Review Article Yonago Acta medica 2001;44:91–105

Internal Standard Compounds for Quantitative Determination of Bile Acids by Gas Chromatography

Nobuo Yamaga, Yoshio Ogura, Kazuo Yamada, Hiromi Kohara* and Kiyohisa Uchida†

*Department of Biochemistry and *Department of Pathological Biochemistry, Tottori University Faculty of Medicine, Yonago 683-0826, and †The Cell Science Research Foundation, Osaka 541-0045 Japan*

Gas chromatography is well recognized as a useful tool with several advantages for the analysis of bile acids as well as various compounds. In gas chromatographic analysis, bile acids in an analytical sample are subjected to a number of complicated procedures involving many steps such as extraction, fractionation, solvolysis, hydrolysis, derivatization and injection to the gas chromatograph. These procedures result in the loss of bile acids in the analytical sample. The addition of suitable internal standard compound(s) into the analytical sample prior to the extraction of bile acids is indispensable for an accurate determination of bile acids. There are two methods for the quantitative determination of bile acids in a biological sample by gas chromatography: one is the determination of total bile acid amounts in the sample. The other is the determination of bile acid amounts in each fraction after group separation of bile acids in the biological sample using an ion exchange gel column. The addition of 7β,12α-dihydroxy-5β-cholanoic acid or 7β,12β-dihydroxy-5β-cholanoic acid as an internal standard compound is useful for the former method. On the other hand, the addition of 7β,12β-dihydroxy-5β-cholanoic acid, glyco-7α,12α-dihydroxy-5β-cholanoic acid, tauro-7α,12β-dihydroxy-5β-cholanoic acid and glyco-7β,12α-dihydroxy-5β-cholanoic acid 7-sulfate is a suitable combination as internal standard compounds for the latter method.

Key words: bile acids; biological sample; gas chromatographic analysis; internal standard compounds; quantitative determination

The use of gas chromatography in investigation of bile acids

Gas chromatography is well recognized as a useful tool with several advantages for the analysis of bile acids as well as various compounds. It especially has good sensitivity and is able to determine a number of different compounds in a class simultaneously. In 1960, Vanden Heuvel and coworkers (1960) for the first time applied gas chromatography to the analysis of bile acids after converting them into methyl ester derivatives. Since then, gas chromatography has contributed greatly to the investigation of bile acid metabolism, analyzing bile acids in several biological samples such as bile, feces, serum, urine, gallstones or tissues.

Abbreviations: αα, 7α,12α-dihydroxy-5β-cholanoic acid; αβ, 7α,12β-dihydroxy-5β-cholanoic acid; βα, 7β,12α-dihydroxy-5β-cholanoic acid; ββ, 7β,12β-dihydroxy-5β-cholanoic acid; Glyco-αα, glyco-7α,12α-dihydroxy-5β-cholanoic acid; Glyco-αα 7-sulfate, glyco-7α,12α-dihydroxy-5β-cholanoic acid 7-sulfate; Glyco-βα 7-sulfate, glyco-7β,12α-dihydroxy-5β-cholanoic acid 7-sulfate; Tauro-αα, tauro-7α,12α-dihydroxy-5β-cholanoic acid; Tauro-αβ, tauro-7α,12β-dihydroxy-5β-cholanoic acid; Tauro-ββ, tauro-7β,12β-dihydroxy-5β-cholanoic acid

Metabolism and movement of bile acids

Bile acids in the human body are mainly composed of cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and ursodeoxycholic acid (Beppu et al., 1982; Yamaga et al., 1994a; Yamaga et al., 1996). Chenodeoxycholic acid and cholic acid are primary bile acids synthesized from cholesterol in the liver, and they are major catabolic products of cholesterol (Danielsson, 1973). Deoxycholic acid, lithocholic acid and ursodeoxycholic acid are secondary bile acids converted from primary bile acids by the action of intestinal flora (Hill and Drasar, 1968; Shimada et al., 1969; Aries and Hill, 1970). Most free bile acids are easily conjugated with glycine or taurine in the liver. Bile containing glycine- and taurine-conjugated bile acids is stored and concentrated in the gallbladder, and then released into the duodenum. Primary and secondary bile acids are absorbed almost exclusively from the ileum, return quantitatively to the liver by way of the portal circulation and are secreted into bile. This is called enterohepatic circulation of bile acids (Lack and Weiner, 1967; Dietschy, 1968; Dowling, 1972; Heaton, 1972). During enterohepatic circulation, some parts of bile acids are not absorbed to any significant extent from the intestine and excreted into feces (Eneroth et al., 1966; Dietschy, 1968; Tyor et al., 1971; Heaton, 1972). A part of bile acids leaks into the systemic circulation (van Berge-Henegouwen et al., 1974), and then excretes into the urine through the kidney.

