Fasting levels of monoketonic bile acids in human peripheral and portal circulation

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Abstract It has been suggested that large amounts of ketonic bile acids may be present in portal venous blood. We have therefore determined the approximate concentration of 3-oxo-, 7-oxo-, and 12-oxo-bile acids (monoketonic bile acids) in human peripheral and portal circulation. These compounds were converted into the corresponding 3α-, 7α-, and 12α-hydroxy bile acids by treatment with sodium borodeuteride, thus increasing the molecular weight of each bile acid formed by one mass unit. The ratio between deuterated and nondeuterated bile acid was determined by combined gas–liquid chromatography–mass spectrometry with use of selected ion monitoring. From the ratio obtained and from the concentration of unlabeled bile acid, determined by isotope dilution–mass spectrometry, the approximate concentration of the different ketonic bile acids could be calculated. This method underestimates 3-oxygenated bile acids by 4–8%, 7-oxygenated bile acids by 2–3%, and 12-oxygenated bile acids by about 25%. The approximate concentration of monoketonic 3,7-oxygenated bile acids was found to be 0.08 ± 0.02 and 0.37 ± 0.25 μmol/l in the peripheral venous serum and the portal venous serum, respectively. The approximate concentration of monoketonic 3,12-oxygenated bile acids was found to be 0.07 ± 0.02 and 0.32 ± 0.12 μmol/l in the peripheral venous serum and the portal venous serum, respectively. The approximate concentration of monoketonic 3,7,12-oxygenated bile acids was found to be 0.03 ± 0.01 and 0.14 ± 0.05 μmol/l in the peripheral venous serum and in the portal venous serum, respectively. The total concentration of the ketonic bile acids constituted only 9 ± 1% and 8 ± 3% of the nonoxidized bile acids in the peripheral venous serum and in the portal venous serum, respectively. Thus it seems less likely that the portal inflow of ketonic bile acids is of significant physiological importance under normal conditions.—**Björkhem, I., B. Angelin, K. Einarsson, and S. Ewerth.** Fasting levels of monoketonic bile acids in human peripheral and portal circulation. *J. Lipid Res.* 1982. **23:** 1020–1025.

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In a previous work (1), we assayed the concentration of individual bile acids in peripheral venous and portal venous blood by isotope dilution–mass spectrometry. In a comment to that publication, Barnes and Siegel (2) reported that they had found much higher levels of bile acids in portal blood when assayed by the 3α-hydroxysteroid dehydrogenase method than when assayed by gas–liquid chromatography. They suggested that the difference might be due to the presence of large amounts of ketonic bile acids. Bile acids with an oxo group in 7- or 12-position are detected in the enzymatic assay, but are not readily detected by gas–liquid chromatography because of their long retention time under most conditions used. In addition, some ketonic bile acids, in particular 3-oxo bile acids, are known to be vulnerable to the alkaline hydrolysis procedure commonly used for deconjugation prior to gas–liquid chromatography (3). Since 3-oxo bile acids are not detected by the enzymatic assay, such bile acids may thus be completely missed both when using the enzymatic assay and the gas–liquid chromatography assays.

Ketonic bile acids are major constituents of human feces (4–6) and have also been isolated from human bile (7). Hepner et al. (7) reported that ketohydroxy bile acids composed 7–15% of the bile acid pool in cholecystectomized patients. Since these authors used alkaline hydrolysis, however, the true concentration of ketonic bile acids may have been even higher than reported.

From the above facts, it seems possible that substantial amounts of the ketonic bile acids formed in the intestine may be transported to the liver in the portal blood. The ketonic bile acids may be of regulatory importance for total bile acid biosynthesis and it has been shown that such bile acids may increase the bile flow (8). Thus it was considered important to determine at least the approximate level of the ketonic bile acids in portal venous and peripheral venous blood.

In view of the methodological difficulties in the direct assay of the ketonic bile acids in serum by gas–liquid chromatography, we have reduced them with sodium borodeuteride to yield the corresponding monodeuterated bile acids. The ratio between monodeuterated bile acids and unlabeled bile acids has then been determined by combined gas–liquid chromatography–mass spectrometry, using selected ion monitoring.

