Presence of monohydroxy bile acids in the urinary precipitates: a pitfall in the analysis of urinary bile acids

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Summary The importance of the method of handling the urinary precipitates frequently present in urine samples, especially after freezing-thawing, for bile acid analysis is emphasized because of the presence of a considerable proportion of monohydroxy bile acids such as lithocholic and 3β-hydroxy-5-cholenoic acids. Filtration of the urinary precipitates may lead to the underestimation of these important bile acid species.—**Yanagisawa, J., H. Ichimiya, M. Nagai, and F. Nakayama.** Presence of monohydroxy bile acids in the urinary precipitates: a pitfall in the analysis of urinary bile acids. *J. Lipid Res.* 1984. **25:** 750–753.

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Analysis of urinary bile acid gives much information on the state of bile acid metabolism in disease states. The change of urinary bile acid composition may at times be the first sign of altered bile acid metabolism, since urinary excretion of bile acid provides alternative route of disposal, as in obstructive jaundice. The precipitates are often encountered in urine samples left at room temperature or after freezing and thawing. However, to our knowledge, the handling of the urinary precipitates for bile acid analysis has not been standardized, much less vigorously investigated. In the present communication, we report the presence of considerable amounts of monohydroxy bile acids such as lithocholic (LCA) and 3β-hydroxy-5-cholenoic acids in urinary precipitates. These bile acids are thought to be important bile acid metabolites because of their hepatotoxicity (1, 2).

MATERIALS AND METHODS

Chemicals

3β-Hydroxy-5-cholenoic acid was obtained from Steraloids Inc., Wilton, NH. [23,23-$^2H_2$]3β-Hydroxy-5-cholenoic acid was kindly supplied by Research Laboratories of Nippon Kayaku Co., Tokyo, Japan. The deuterium content was $^2H_0$: 0.8%, $^2H_1$: 12.9%, $^2H_2$: 86.2%. Other authentic and deuterated bile acids used were the same as in the previous paper (3). Bond-Elut cartridge containing 500 mg C_{18} sorbent, reverse-phase octadecylsilane-bonded silica (Analytichem International, Harbor City, CA) was washed with water, ethanol, ethyl acetate, and water, successively, prior to use. Partially purified cholylglycine hydrolase was purchased from Sigma Chemical Co., St. Louis, MO; dimethylethylsilyl imidazole from Tokyo Kasei Kogyo Co., Tokyo, Japan; and Sephadex LH-20 from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Urinary samples

Urinary samples were collected in the early morning from the patients admitted to the Department of Surgery I, Kyushu University Hospital for surgical treatment. All patients except patient 4 had normal liver and renal function tests.

Effect of repeated washing on bile acid in urinary precipitates

Ten ml of urine from patient 1 was frozen at −20°C for 24 hr, thawed at room temperature, cooled in ice water for 30 min, and centrifuged at 1,500 *g* for 10 min. The precipitate (P-1) was resuspended in 10 ml of water by vigorous shaking with Thermo-mixer, left in ice water for 30 min, and centrifuged, yielding S-2 and P-2 fractions. The P-2 fraction was treated in the same way two more times, yielding subsequently S-3, P-3, S-4, and P-4 fractions. The final precipitate (P-4) was dissolved with internal standards in 1 ml of 0.1 N NaOH at 70°C for 10 min and passed through a Bond-Elut cartridge.

Effect of freezing and thawing on the bile acid content of urinary precipitates

Immediately after the collection, a fresh clear urine sample from patient 2, without the presence of any precipitate, was divided into six 3-ml aliquots. Three aliquots were analyzed immediately as such. The other three were frozen at −20°C for 24 hr and thawed. They were mixed vigorously with Thermo-mixer, ultrasonified, and incubated at 37°C for 10 min with constant shaking and occasional vigorous mixing, resulting in the complete disappearance of the precipitates produced after freezing-thawing. Both fresh and frozen-thawed samples were analyzed in an identical manner.

Effect of filtration on bile acid composition in urine

The widely used method of passing urine through filter paper prior to Amberlite XAD-2 extraction (4) was assessed with respect to the possible loss of bile acid with the precipitates. Urine from patients 3, 4, 5, 6, and 7 were studied. Each fresh urine sample was divided into six 10-ml aliquots, frozen at −20°C for 24 hr, thawed,

Abbreviations: LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid.

and left at room temperature for 30 min. Three were treated by mixing, ultrasonification, and incubation. The remaining three were filtered through filter paper after mixing briefly by hand. The precipitates remaining on the filter paper were washed with the same volume of water. The wash was combined with the filtrate and subjected to bile acid analysis.

