Quantitative Determination of D-Glucaric Acid in Bile in Relation to Inhibitory Effect of Bile on Bacterial β -Glucuronidase*

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(Received for publication, July 22, 1965)

Normal bile has a considerable effect to inhibit the activity of bacterial β -glucuronidase, and such a property of bile is of practical interest since this enzyme is concerned with formation of calcium bilirubinate gallstones. In relation to this fact, it was demonstrated qualitatively in the preceding paper that bile might contain D-glucaro-1,4-lactone, a lactone of D-glucaric acid and strong inhibitor of this enzyme. To confirm the fact on a quantitative basis, the method of Ishidate *et al.* for quantitative estimation of D-glucaric acid in urine was modified so that it was made applicable to bile specimens. The D-glucaric acid content of human gallbladder bile as measured by this method ranged from 38 to 634 $\mu\text{g/ml}$. Furthermore, a significant positive correlation was pointed out between the D-glucaric acid content and the inhibitory power of bile over bacterial β -glucuronidase.

Maki and his associates¹⁻³ of our laboratory found that the bacterial β -glucuronidase activity of bile, originating mostly from infecting *Escherichia coli*, played a substantial role in the development of calcium bilirubinate stones. This implies that an inhibitor of this enzyme, if any in bile, provides a complicating factor to the pathogenesis of this type of gallstones. In this relation, one of us (T.M.) demonstrated earlier that normal human bile had a considerable effect to inhibit the activity of bacterial β -glucuronidase.⁴ However, none of already known components of bile was found to be significantly responsible for such an effect,⁵ and it has been inferred, on a qualitative basis, that D-glucaro-1,4-lactone, a lactone of D-glucaric acid and specific inhibitor of this enzyme,⁶ is present in bile as a

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* Fourth Report on Inhibitory Effect of Bile Constituents upon Bacterial β -Glucuronidase Activity. Read in part before The 10th Anniversary Symposium on Glucuronic Acid Research (Tokyo Biochemical Research Foundation), December 2-3, 1964, Tokyo.

minor but normal constituent.⁷ As a continuation of this series of study, we have made attempts at quantitative determination of D-glucaro-1,4-lactone of human bile.

In 1963, Marsh⁸ succeeded for the first time in quantitatively estimating D-glucaro-1,4-lactone of human urine. But this method did not serve for the present purpose since it was based on the specific activity of this compound to inhibit β -glucuronidase. Recently, Ishidate and his collaborators⁹ have devised a method for chemical quantitative determination of D-glucaric acid in urine. Although their method is not for separative determination of D-glucaro-1,4-lactone but deals with total D-glucaric acid including glucarate and other lactones, it should be a great advantage of this method that D-glucaric acid is quantitatively determined by purely chemical procedure, unrelated to its inhibitory power over β -glucuronidase. Accordingly, this method was chosen as the prototype for our modification.

MATERIALS AND METHODS

Materials

Bile specimens were collected, by puncture of the gallbladder at laparotomy, from surgical patients without disorder of the hepato-biliary system. Each sample was confirmed by culture to be free of bacterial infection. D-Glucaro-1,4-lactone used as the authentic compound was a preparation of Tanabe Seiyaku Co., Osaka.

Methods

Determination of the inhibitory power: The inhibitory power for β -glucuronidase of bile specimens and of authentic D-glucaro-1,4-lactone solutions was estimated by an already described method^{4,5} using Bacterial β -Glucuronidase Sigma, and expressed by the "inhibition ratio", the per cent decrease of the activity of the above enzyme material induced by application of the sample.