MATERIALS AND METHODS

Subjects

The subjects of the study were five females and one male (ages 32–79 years) suffering from cholelithiasis and admitted for elective cholecystectomy. Informed consent was given. The gallbladder was functioning at the time of the study. There was no history of previous liver disease or of common bile duct stones, and all patients displayed normal liver function tests.

Experimental procedure

The patients were hospitalized in the surgical ward where routine laboratory tests and a clinical examination were performed. All operations were performed between 8 and 9 AM after a 12-hour fast. Anesthesia was induced by thiopentothal and continued with nitrous oxide, diazepam, and fentanyl. After access to the biliary region had been obtained, the cystic duct was identified and clamped in order to avoid leakage of gallbladder bile into the common bile duct. The portal vein was punctured with a fine needle and blood was aspirated, carefully avoiding contamination from the common bile duct. Simultaneously, peripheral blood was sampled from the antecubital vein. Thereafter, a regular cholecystectomy was performed. No complications were encountered in any of the patients during operation or postoperatively. The ethical aspects of the study were approved by the Ethical Committee of the Karolinska Institutet, Stockholm, Sweden.

Materials

Unlabeled bile acids were obtained from Sigma Chemical Co. (St. Louis, MO). The 3-oxo bile acids were obtained by Oppenhauer oxidation of respective bile acid (9). The 7-oxo bile acids were obtained by oxidation with N-bromosuccinimide of respective bile acid (10). 3α-Hydroxy-12-oxo-5β-cholan-24-oic acid was obtained by oxidation of deoxycholic acid with chromic acid as described by Bergström and Haslewood (11). All the different ketonic bile acids had the expected properties when subjected to gas–liquid chromatography as methyl esters, using 1.5% SE-30 as stationary phase (cf. ref. 12) or when subjected to thin-layer chromatography, using system S12 (13). The deuterated bile acids used as internal standard (2,2,3,4,4-$^2H_5$) cholic acid, (2,2,3,4,4-$^2H_5$) chenodeoxycholic acid, and (11,11,12-$^2H_3$) deoxycholic acid, were those used in a previously published study (14).

It should be pointed out that the major molecular species of the preparation of 2,2,3,4,4-$^2H_5$-labeled chenodeoxycholic acid contained only three atoms of deuterium.

After clotting of the blood at room temperature, serum was obtained by centrifugation and was stored frozen at −20°C for later analysis.

Analysis of unreduced serum

The mass fragmentographic technique for determination of the individual bile acids, cholic acid, chenodeoxycholic acid, and deoxycholic acid in serum previously used (14) was modified and will be published in detail elsewhere.[1]

With the modified technique, more specific ions for each bile acid derivative are used. As a consequence, however, with the particular instrument used here (LKB 9000), each bile acid must be determined separately.

To 0.5 ml of serum (0.1 ml in the analysis of portal serum), 1 μg of (2,2,3,4,4-$^2H_5$) cholic acid, 0.5 μg of (2,2,3,4,4-$^2H_5$) chenodeoxycholic acid, and 2.5 μg of (11,11,12-$^2H_3$) deoxycholic acid were added at the same time. The serum, together with the internal standards, was hydrolyzed with 1 M KOH at 110°C for 12 hr. The alkaline solution was extracted three times with ethyl ether in order to remove most of the neutral steroids. The bile acids were then extracted from the acidified water phase with ethyl ether, methylated with diazomethane, and converted into trimethylsilyl derivatives. The derivatives were analyzed by gas–liquid chromatography–mass spectrometry using an LKB 9000 instrument equipped with a MID unit (multiple ion detector). A 1.5% SE-30 column was used and the operating temperature was 260–290°C. Cholic acid was analyzed with two of the channels focused at m/e 623 and m/e 628, corresponding to the M-15 peak in the mass spectrum of trimethylsilyl derivative of methyl ester of unlabeled and deuterium-labeled cholic acid, respectively. Chenodeoxycholic acid was analyzed with two of the channels focused on m/e 370 and m/e 373, corresponding to the M-2 × 90 peak in the mass spectrum of trimethylsilyl derivative of methyl ester of unlabeled and deuterium-labeled chenodeoxycholic acid, respectively. Deoxycholic acid was analyzed with two of the channels focused on m/e 255 and m/e 258, corresponding to the base peak in the mass spectrum of trimethylsilyl derivative of methyl ester of unlabeled and deuterium-labeled deoxycholic acid, respectively.

