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An Examination of Alkaline Hydrolyzing Conditions of Conjugated Bile Acids with Carbonyl Groups

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The conditions for alkaline hydrolysis of conjugated bile acids with carbonyl groups were investigated using several oxo bile acids and conjugated bile acids. Under the condition using a 1 N NaOH solution for 4 h in an autoclave, glycine- and taurine-conjugated bile acids were hydrolyzed and also, the amounts of oxo bile acids used were recovered without any significant decomposition.

Key words: alkaline hydrolyzing conditions; capillary gas chromatography; conjugated bile acids; 1 N NaOH solution; oxo bile acids

Hydrolysis is often applied for the analysis of bile acids and is performed either in an alkaline solution or using cholylglycine hydrolase. Alkaline hydrolysis is usually performed with 2 N NaOH solution for 4 h at 130°C under pressure, but oxo bile acids are vulnerable during this process (Lepage et al., 1978; Yamamoto et al., 1977).

In this study, we examined conditions for alkaline hydrolysis of conjugated bile acids and found a suitable condition which caused no significant decomposition of oxo bile acids.

Materials and Methods

The unconjugated oxo bile acids and glycine- and taurine-conjugated bile acids used in this study were those kept in our laboratory. 7β, 12β-Dihydroxycholanoic acid and glyco-7β, 12α-dihydroxycholanoic acid as internal standards for analysis by capillary gas chromatography were synthesized as previously described (Arimoto et al., 1982; Yamaga et al., 1994). These bile acids were estimated to be more than 96% pure by capillary gas chromatography.

Each of four bile acid mixtures, as listed in Table 1, was separately hydrolyzed in a glass tube by four conditions consisting of a combination of 2.0 mL of 1 N or 2 N NaOH solutions for a period of 2 h or 4 h in an autoclave (120°C, 1 to 1.2 kgf/cm^2). Particularly, two oxo bile acids, 7-oxo-3α-hydroxycholanoic acid and 12-oxo-3α-hydroxycholanoic acid were grouped separately (1-a and 1-b in Table 1), since these bile acids show the same retention time on the present capillary column of gas chromatography.

After acidification to pH 1 with 3 N HCl on ice, bile acids were extracted 3 times with diethyl ether. The extracted bile acids were converted into methyl ester dimethylethylsilyl ether derivatives as reported previously (Yamaga et al., 1994). The procedure for analysis of urinary bile acids was described elsewhere (Yamaga et al., 1994). Capillary gas chromatograms and analytical data of the extracted bile acid derivatives were obtained using a gas chromatograph (Model GC-14A, Shimadzu, Kyoto, Japan) with a computerized data system (Model C-R4A Chromatopac, Shimadzu). The gas chromatograph was equipped with a HiCap CBP-1 capillary column (25 m × 0.25 mm I.D., Shimadzu) and a solventless injector. The capillary gas chromatographic conditions were described elsewhere (Yamaga et al., 1994).

Results and Discussion

Oxo bile acids are formed by the action of the dehydrogenase of intestinal flora in mammalian species (Matern et al., 1975; Eneroth et al.,

1966; Fromm et al., 1980, 1983). Oxo bile acids such as 12-oxo-3α-hydroxycholanoic acid and 12-oxo-3β-hydroxycholanoic acid have been detected as major bile acids in human feces (Danielson et al., 1963; Eneroth et al., 1968; Hill and Aries, 1971). Recently, 12-oxo-3α-hydroxycholanoic acid was determined as a major bile acid among the many species of bile acids in urine specimens from healthy humans, and also, no other species of oxo bile acids except for 12-oxo-3α-hydroxycholanoic acid were detected in them (Yamaga et al., 1996). On the other hand, oxo bile acids are reported to be vulnerable to alkaline hydrolysis (Lepage et al., 1978; Yamamoto et al., 1977), and moreover the chemical mode of oxo bile acids in biological specimens is unknown. Therefore, it remains a possibility that oxo bile acids in biological specimens have not been measured completely after alkaline hydrolysis. In this study, suitable conditions for alkaline hydrolysis of conjugated bile acids including conjugated oxo bile acids were investigated using several oxo bile acids and common conjugated bile acids.