Some parts of bile acids, especially secondary bile acids in the blood and urine are found as their sulfates (Palmer, 1967; Makino et al., 1973; Back, 1974), glucosides (Marschall et al., 1988; Marschall et al., 1989), glucuronides (Palmer,1967; Stiehl, 1974; Alme and Sjövall, 1980), and/or *N*-acetylglucosaminides (Marschall et al., 1988; Marschall et al., 1989; Takikawa et al., 1982; Yamaga et al., 1994a). The formation of unusual bile acids and the ratios of various conjugated bile acids offer useful information on hepatobiliary and gastrointestinal diseases (Garbutt et al., 1969; Neale et al., 1971; Takikawa et al., 1983a, 1983b).

Derivatizations of bile acids for gas chromatography

For gas chromatographic analysis the compounds analyzed are required to be volatile. Therefore, nonvolatile compounds should be converted into volatile derivatives quantitatively. It is true in the case of bile acids since they are polar compounds having a carboxyl group and some hydroxyl groups in their molecules. Usually, the carboxyl group of bile acids is methylated with diazomethane or hexafluoroisopropylated with hexafluoroisopropanol (Imai et al., 1976). The hydroxyl group is acetylated with acetic anhydride (Roovers et al., 1968) or trifluoroacetylated with trifluoroacetic anhydride (Endo et al., 1979). In addition, the hydroxyl group is silylated with both hexamethyldisilazane and trimethylchlorosilane (Makita and Wells, 1963), *N*-trimethylsilylimidazole (Karlangnis and Paumgartner, 1979; Amuro et al., 1983) or dimethylethylsilylimidazole (Miyazaki et al., 1977; Arimoto et al., 1982). In these situations, direct conversion of original conjugated bile acids into volatile derivatives has not been succeeded up to the present.

Fractionation and gas chromatographic analysis by difference in conjugated bile acid forms

As mentioned above, bile acids in human and animal bodies are present as a nonamidated form (free form) and forms conjugated with glycine, taurine, sulfuric acid, glucuronic acid and/or others. Therefore, in gas chromatographic analysis, bile acids must be converted into volatile derivatives after carrying out a number of complicated procedures. Bile acids extracted from an analytical sample are converted into free bile acids by solvolysis with hydrochloric acid (Back, 1937; van Berge-Henegowen et al., 1976; Alme et al., 1977; Yamaga et al., 1994a), followed by alkaline

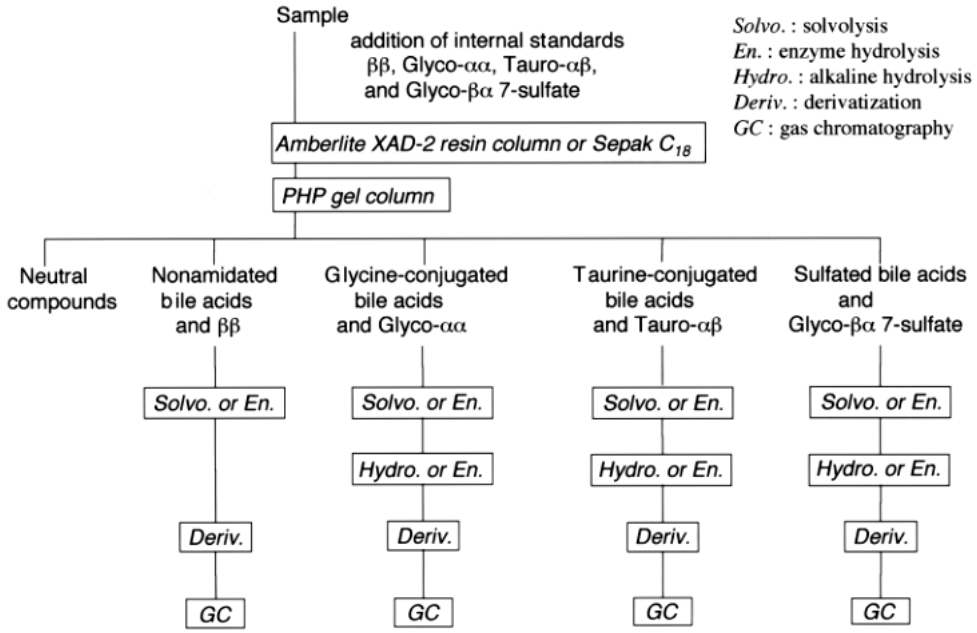

Fig. 1. Outline of procedures for systematic analysis of bile acids in a sample.

hydrolysis with 2 M sodium hydroxide solution (Nair and Garcia, 1969; Campbell et al., 1975; Yamaga et al., 1997) or enzymatic hydrolysis (Nair and Gordon, 1967; Cantafora et al., 1979), and free bile acids are converted into volatile derivatives. Bile acid derivatives are analyzed by gas chromatography.