The concentration of cholic acid was thus calculated from the ratio between the tracing at m/e 623 and the tracing at m/e 628. The concentration of chenodeoxycholic acid was calculated from the ratio between the tracing at m/e 370 and the tracing at m/e 373. The concentration of deoxycholic acid was calculated from the ratio between the tracing at m/e 255 and the tracing at m/e 258. Standard curves for each bile acid were

[1] Björkhem, I., and O. Falk. To be published.

used. The relative standard deviation of the method, as calculated from duplicate samples, was 2–3%.

Analysis of reduced serum

Sodium borodeuteride, 50 mg (Merck, Darmstadt, West Germany, 99% pure with respect to ^2H), was added to 0.25 ml of serum in 5 ml of methanol. The mixture was left at room temperature for 30 min. Additional sodium borodeuteride, 50 mg, was then added. After an additional 30 min, the mixture was neutralized with aqueous hydrogen chloride and centrifuged. The supernatant was then hydrolyzed with KOH under the same conditions as above. The free bile acids obtained were extracted and derivatized as above.

The ratio between monodeuterated and unlabeled cholic acid was determined by mass fragmentography, using the ions at m/e 624 and m/e 623. The ratio between monodeuterated chenodeoxycholic acid and unlabeled chenodeoxycholic acid was determined with the use of the ions at m/e 371 and m/e 370. The ratio between monodeuterated deoxycholic acid and unlabeled deoxycholic acid was determined with the use of the ions at m/e 256 and m/e 255. The different ratios obtained were compared with the ratios obtained in the analysis of the corresponding unlabeled acids.

The analysis of the standard and the unreduced serum sample was performed immediately before and after analysis of each reduced serum sample.

From the difference obtained in the analysis of reduced and unreduced serum, and from the known concentration of unlabeled bile acid, the approximate concentration of each monodeuterated bile acid could be calculated (cf. Results).

In this calculation it was assumed that a fixed amount of unlabeled bile acid gave the same response in the M channel as that obtained in the M + 1 channel in the analysis of the same amount of monodeuterated bile acid. When the ratio between monodeuterated bile acid and unlabeled bile acid is high, this may give a small underestimation due to incomplete discrimination between the two channels (cf. Fig. 1). With a ratio between monodeuterated bile acid and unlabeled bile acid less than 0.1, the underestimation due to this should, however, be 1% or less.

It was shown that the ratio between the different tracings could be determined with a coefficient of variation of 1% or less (within-assay variation, cf. ref. 15).

It should be pointed out that a deuterium label in the 3β-position in a bile acid is completely stable under the conditions employed. In the experiments with a fully 3β-deuterated bile acid, no evidence was obtained for hydrogen-deuterium exchange in the solvents or on the column.

Analysis of reduced ketonic standards

In some experiments, ketonic bile acids (7 α-hydroxy-3-oxo-5β-cholan-24-oic acid, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid, 3α,12α-hydroxy-7-oxo-5β-cholan-24-oic acid, 3α-hydroxy-7-oxo-5β-cholan-24-oic acid, and 3α-hydroxy-12-oxo-5β-cholan-24-oic acid) were reduced with sodium borohydride under the same conditions as above. The yield of the 3α-epimer, 7α-epimer, or 12α-epimer was calculated by analysis by gas–liquid chromatography of the trimethylsilyl ether, using the same conditions as above (cf. ref. 12). In some cases the yield of the epimer was obtained by thin-layer chromatography in system S12 (13), followed by development with sulfuric acid and transmittance densitometry, using a type TLD 100 Vitatron instrument (16).

RESULTS

According to the general rule, reduction of sterically unhindered steroidal ketones with sodium borohydride should preferentially give the equatorial alcohol (17, 18). In the case of sterically hindered ketones, however, the axial alcohol is the predominant product (17). The 3-oxo group in 7α-hydroxy-3-oxo-5β-cholan-24-oic acid is unhindered, and in accordance with this reduction of the methylated steroid with sodium borohydride, predominantly gave the equatorial 3α-epimer (92–96%). The same results were obtained when substituting sodium borodeuteride for sodium borohydride. It has previously been reported that 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid is reduced by sodium borohydride to the axial 7α-epimer, presumably due to bending around of ring A, which may shield the α-side (17). In accordance with that work, reduction of the methyl ester of 3α-hydroxy-7-oxo-5β-cholan-24-oic acid with sodium borohydride, almost exclusively gave the 7α-hydroxy epimer (97–98%). The 12-position is partially sterically hindered (17). In accordance with this, reduction of 3α-hydroxy-12-oxo-5β-cholan-24-oic acid with sodium borohydride predominantly gave the axial 12α-hydroxy epimer (74–77%).