Purification and derivatization of bile acid present in supernatant and urinary precipitates

Following addition of internal standards, aliquots of urine samples were diluted with ten volumes of 0.1 M phosphate buffer (pH 7.0) and applied onto Bond-Elut cartridge. The preliminary experiment showed that the recovery of bile acids suspended in neutral buffer by Bond-Elut extraction was satisfactory (LCA, 80%; glycocholic acid, 94%; taurocholic acid, 95%; and taurolithocholic acid-3-sulfate, 80%). The cartridge was washed with 15 ml of water followed by elution with 5 ml of ethanol. After evaporation to dryness, enzymatic hydrolysis was carried out (5, 6) followed by extraction of bile acid on Bond-Elut cartridge and solvolysis (4). Cleavage of the amide linkage in amidated sulfate-conjugates was done by the use of cholylglycine hydrolase, which gave good yield in concordance with others (7). Dimethylethylsilyl ether derivatives of bile acid ethyl esters were prepared as described previously (3).

Gas–liquid chromatography–mass spectrometry

Gas–liquid chromatography–mass spectrometric analysis was carried out using a Shimadzu Auto GC-MS 9020DF (Kyoto, Japan) equipped with a Van den Berg solventless injector, SE-30 glass capillary column, and data processing system, SCAP 1123. Each bile acid was quantitated by calculating the peak area ratio to the corresponding deuterated bile acid at $[M\text{-}DMESOH\text{-}29]^+$ ion (chenodeoxycholic acid, CDCA) or $[M\text{-}29]^+$ ion (others).

Upon selected ion recording, the dimethylethylsilyl ether derivative of 3β-hydroxy-5-cholenoic acid ethyl ester in urine samples was sometimes interfered by a co-eluting substance, whose mass spectrum appeared to indicate the presence of a steroid nucleus other than the C-24 bile acid structure.

RESULTS

Distribution of bile acid in urinary precipitates

The urine used to test the effect of washings on the bile acid present in the precipitates contained a brown precipitate occupying about one-twentieth the height of the urine sample in a glass tube upon thawing. The height of the precipitates decreased by about one-third after each washing. Therefore, the amount of the final precipitate (P-4) was very small but still persisted. **Table 1** shows the percentage of bile acid extracted by each washing. Dihydroxy and trihydroxy bile acids, i.e., deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and cholic acid (CA) were almost completely extracted in the first (S-1) and the second (S-2) supernatants, while monohydroxy bile acid, i.e., LCA and 3β-hydroxy-5-cholenoic acid, were extracted only by 40–60% into the first supernatant. Even after the third washing, a small amount of LCA was found to be present in the precipitate (P-4).

Effect of freezing and thawing on the analysis of urinary bile acid

As shown in **Table 2,** the composition of bile acids was almost identical in fresh and frozen-thawed samples except for LCA, which gave a higher value in the frozen-thawed sample than in the fresh sample; but the difference was not statistically significant because of the large between-assay coefficient of variation. The result indicated that even with the presence of precipitate in urine samples, as often encountered with frozen-thawed samples, bile acid can be adequately quantitated without apparent loss of accuracy by vigorous mixing, followed by ultrasonification and incubation before analysis, as practiced in our laboratory.

Effect of filtration on the bile acid in urinary precipitates

The concentrations of LCA and 3β-hydroxy-5-cholenoic acid found in two sets of triplicate samples analyzed after filtration or after mixing, ultrasonification, and incubation without filtration were compared (**Table 3**). Although a large analytical error was expected with filtration, variance between assays was small, making it possible to compare two analytical methods. Except in one instance (3β-hydroxy-5-cholenoic acid in patient 7), the values for monohydroxy bile acid obtained after filtration of the

TABLE 1. Effect of repeated washings of precipitates on the distribution of bile acid in urine

Bile Acid	S-1[a]	S-2	S-3	S-4	P-4[b]
Lithocholic acid	59.5[c]	23.9	8.7	3.6	4.3
3β-Hydroxy-5-cholenoic acid	44.6	24.3	21.6	9.4	0
Deoxycholic acid	87.3	9.0	3.7	0	0
Chenodeoxycholic acid	91.2	8.8	0	0	0
Ursodeoxycholic acid	97.0	3.0	0	0	0
Cholic acid	91.7	8.3	0	0	0
Total bile acid[d]	64.5	20.6	8.6	3.5	2.9

[a] Supernatant.
[b] Precipitate.
[c] Percentage of each bile acid present in different fractions.
[d] Sum of lithocholic, 3β-hydroxy-5-cholenoic, deoxycholic, chenodeoxycholic, ursodeoxycholic, and cholic acids present in each fraction.