However no information on the original conjugated forms of bile acids is obtained from the gas chromatogram. In order to solve this problem, crude bile acid extract from the analytical sample is first subjected to column chromatography with an ion exchange gel such as diethylaminohydroxypropyl-Sephadex-LH-20 (DEAP-LH-20) (Alme et al., 1977) or piperidinohydroxypropyl-Sephadex- LH-20 (PHP-LH-20) (Goto et al., 1978), fractionating into five fractions; each containing neutral compounds, nonamidated bile acids, glycine-conjugated bile acids, taurine-conjugated bile acids and sulfated bile acids. Each of all the fractions is subjected to solvolysis with hydrochloric acid, followed by hydrolysis with enzyme or 2 M sodium hydroxide solution, and then free bile acids thus obtained are converted into volatile derivatives for gas chromatographic analysis. Outline of common procedures for systematic analysis of bile acids in sample is shown in Fig. 1. By these procedures, the information on original conjugated bile acids is available.

Glucosides, glucuronides and *N*-acetylglucosaminides of bile acids (particular bile acid conjugates) are present in only their forms or double conjugated forms with glycine or taurine. When bile acid extract from biological sample was fractionated with PHP-LH-20 gel or DEAP-LH-20 gel column, these particular bile acid conjugates are not fractionated independently. No fractions are restricted where the particular bile acid conjugates are fractionated. For example, bile acid *N*-acetylglucosaminides are fractionated into both nonamidate fraction and glycine-conjugate fraction (Yamaga and Kohara, 1994). There are no reports describing the quantitative determination of bile acid glucosides, bile acid glucuronides and/or bile acid *N*-acetylglucosaminides in systematic analysis. The method for their analysis is referred to the individual reports (Takikawa et al., 1982; Marschall et al., 1988, 1989; Yamaga and Kohara, 1994).

Indispensability of internal standard compounds

A part of bile acids in an analytical sample, especially in a small volume sample, is certainly lost during procedures such as extraction, fractionation and other treatments. The usual method for correcting the loss of bile acids during analytical procedures is based on either adding an internal standard compound to the sample in the course of the systematic analysis (Fig. 1) or performing a recovery test using labeled and non-labeled bile acids under a condition similar to the analytical condition. For gas chromatographic analysis, the latter method does not ensure that the recovery from a biological sample will be the same as that from an artificial sample that has been prepared with labeled or non-labeled bile acids. Moreover, recovery may differ from assay to assay. Therefore, the use of internal standard compounds is entirely indispensable for correcting the loss of bile acids.

The minimum requirements for selecting internal standard compound for gas chromatography of bile acids

The minimum requirements for selecting a suitable internal standard compound for the determination of bile acids by gas chromatography are as follows.

i) Internal standard compound(s) must be added to the analytical sample before the extraction of bile acids. Then, the peak of characteristic internal standard compound must appear together with peaks of bile acids in the analytical sample on the same gas chromatogram.

ii) It is not necessary for internal standard compound(s) to be bile acid(s). However, it is better for them to be non-natural 5β-cholanoic acid homologues with some hydroxyl groups.

iii) Internal standard compound(s) should be stable. That is, during the analysis, they must not decompose to other compounds.

iv) After the fractionation with PHP-LH-20 gel column or DEAP-LH-20 gel column, the characteristic internal standard compound(s) should be transferred into each of all fractions. For this reason, four internal standard compounds consisting of non-amidated, glycine-conjugated, taurine-conjugated and sulfated forms, are necessary for the quantitative and qualitative determination of bile acids in each fraction after group separation.

v) The peak(s) of internal standard compound(s) must not pile up on the peaks of bile acids and the peaks of other mixed compounds in the biological sample.

vi) The retention time of the internal standard compound peak in each fraction must not be largely distant from those of bile acid peaks. Preferably, the peak of the internal standard compound should appear in the range between the first and the last bile acid peaks detectable on the gas chromatogram.

vii) Some linear relationship should be kept between the peak high or area and the weight of each bile acid to the internal standard compound.