Fig. 1 illustrates the principle of the assay. On the left side of the figure, a selected ion chromatogram is shown of the trimethylsilyl ether of the methyl ester of unlabeled chenodeoxycholic acid. The most prominent peak was obtained in the tracing at m/e 370, corresponding to the M-2 × 90 ion in the mass spectrum. As expected, the ion at m/e 371 was less prominent, and the ratio between the two peaks (m/e 371/m/e 370) was 0.29. Exactly the same ratio was obtained in the simultaneous analysis of chenodeoxycholic acid in a

serum extract. On the right, a selected ion chromatogram is shown of the trimethylsilyl ether of methyl ester of monodeuterated chenodeoxycholic acid, formed from methyl ester of unlabeled 7α-hydroxy-3-oxo-5β-cholanoic acid by reduction with sodium borodeuteride under the conditions given in Materials and Methods. The ratio (m/e 371/m/e 370) was now about 9. If the nondeuterated derivative had an isotopic purity of 100% and if there were 100% discrimination between the two channels, there should have been no peak at all in the tracing at m/e 370.

If a serum containing 7α-hydroxy-3-oxo- or 3α-hydroxy-7-oxo-5β-cholan-24-oic acid is reduced by sodium borodeuteride prior to hydrolysis and selected ion monitoring, it is evident that the ratio between the peak at m/e 371 and the peak at m/e 370 can be expected to increase above 0.29. From the difference in ratio after and prior to sodium borodeuteride reduction, the approximate amount of ketonic bile acid in serum can be calculated, provided that the concentration of chenodeoxycholic acid is known.

The accuracy of the assay was tested by addition of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid, 167 ng, to 0.25 ml of a specific serum sample containing 285 ng of chenodeoxycholic acid as measured by the isotope dilution technique. The mixture was then reduced with sodium borodeuteride, hydrolyzed, derivatized, and subjected to selected ion monitoring. The ratio between the peak in the tracing at m/e 371 and the peak in the tracing at m/e 370 was now 0.92. The corresponding ratio obtained in the analysis of unreduced serum and serum reduced with sodium borodeuteride was 0.27 and 0.34, respectively. It is evident that the contribution at m/e 371 from the added 3-oxosteroid was 0.92 − 0.34 = 0.58. From this ratio, and from the amount of unlabeled chenodeoxycholic acid present in the serum sample, 285 ng, the amount of ketonic acid added could be calculated to be 165 ng. The difference between expected and calculated value was thus only 1%.

Reduction of serum samples with sodium borodeuteride was, in general, found to increase the ratio m/e 371/m/e 370 in the chenodeoxycholic acid fraction, indicating the presence of small amounts of 3α-hydroxy-7-oxo-5β-cholan-24-oic acid and/or 7α-hydroxy-3-oxo-5β-cholan-24-oic acid (**Table 1**). In the analysis of one specific serum, this increase was 0.37; in all the other cases, the increase was less than 0.15. The concentration of 3α-hydroxy-7-oxo-5β-cholan-24-oic acid and 7α-hydroxy-3-oxo-5β-cholan-24-oic acid together in peripheral venous serum was calculated to be 0.08 ± 0.02 (mean ± S.E.M.) μmol/l, whereas the corresponding concentration in portal venous serum was 0.37 ± 0.25 μmol/l. The concentration of chenodeoxycholic acid