Table 1. The composition and weight of bile acids used for alkaline hydrolysis

1-a) Unconjugated oxo bile acid mixture	(μg/tube)
7β,12β-Dihydroxycholanoic acid as internal standard	5.1
3-Oxo-cholanoic acid	5.1
3-Oxo-12α-hydroxycholanoic acid	5.3
3-Oxo-7α-hydroxycholanoic acid	4.9
12-Oxo-3α-hydroxycholanoic acid	4.9
3-Oxo-7α,12α-dihydroxycholanoic acid	5.0
7-Oxo-3α,12α-dihydroxycholanoic acid	5.2
12-Oxo-3α,7α-dihydroxycholanoic acid	5.1
1-b) Unconjugated oxo bile acid mixture	(μg/tube)
7β,12β-Dihydroxycholanoic acid as internal standard	5.1
3-Oxo-cholanoic acid	5.1
3-Oxo-12α-hydroxycholanoic acid	5.3
3-Oxo-7α-hydroxycholanoic acid	4.9
7-Oxo-3α-hydroxycholanoic acid	5.0
3-Oxo-7α,12α-dihydroxycholanoic acid	5.0
7-Oxo-3α,12α-dihydroxycholanoic acid	5.2
12-Oxo-3α,7α-dihydroxycholanoic acid	5.1
2) Glycine-conjugated bile acid mixture	[μg (as free)/tube]
7β,12α-Dihydroxycholanoic acid as internal standard	4.8
Lithocholic acid	3.4
Deoxycholic acid	4.4
Chenodeoxycholic acid	3.5
Cholic acid	6.0
3) Taurine-conjugated bile acid mixture	[μg (as free)/tube]
Glyco-7β,12α-Dihydroxycholanoic acid as internal standard	4.5
Lithocholic acid	2.6
Deoxycholic acid	3.4
Chenodeoxycholic acid	3.5
Cholic acid	4.6

Table 2 summarizes the effects of alkaline hydrolysis on several oxo bile acids, under the four conditions described in Materials and Methods. Obviously, the oxo bile acids were labile to alkaline hydrolysis, particularly under conditions which using a 2 N NaOH solution for 4 h in an autoclave. The four 3-oxo bile acids (3-oxocholanoic acid, 3-oxo-7α-hydroxycholanoic acid, 3-oxo-12α-hydroxycholanoic acid and 3-oxo-7α,12α-dihydroxycholanoic acid) were significantly sensitive. Artificial products from the oxo bile acids were not detected at their peaks on the gas chromatogram, although Lepage and colleagues (1978) detected some identified and unidentified peaks of artificial products from corresponding oxo bile acids. Decreased recoveries of oxo bile acids were dependent on the concentration of NaOH solution, but not on the period of hydrolysis. The recovery for all of oxo bile acid species including the four labile oxo bile acids mentioned above, was over 80% when a 1 N NaOH solution was used. The most excellent recovery for all of oxo bile acid species examined was obtained under conditions hydrolyzing in a 1 N NaOH solution for 2 h. These results indicate that the decomposition of oxo bile acids is related to the position of carbonyl groups in the steroid nucleus and to the condition of alkaline hydrolysis.