Quantitative determination of D-glucaric acid in bile: The method of Ishidate *et al.*⁹ which was originally devised for determination of D-glucaric acid in urine consists of three consecutive steps: (1) conversion of D-glucarolactones into non-lactone D-glucaric acid (D-glucarate), (2) chromatographic isolation of a D-glucaric acid-containing fraction, and (3) determination of D-glucaric acid by color reaction of McFadden and Howes (Fig. 1).¹⁰ In application of this method to bile specimens, the first two steps were modified as follows: The bile specimen was diluted 1:2 with distilled water. A 0.1 ml aliquot of the diluted bile was mixed with 1-4 ml of 0.1 M sodium borate to bring pH of the mixture to 8.5-9.0, allowed to stand at room temperature for 15 minutes, heated for 30 minutes in boiling water bath and then subjected to chromatographic separation after being cooled by running water. The chromatographic column for isolation

Fig. 1. Principle of quantitative determination of D-glucaric acid by McFadden and Howes.¹⁰ D-Glucaric acid is oxidized by periodic acid into glyoxylic acid. Glyoxylic acid is condensed with phenylhydrazine to its phenylhydrazone and, in the presence of potassium ferricyanide and excess phenylhydrazine, eventually converted into a color substance (1,5-diphenylformazan¹¹). The latter is quantitatively determined by spectrophotometry.

of D-glucaric acid was prepared in a glass tube of 9 mm in diameter with 3.0 ml of anion exchange resin Dowex 1-X8 (50–100 mesh) that had been preliminarily washed first with 5–6 parts of 2*N* hydrochloric acid, then with distilled water and finally with 0.1*M* sodium borate until the washing had become negative for Cl[−]. After application of the bile specimen, the column was eluted first with 150 ml of 0.05*M* sodium borate–0.02*M* sodium sulfate and then with 25.0 ml of 0.05*M* sodium borate–0.1*M* sodium sulfate, both at a velocity of 1.0 ml per minute. The latter 25 ml eluate was utilized for determination of D-glucaric acid.

Subsequent procedure, i.e., quantitative analysis of D-glucaric acid in the separated fraction, exactly followed the original method. A 5.0 ml aliquot of the eluate was mixed with 1.0 ml of 1*M* potassium biphosphate and 0.1 ml of 0.01*M* periodic acid, and allowed to stand at room temperature for 15 minutes. To the mixture were then added 0.1 ml of 1% sodium bisulfate, 1.0 ml of 1*M* oxalic acid and 1.0 ml of 1% phenylhydrazine hydrochloride; the mixture was heated for 5 minutes in boiling water bath and then cooled for 5 minutes by running water. Finally, 4.0 ml of concentrated hydrochloric acid and 1.0 ml of 5% potassium ferricyanide were added to the mixture, which was then left standing at room temperature for 10 minutes. The optical density of the final mixture was measured at 520 mμ with a Beckman spectrophotometer, using a similarly treated solution of 0.05*M* sodium borate–0.1*M* sodium sulfate as a blank. The optical density was converted into the D-glucaric acid concentration of original bile (μg/ml) using a pre-established calibration curve.

Quantitative determination of L-ascorbic acid in bile: L-Ascorbic acid was sug-

gested to interfere with the above quantitative determination of D-glucaric acid.¹¹ To estimate the error due to presence in bile of L-ascorbic acid, its normal concentration in human bile was measured as follows by a modification of the hydrazone method of Roe *et al.*¹² A 5.0 ml aliquot of bile was mixed with the equal amount of 12% trichloroacetic acid, allowed to stand at room temperature for 5 minutes, and centrifuged. The deproteinized bile was then vigorously shaken for 2 minutes with 0.5 g charcoal to discard bile pigments and to convert L-ascorbic acid into L-dehydroascorbic acid, and filtered. To 2.0 ml of the filtrate were added two drops of 10% thiourea (in 50% v/v ethanol) and 0.5 ml of a 2% 2,4-dinitrophenol hydrazine reagent (one part of concentrated sulfuric acid was mixed with three parts of distilled water to make approximately 9N sulfuric acid, into 100 ml of which was dissolved 2.0 g 2,4-dinitrophenol hydrazine), and the mixture was incubated at 37°C for exactly 3 hours to complete hydrazone formation. The mixture was then cooled in an iced bath while 2.5 ml of 85% sulfuric acid was pipetted in. After 30 minutes' standing at room temperature, optical density of the colored mixture was measured at 520 m μ in a Beckman spectrophotometer. The blank in this case was prepared from another 2.0 ml portion of the deproteinized and charcoal-treated sample by adding two drops of 10% thiourea, 2.5 ml of 85% sulfuric acid and 0.5 ml of the 2,4-dinitrophenol hydrazine reagent and leaving the mixture for 30 minutes at room temperature. The calibration curve was prepared with authentic L-ascorbic acid (J.P.) dissolved in 6% trichloroacetic acid, which revealed satisfactory linear proportionality at concentrations below 16 μ g/ml. D-Glucaro-1,4-lactone, D-glucuronic acid or D-glucuronolactone did not interfere with this assay procedure for L-ascorbic acid.