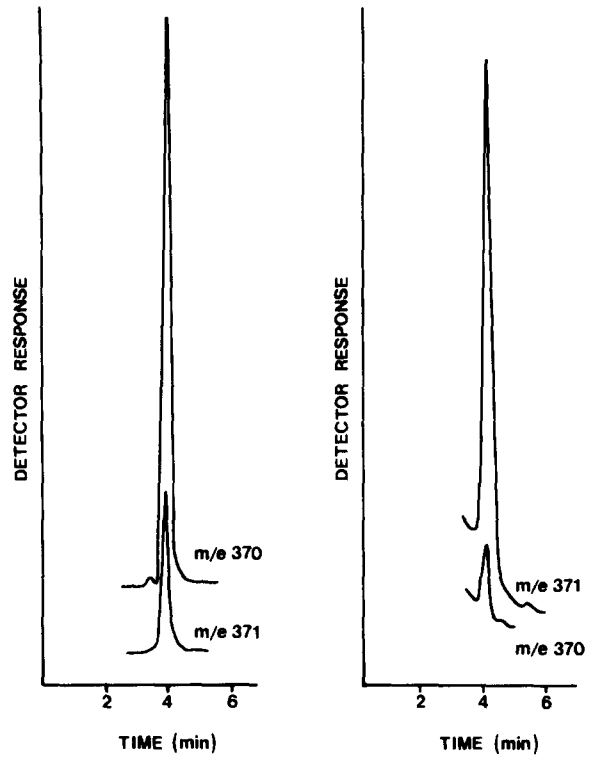

Fig. 1. Selected ion chromatogram obtained in analysis of trimethylsilyl derivative of methyl ester of unlabeled chenodeoxycholic acid (left) and monodeuterated chenodeoxycholic acid (right). The latter acid was formed from the methyl ester of 7 α-hydroxy-3-oxo-5β-cholan-24-oic acid by reduction with sodium borodeuteride. For further experimental details, see Materials and Methods.

was 1.07 ± 0.18 μmol/l and 5.92 ± 1.36 μmol/l, respectively.

It should be pointed out that in some of the serum samples, where the difference in the ratio between m/e 371 and m/e 370 before and after reduction with sodium borodeuteride was very low, the imprecision in the estimation of the concentration of the ketonic bile acid was great. If the difference in ratio is only 0.02 (corresponding to a concentration of the monoketonic bile acids of about 0.02 μmol/l) it can be calculated that the absolute error in the estimation of the difference in ratio should be about 0.005. This would give an analytical imprecision in the calculated concentration of the monoketonic bile acid at that level of about ±25%.

Using the ions at m/e 256 and m/e 255, the concentration of 12α-hydroxy-3-oxo-5β-cholan-24-oic acid and 3α-hydroxy-12-oxo-5β-cholan-24-oic acid together was calculated to be 0.07 ± 0.02 μmol/l and 0.32 ± 0.12 μmol/l in peripheral venous serum and in portal venous serum, respectively (Table 1). The concentration of deoxycholic acid in the two compartments was 0.75 ± 0.14 μmol/l and 3.63 ± 1.50 μmol/l, respectively.

TABLE 1. Concentrations of monoketonic bile acids in peripheral venous and portal venous serum of six fasting subjects.

	Peripheral Venous Serum[a]	Portal Venous Serum[a]
	μmol/l	
Concentration of chenodeoxycholic acid	1.07 ± 0.18 (0.50–1.60)	5.92 ± 1.36 (1.0–9.6)
Calculated concentration of ketonic 3,7-oxygenated bile acids	0.08 ± 0.02 (0.00–0.18)	0.37 ± 0.25 (0.00–1.55)
Concentration of deoxycholic acid	0.75 ± 0.14 (0.19–1.18)	3.63 ± 1.50 (0.9–10.8)
Calculated concentration of ketonic 3,12-oxygenated bile acids	0.07 ± 0.02 (0.00–0.13)	0.32 ±0.12 (0.05–0.50)
Concentration of cholic acid	0.23 ± 0.06 (0.10–0.50)	4.21 ± 1.58 (0.65–9.60)
Calculated concentration of ketonic 3,7,12-oxygenated bile acids	0.03 ± 0.01 (0.00–0.06)	0.14 ± 0.05 (0.03–0.24)

[a] The figures given are the means ± S.E.M. The figures within parentheses correspond to the ranges.

Using the ions at m/e 624 and m/e 623, the concentration of ketonic 3,7,12-oxygenated bile acids together was calculated to be 0.03 ± 0.01 μmol/l and 0.14 ± 0.05 μmol/l in peripheral venous and portal venous serum, respectively (Table 1). The concentration of cholic acid in the two compartments was calculated to be 0.23 ± 0.06 μmol/l and 4.21 ± 1.58 μmol/l, respectively.