TABLE 2. Effect of freezing and thawing on bile acid composition in urine

Bile Acid	Fresh Sample	Frozen and Thawed Sample
Lithocholic acid	14.3 ± 2.3 (16.0)	17.3 ± 2.0 (10.6)
Deoxycholic acid	10.3 ± 0.3 (2.8)	10.5 ± 0.5 (5.1)
Chenodeoxycholic acid	17.2 ± 0.5 (2.9)	17.9 ± 1.8 (9.8)
Ursodeoxycholic acid	13.8 ± 0.9 (6.7)	14.2 ± 1.6 (11.0)
Cholic acid	12.7 ± 0.9 (7.5)	12.7 ± 0.4 (3.4)

Urine was obtained in the morning from patient 2. 3β-Hydroxy-5-cholenoic acid was not determined due to the existence of other interfering peaks upon chromatography. Values are in nmol/ml, expressed as mean ± standard deviation (n = 3) with coefficient of variation (%) in parentheses.

precipitates were 60–80% of those obtained by proper handling of the precipitates, irrespective of the concentration of bile acid present. The differences were statistically significant ($P < 0.01$). For 3β-hydroxy-5-cholenoic acid in patient 7, a low concentration of the bile acid present may have influenced the analysis, done near the lowest detection limit of the procedure. Concentrations of di- and trihydroxy bile acids found were almost the same with or without filtration of the precipitates.

DISCUSSION

The presence of a considerable amount of monohydroxy bile acids in the urinary precipitates may be related to their poor solubility in aqueous media. According to Small and Admirand (8), the maximum solubility of LCA or LCA conjugate sodium salt in water was very low at 20°C (probably 2–5 mM, as judged from the figure in reference 8). For their sulfated disodium salts, several times greater solubility was observed, though the maximal solubility of sulfated glycolithocholic acid disodium salt was similar to that of the corresponding nonsulfate (9). Carey, Wu, and Watkins (9) also showed that the maximal solubilities of sulfated glyco- and tauro-3β-hydroxy-5-cholenoic acid disodium salts were 80 and 40 mM in 0.15 M NaCl at 37°C, respectively. The nonsulfates were quite insoluble, i.e., 1.5 and 0.05 mM in 0.15 M NaCl at 37°C for aqueous solubilities of glyco- and tauro-3β-hydroxy-5-cholenoic acid sodium salts, respectively. Since preliminary experiments with diethylaminohydroxypropyl Sephadex LH-20 column chromatography (4) showed that monohydroxy bile acid in the precipitates is mostly present in sulfate form (i.e., about 80–90%), the maximum aqueous solubility of monohydroxy bile acids, LCA and 3β-hydroxy-5-cholenoic acid, either sulfated or nonsulfated, thus appeared to be far above the actual concentration present in urine of our patients (at most 17 μM for LCA and 4 μM for 3β-hydroxy-5-cholenoic acid). Small and Admirand (8) also found that the solubility of LCA was greatly enhanced by the presence of di- and trihydroxy bile acids and their conjugates, as in the case of urinary bile acid. Furthermore, the pH of urine ranges approximately from 4.8 to 7.6 and contains various counterions as various biological metabolites. All these factors may well affect the solubility of monohydroxy bile acid. However, the percentage of monohydroxy bile acids remaining in the precipitates was found not to be related to their concentration in urine (Table 3), suggesting that their solubility alone is not responsible for the presence of monohydroxy bile acid in the precipitates. Mucus may be partly responsible for the formation of urinary precipitates. During the formation of precipitates, monohydroxy bile acid seems to be co-precipitated.

When the precipitates still persist after incubation at 37°C for 10 min, addition of 5% NaOH and heating at 70°C for 10 min should dissolve the precipitate almost completely. Although the addition of methanolic or ethanolic NaOH or KOH may also bring the precipitates

TABLE 3. Effect of filtration of precipitates on the monohydroxy bile acid concentrations in urine

Patient Number	Lithocholic Acid			3β-Hydroxy-5-cholenoic Acid		
	A[a]	B[b]	B/A	A	B	B/A
3	0.67 ± 0.05	0.43 ± 0.03	64%	N D[c]		
4	5.50 ± 0.43	4.44 ± 0.54	81%	3.75 ± 0.08	2.59 ± 0.15	69%
5	2.18 ± 0.22	1.58 ± 0.08	73%	0.25 ± 0.02	0.19 ± 0.02	76%
6	7.07 ± 0.85	5.43 ± 0.47	77%	0.70 ± 0.05	0.55 ± 0.04	79%
7	0.088 ± 0.014	0.067 ± 0.002	77%	0.068 ± 0.003	0.067 ± 0.012	102%

Values are in nmol/ml, expressed as mean ± standard deviation (n = 3).
[a] A, Without filtration.
[b] B, With filtration.
[c] Not determined due to co-elution of other substance with 3β-hydroxy-5-cholenoic acid upon chromatography.

into solution, the presence of alcohol interferes with the subsequent extraction of bile acid on Bond-Elut cartridge, leading to the unsatisfactory recovery of bile acid.

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