RESULTS

1. Examination of the assay method

Absorption spectrum: Fig. 2 shows the result of spectrophotometric scanning of the final solutions derived from a bile specimen and from a solution of authentic D-glucaro-1,4-lactone. Both curves ran parallel and had an absorption maximum at 520 m μ .

Calibration curve: To prepare the calibration curve, calculated amounts of D-glucaro-1,4-lactone were dissolved in 1.0 ml each of 0.1M sodium borate and allowed to stand at room temperature for 15 minutes; each solution was then mixed with 4.0 ml of 0.05M sodium borate-0.1M sodium sulfate, and subjected to the color reaction and subsequent spectrophotometry at 520 m μ . The abscissa of the calibration chart was settled to directly indicate the concentration of D-glucaric acid in bile, all dilution factors being taken into consideration. The result is shown in Fig. 3. It has been demonstrated that the law of Beer-Lambert is obeyed when the D-glucaric acid content of bile is 500 μ g/ml or less.

Fig. 2

Fig. 2. Optical density curves for samples derived from authentic D-glucaro-1,4-lactone (dotted line) and normal bile (solid line).

Fig. 3

Fig. 3. Calibration curve for determination of D-glucaric acid in bile. Calibrated with authentic D-glucaro-1,4-lactone.

Fig. 4. Fractional recovery test with 200 μg D-glucaro-1,4-lactone.

Recovery tests: One milliliter of a 200 $\mu\text{g/ml}$ aqueous solution of authentic D-glucaro-1,4-lactone was subjected to the chromatographic procedure and the eluate was collected in fractions of 5.0 ml. Each fraction was then analyzed by the above-mentioned method for the D-glucaric acid content. As shown in Fig. 4, the result indicated that 197 μg , or 98.5 per cent of the 200 μg , of D-glucaric acid was recovered in the first 25 ml of the eluate. When 10–300 μg samples of D-glucaro-1,4-lactone were added to 1.0 ml portions of a 1:2 diluted bile specimen and the mixtures were tested for recovery of D-glucaric acid, the rate of recovery fell into the range of 100 ± 5 per cent.

Examination of interfering substances: When 1.0 mg aliquots of free D-glucuronic acid were tested in the above manner either as an aqueous solution or a solution in bile, the amounts of the color substance appearing in the final reaction mixture were equivalent to less than 10 μg of D-glucaric acid. This means that in determination of the D-glucaric acid content, the magnitude of error due to coexisting free D-glucuronic acid corresponds to below 1 per cent of its concentration. Since the upper limit of free D-glucuronic acid content of gallbladder bile is about 1 mg/ml,¹³ the actual size of this error is estimated to be 10 $\mu\text{g/ml}$ or less. Phenolphthalein glucuronide (Sigma) did not interfere with determination of D-glucaric acid by this method, suggesting that the error due to endogenous O-glucuronides may be negligible in estimation of D-glucaric acid in bile.