DISCUSSION

The present assay is based on the fact that reduction of ketonic bile acids with sodium borohydride deuteride in methanol predominantly gives the α-hydroxy bile acids. Based on the relative yield of the above hydroxy epimers in the reduction of the corresponding ketonic acids, it can be calculated that the assay should underestimate 3-oxo bile acids by about 4–8%, 7-oxo acids by about 2–3%, and 12-oxo bile acids by about 23–26%. In addition to this underestimation, there may be a small underestimation (about 1% or less) due to incomplete discrimination between the two channels of the MID-unit (cf. Results). Another source of error in the assay is the fact that the ratio between the different tracings could not be calculated with a higher precision than about 1%. In view of the fact that the ratio between monodeuterated and unlabeled bile acids was calculated from two different tracings, the detection limit of the assay should be about 0.01 with respect to the ratio between the ketonic bile acids and the corresponding nonoxidized bile acids.

It may be mentioned that Beher et al. (19) have analyzed ketonic bile acids after previous reduction in order to avoid destruction during the hydrolysis. These authors used unlabeled sodium borohydride, however. Thus, it was not possible to establish the original ratio between ketonic bile acids and normal bile acids with their technique.

It should be pointed out that the present assay is specific for bile acids with one oxo group. Thus reduction with sodium borodeuteride of bile acids with two and three oxo-groups gives di- and tri-deuterated bile acids, which are not detected in the assay. It seems however less likely that di- and tri-ketonic bile acids should be important from a quantitative point of view.

In spite of the limitations of the present assay, the results obtained seem to exclude the possibility that there are high concentrations of ketonic bile acids in the systemic and portal circulation. The mean concentration of the ketonic bile acids determined was in general only about 10% or less of the concentration of the corresponding nonoxidized bile acids. Thus the total concentration of the ketonic bile acids constituted 9 ± 1% and 8 ± 3% in the peripheral venous serum and in the portal venous serum, respectively. Only in one of the patients studied was the concentration of ketonic bile acids relatively higher. In this case the concentration of 3α-hydroxy-7-oxo-5β-cholan-24-oic acid and 7α-hydroxy-3-oxo-5β-cholan-24-oic acid in portal blood was about 30% of the concentration of chenodeoxycholic acid. It was only possible to study fasting levels of the ketonic acids in portal blood. It seems unlikely, however, that the relative amount of this bile acid fraction should increase after a meal.

The patients participating in the present study had

a functioning gallbladder. With a nonfunctioning gallbladder, or after cholecystectomy, the bile acid pool is exposed to the intestinal microflora to a greater extent than normally, which may result in increased formation of ketonic bile acids (cf. ref. 7).

It should be pointed out, that there are oxidoreductases present in the liver and part of the ketonic bile acids present in the peripheral circulation could thus have been formed by oxidation in the liver. In addition, the ketonic bile acids in the portal blood may be reduced in the liver, as has been shown to be likely in the metabolism of triketocholanic acid (8). It has been shown in vivo that 7-ketolithocholic acid is efficiently converted into chenodeoxycholic acid and to a smaller extent into ursodeoxycholic acid in human liver (20, 21).

In addition to the report by Barnes and Siegel (2), we have found only one previous report concerned with ketonic bile acids in portal blood. In preliminary form, Holzbach and Marsh (22) reported that according to their gas chromatographic investigation, cholic acid, chenodeoxycholic acid, and deoxycholic acid predominated in portal blood (75%) and lithocholic acid was present only in small quantities (3%). The remaining fractions consisted of a ketonic bile acid, believed to be 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid, a significant bile acid fraction less polar than lithocholate, and one or two minor unknown fractions. No definite identification of the ketonic bile acid was performed. Although no exact figure was given for the concentration of the ketonic bile acid, it seems to have been within the concentration range obtained in the present work. Based on the difference between the enzymatic assay and the gas–liquid chromatographic assay of bile acids reported by Barnes and Siegel (2), about twice as much ketonic bile acids as nonoxidized bile acids would have been expected in portal blood. From the results of the present work it seems likely that some compound(s) other than bile acids may have interfered in the assay.

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