human-I	6.4	96.2	—	—
Human-II	1.2	35.0	—	—

together with their ratios. The ratios Cda/Cca for the bovine and dog bile samples were measured simultaneously by GLC and are listed in comparison. As shown in the table, the Cda/Cca ratios obtained by the present method were well coincident with those obtained by GLC, suggesting that the present method is applicable to quantitative determination of deoxycholic and chenodeoxycholic acids in such a dihydroxy-bile acid fraction, as prepared from human bile. Deoxycholic acid in fistula bile samples of human being and rats was found in a small amount or not detectable. The finding is well accordant with the fact that the acid is a secondary bile acid.

As deoxycholic and chenodeoxycholic acids display almost the same behavior on a TLC plate, it is not easy to be separately detected. But application of the present method can afford a differential determination of the individual acids. Interferences may be caused by the presence of such other dihydroxy-bile acids as hyodeoxycholic acid and ursodeoxycholic acid. Therefore, the application of this method is preferable to the determination of deoxycholic and chenodeoxycholic acids in bile samples having these acids as the major components in the dihydroxy-bile acid fraction as is the case with human bile.

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Trivial names used are as follows: cholic (3α, 7α, 12α-trihydroxycholan-24-oic), deoxycholic (3α, 12α-dihydroxycholan-24-oic), chenodexycholic (3α, 7α-dihydroxycholan-24-oic) and lithocholic (3α-hydroxycholan-24-oic) acids.