The L-ascorbic acid content of normal gallbladder bile as measured by the above-mentioned method varied in five individuals from 4.8 $\mu\text{g/ml}$ to 13.9 $\mu\text{g/ml}$. Another supplementary experiment showed that in the last step of the present assay method for D-glucaric acid, L-ascorbic acid gave the same color compound but the yield, in terms of the optical density at 520 m μ , was about one-fifth that yielded by equal quantity of D-glucaric acid. Accordingly, the interference of endogenous L-ascorbic acid with quantitative analysis of D-glucaric acid of bile is expected to be of a magnitude of 2 $\mu\text{g/ml}$ at most, even assuming that a considerable proportion of L-ascorbic acid leaks into the D-glucaric acid fraction in chromatography of the bile specimen.

2. Inhibitory effect of authentic D-glucaro-1,4-lactone

The inhibitory effect of D-glucaro-1,4-lactone on Bacterial β -Glucuronidase Sigma is shown in Fig. 5 as a function of the concentration in the assay system.⁵ The effect was perceptible even at 1 $\mu\text{g/ml}$ and increased, though not in a linear fashion, as the concentration increased.

3. Results with bile specimens

In a total of 14 bile specimens examined in this series, the D-glucaric acid content ranged between 38 $\mu\text{g/ml}$ and 634 $\mu\text{g/ml}$, the average and the standard deviation being 266 ± 159 $\mu\text{g/ml}$. The inhibition ratio of the same specimens, on

Fig. 5. Inhibitory effect of authentic D-glucaro-1,4-lactone on Bacterial β -Glucuronidase Sigma.

Fig. 6. Relation between the D-glucaric acid content and the inhibition ratio of bile. $r=+0.88$. The straight line in the graph is the line of linear regression of the inhibition ratio (y , in %) over the D-glucaric acid content (x , in $\mu\text{g/ml}$).

the other hand, was 5.0–48.1%. As indicated in Fig. 6, there was a significant positive correlation between the content of D-glucaric acid and the inhibition ratio, the coefficient of correlation being +0.88.

In determination of the inhibition ratio of bile, a 0.1 ml aliquot of 1:10 diluted bile was put in the assay system and the total amount made 1.0 ml.⁴ Therefore, in this case, the concentration of a bile constituent in the assay system would be one-hundredth that in the original bile specimen. In this connection, Fig. 7 was prepared by converting the abscissa of Fig. 6 into the D-glucaric acid concentration of the assay system and superimposing the curve of Fig. 5. It was

Fig. 7. Comparison between the inhibitory power of bile and that of aqueous solution of authentic D-glucaro-1,4-lactone. See text for explanation.

thus revealed that the inhibitory power of a bile specimen was generally stronger than was expected from its D-glucaric acid content.

DISCUSSION

The method of Ishidate *et al.*⁹ for chemical quantitative determination of D-glucaric acid in urine includes the following three steps: (1) conversion of lactones of D-glucaric acid into D-glucarate, which is achieved by incubation with alkali, (2) separation of D-glucaric acid by means of column chromatography, and (3) oxidative decomposition of D-glucaric acid into glyoxylic acid and quantitative determination of the latter by the method of McFadden and Howes.¹⁰ According to Ishidate *et al.*, the original authors, the accuracy of this method was satisfactory as far as urine samples were concerned. However, some modifications were required in application of the method to bile specimens.

The alkali incubation of the sample for conversion of D-glucaro-lactones into D-glucarate is based on the fact that in aqueous solution the lactones and glucarate form an equilibrium which is favorable for the latter in alkaline reaction. In the original method, the sample (urine) is mixed with the same amount of 0.1M sodium borate and left standing at room temperature for 15 minutes to accomplish the conversion. However, a preliminary experiment indicated that this method was not sufficient for conversion of D-glucaro-lactones of bile into D-glucarate, probably due to greater buffer action of bile than urine. Therefore, this step was altered to such a procedure that one part of 1:2 diluted bile was mixed with 1-4 parts of 0.1M sodium borate to make pH of the mixture 8.5-9.0, allowed to stand at room temperature for 15 minutes, and heated in boiling water bath for 30

minutes. Furthermore, on the basis of the result of a fractional recovery test, the amount of the eluting solution in chromatographic isolation of D-glucaric acid was increased from 20 ml in the original method to 25 ml.