However, the question remains as to whether glycine- and taurine-conjugated bile acids, including conjugated oxo bile acids, are completely hydrolyzed by the conditions mentioned

Table 2. Effects of alkaline hydrolysis on several unconjugated oxo bile acids

Bile acids	2 h*		4 h	
	1 N NaOH†	2 N NaOH	1 N NaOH	2 N NaOH
	μg (%)	μg (%)	μg (%)	μg (%)
3-Oxocholanoic acid	5.0 (98.0)	3.8 (74.5)	4.8 (94.1)	3.3 (64.7)
3-Oxo-12α-hydroxycholanoic acid	5.2 (98.1)	3.8 (71.7)	5.1 (96.2)	3.7 (69.8)
3-Oxo-7α-hydroxycholanoic acid	4.6 (93.9)	2.6 (53.1)	4.0 (81.6)	2.4 (53.1)
7-Oxo-3α-hydroxycholanoic acid	4.5 (91.8)	4.8 (98.0)	4.7 (95.9)	4.8 (98.0)
12-Oxo-3α-hydroxycholanoic acid	4.9 (98.0)	4.7 (94.0)	4.8 (96.0)	4.7 (94.0)
3-Oxo-7α,12α-dihydroxycholanoic acid	5.3 (106.0)	2.7 (54.0)	4.4 (88.0)	2.7 (54.0)
7-Oxo-3α,12α-dihydroxycholanoic acid	4.7 (90.4)	4.1 (78.8)	4.2 (80.8)	4.0 (76.9)
12-Oxo-3α,7α-dihydroxycholanoic acid	4.6 (90.2)	4.3 (84.3)	4.2 (82.4)	4.2 (82.4)

Data represent mean value of five experiments and numbers in ( ) represent recovery % from the five experiments.
* Period of alkaline hydrolysis.
† Concentration of NaOH solution.

above. For the recovery tests, we used common glycine- and taurine-conjugated bile acids instead of conjugated oxo bile acids (unavailable) and their alkaline hydrolysis was performed under 1 N and 2 N NaOH solutions for 2 and 4 h. The results are shown in Table 3. The glycine-conjugated bile acids were hydrolyzed sufficiently under all the conditions described above. The recoveries of taurine-conjugated bile acids were almost constant, although the amount recovered was slightly decreased, under the three conditions except for the 1 N NaOH solution for 2 h. The decreased recovery is due to the difficulty of the hydrolysis of taurine-conjugated bile acids compared with that of glycine-conjugated bile acids, and also to the use of glyco-7β,12α-dihydroxycholanoic acid as an internal standard for analysis of taurine-conjugated bile acids. These results indicate that authentic conjugated bile acids were obviously hydrolyzed by a 1 N NaOH solution for 4 h in an autoclave.

Table 3. Effects of alkaline hydrolysis on glycine-conjugated bile acids and taurine-conjugated bile acids

Conjugated bile acids	4 h*		2 h	
	1 N†	2 N	1 N	2 N
	μg (%)	μg (%)	μg (%)	μg (%)
G-LA	3.9 (114.7)	3.9 (114.7)	4.1 (120.6)	3.9 (114.7)
G-DOCA	3.7 (84.1)	3.8 (102.7)	3.8 (86.4)	4.0 (90.9)
G-CDCA	3.3 (94.3)	3.4 (97.1)	3.3 (94.3)	3.6 (102.9)
G-CA	6.0 (100.0)	6.1 (101.7)	6.1 (101.7)	5.9 (98.3)
Total	16.9	17.2	17.3	17.4
T-LA	2.2 (84.6)	2.4 (92.3)	2.2 (84.6)	2.2 (84.6)
T-DOCA	2.7 (79.4)	3.1 (91.2)	2.5 (73.5)	2.5 (73.5)
T-CDCA	2.8 (80.0)	2.8 (80.0)	2.7 (77.1)	3.0 (85.7)
T-CA	4.0 (87.0)	4.0 (87.0)	3.7 (80.4)	3.9 (84.8)
Total	11.7	12.3	11.1	11.8

Data and numbers in ( ) represent mean value and recovery % from the five experiments, respectively.
* Period of alkaline hydrolysis.
† Concentration of NaOH solution.
CA, 3α,7α,12α-trihydroxycholanoic acid; CDCA, 3α,7α-dihydroxycholanoic acid; DOCA, 3α,12α-dihydroxycholanoic acid; G, glycine-conjugated; LA, 3α-hydroxycholanoic acid; T, taurine-conjugated.

Table 4. The amounts (mg) and compositions (%) of bile acids in 5 mL of urine specimens from two healthy subjects (A and B)

Bile acids	Subject A				Subject B			
	1 N, 4 h*		2 N, 4 h		1 N, 4 h		2 N, 4 h	
	μg	(%)	μg	(%)	μg	(%)	μg	(%)
Lithocholic acid	0.98	(2.6)	1.17	(3.1)	2.68	(5.5)	2.73	(5.6)
3β-Hydroxychol-5-enoic acid	ND		ND		0.91	(1.9)	0.82	(1.7)
3α,12β-Dihydroxycholanoic acid	4.93	(13.2)	4.62	(12.3)	0.61	(1.3)	0.77	(1.6)
3β,7α-Dihydroxycholanoic acid	ND		ND		0.48	(1.0)	0.53	(1.1)
Deoxycholic acid	1.86	(5.0)	1.90	(5.1)	1.06	(2.2)	1.08	(2.2)
Chenodeoxycholic acid	ND		ND		2.23	(4.6)	2.06	(4.2)
12-Oxo-3α-hydroxycholanoic acid	25.37	(67.9)	25.38	(67.7)	3.10	(6.4)	3.09	(6.4)
Norcholic acid	ND		ND		1.38	(2.8)	1.34	(2.8)
Ursodeoxycholic acid	0.65	(1.7)	0.95	(2.3)	5.67	(11.6)	5.81	(11.9)
3β,7β-Dihydroxycholanoic acid	ND		ND		5.25	(10.8)	4.95	(10.1)
3α,7α,12β-Trihydroxycholanoic acid	ND		ND		2.66	(5.4)	2.29	(4.7)
Cholic acid	2.27	(6.1)	2.10	(5.6)	3.12	(6.4)	2.98	(6.1)
3α,7β,12α-Trihydroxycholanoic acid	1.31	(3.5)	1.39	(3.7)	14.98	(30.7)	15.29	(31.4)
7-Oxo-3α,12α-dihydroxycholanoic acid	ND		ND		2.83	(5.8)	2.60	(5.3)
12-Oxo-3α,7α-dihydroxycholanoic acid	ND		ND		1.82	(3.7)	2.31	(4.7)
Total	37.37		37.51		48.78		48.65	

Numbers in ( ) represent composition (%) of each bile acid in urine specimens.
* The condition for alkaline hydrolysis. N and h mean the concentration of NaOH solution and a period of alkaline hydrolysis, respectively.
ND, not detectable.

After the extraction of bile acids from urine specimens (5 mL) of two healthy humans, the extracted bile acids were hydrolyzed separately by autoclaving for 4 h in 2.0 mL of 1 N and 2 N NaOH solutions, and then measured. The amounts and compositions of the urinary bile acids coincided well in both conditions, and the oxo bile acid identified tentatively by the retention time on the present capillary column of gas chromatography was only 12-oxo-3α-hydroxycholanoic acid in subject A, and 12-oxo-3α-hydroxycholanoic acid, 7-oxo-3α,12α-dihydroxycholanoic acid and 12-oxo-3α,7α-dihydroxycholanoic acid in subject B, as shown in Table 4. No 3-oxo bile acid was detected in either specimen. The present data on urine specimens suggest that glycine- and taurine-conjugated bile acids were hydrolyzed under condition using a 1 N NaOH solution for 4 h in an autoclave which will not decompose 3-oxo bile acids.

We conclude that the conjugated bile acids and oxo bile acids extracted from biological specimens are hydrolyzed by autoclaving for 4 h in a 1 N NaOH solution instead of a 2 N NaOH solution, without any significant decomposition of oxo bile acids.

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