There was no problem in the last step of the quantitative procedure. A sample derived from commercial preparation of D-glucaro-1,4-lactone and another from normal bile both exhibited an absorption peak at 520 m μ . At this wave length, Beer-Lambert's law was obeyed over a range of D-glucaric acid content between 0 and 500 μ g/ml which almost perfectly covered the normal range in human bile.

With the above-mentioned modifications, the recovery rate of D-glucaric acid was brought into the range of 100 ± 5 per cent, a satisfactory level for this type of quantitative analysis. However, in analysis of bile with this method, possible errors due to presence in bile of interfering substances should be taken into consideration. Among normal constituents of bile, L-ascorbic acid and D-glucuronic acid are known to yield glyoxylic acid when oxidized by periodate, thus possibly interfering with the determination of D-glucaric acid.¹¹ Our experiments as regards this problem showed that the content of L-ascorbic acid in normal bile was so small that the error due to endogenous L-ascorbic acid was negligible. The magnitude of error that could be caused by endogenous free D-glucuronic acid was estimated to be 10 μ g/ml at most at its normal concentration, which level is also small enough when compared with the D-glucaric acid concentration 266 ± 159 μ g/ml. Thus, in normal conditions, one may disregard the effect of coexisting substances in quantitative determination of D-glucaric acid in bile. However, in particular cases such as loading of L-ascorbic acid or D-glucuronic acid, or their precursors, adequate means might be required to lessen the error. In their recent paper, Ishidate *et al.*¹¹ recommended pretreatment of the sample with 2,6-dichlorophenol-indophenol and sodium borohydride to minimize interference of L-ascorbic acid and/or D-glucuronic acid with quantitative determination of D-glucaric acid in urine.

When this method was applied to a total of 14 bile specimens that had been obtained from surgical patients without hepato-biliary diseases, the D-glucaric acid content ranged between 38 μ g/ml and 634 μ g/ml. The individual variation was thus quite large and this should be an interesting subject of future investigation. It was also found that there was a remarkable correlation between the D-glucaric acid content of bile and its inhibitory power over bacterial β -glucuronidase. Considering the fact that only 1,4-lactone among various molecular forms of D-glucaric acid is significantly inhibitory to β -glucuronidase,⁶ the above result is interpreted to show that D-glucaro-1,4-lactone is a definitive factor that is responsible for the inhibitory effect of bile.

A question is aroused here whether D-glucaro-1,4-lactone is the only inhibitor existing in bile. In this relation, the present study revealed that the in-

hibitory power of a bile specimen was invariably greater than that of an aqueous solution of D-glucaro-1,4-lactone when compared at the same concentration of D-glucaric acid. It is thus quite likely that bile includes other inhibitors of β -glucuronidase. However, further studies are required before obtaining a conclusion since it is also possible that some bile constituents enhance the inhibitory effect of D-glucaro-1,4-lactone even though they are not inhibitory to β -glucuronidase.

CONCLUSIONS

1. The method of Ishidate *et al.* for quantitative determination of D-glucaric acid in urine was applied to bile with some modifications. With this method, the D-glucaric acid content of normal gallbladder bile in man was determined to be $266 \pm 159 \mu\text{g/ml}$ (mean $\pm$ SD).

2. There was a statistically significant positive correlation between the D-glucaric acid content and the inhibitory power over bacterial β -glucuronidase of bile. This fact supports the view that D-glucaro-1,4-lactone is mainly responsible for the inhibitory effect of bile upon this enzyme.

Acknowledgment

The authors are largely indebted to Dr. Z. Yoshizawa, Professor of Biological Chemistry, Tohoku University School of Medicine, for his valuable suggestions given in the course of this study. The expense for this study was defrayed, in part, by a grant-in-aid from the Scientific Research Fund, Ministry of Education of Japan, and also by a research grant from Tokyo Biochemical Research Foundation, which is gratefully acknowledged.

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