When the selected internal standard compound(s) satisfy the requirements above mentioned, the area of an internal standard compound peak on the gas chromatogram is regarded as the amount (weight) of the internal standard compound added to the analytical sample, irrespective of the recovery rate during all the procedures. There is no occasion for considering the recovery ratio. It is only required to obtain linear calibration curves between the peak area ratios and the weight ratios of each bile acid to the internal standard compound.

Up to now, some kinds of unique bile acids such as 5β-cholanoic acid (Campbell et al., 1975),

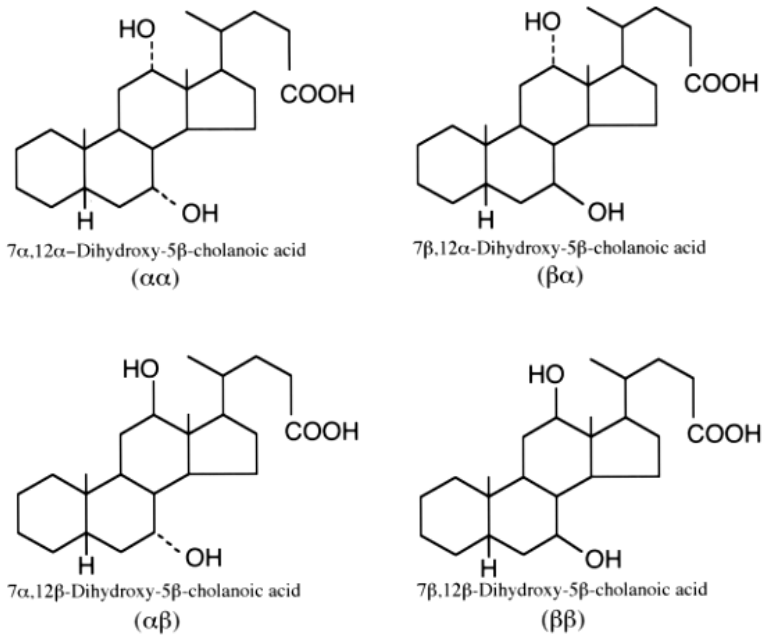

Fig. 2. Artificial bile acids chemically synthesized from cholic acid.

nordeoxycholic acid (van Berge-Henegouwen et al., 1977; Roda et al., 1978), hyodeoxycholic acid (Ali and Javitt, 1970; Karlangnis and Paumgartner, 1978; Cantafora et al., 1979), 7-ketolithocholic acid (Klaassen, 1971), 7-ketodeoxycholic acid (van Berge-Henegouwen et al., 1976) and hyocholic acid (Subbiah, 1973; Yamaga et al., 1983) have been used as an internal standard compound for gas chromatographic analysis. Certainly, these compounds are able to be used as an internal standard compound. But they have some drawbacks. They are sometimes found in samples from healthy subjects, neonates and some patients (Alme et al., 1977, 1978, 1980; Back and Walter, 1980; Sawada, 1981). Furthermore, oxo-5β-cholanoic acids such as 7-ketolithocholic acid and 7-ketodeoxycholic acid are easily vulnerable to decomposition during alkaline hydrolysis with sodium hydroxide solution (Lepase et al., 1978). For these reasons, it is necessary to develop other internal standard compounds.

Internal standard compounds for the determination of total bile acid amounts

Natural bile acids originally have a hydroxyl group at the C-3α position in the steroid nucleus. First, we chemically synthesized four isomers of 7,12-dihydroxy-5β-cholanoic acid lacking a hydroxyl group at C-3α position of cholic acid; 7α,12α-dihydroxy-5β-cholanoic acid (αα), 7β,12α-dihydroxy-5β-cholanoic acid (βα), 7α,12β-dihydroxy-5β-cholanoic acid (αβ) and 7β,12β-dihydroxy-5β-cholanoic acid (ββ) (Fig. 2) (Arimoto et al., 1982). An outline of the chemical synthesis of these artificial bile acids from cholic acid is shown in Fig. 3. These compounds in the derivatives of methyl ester dimethylethylsilyl (DMES) ether have different retention times compared to each other on the gas chromatogram (Fig. 4). In addition, the retention times of these four compounds are different from those of many authentic bile acids. But, only the retention time of αβ is very close to that of lithocholic acid (Table 1) and

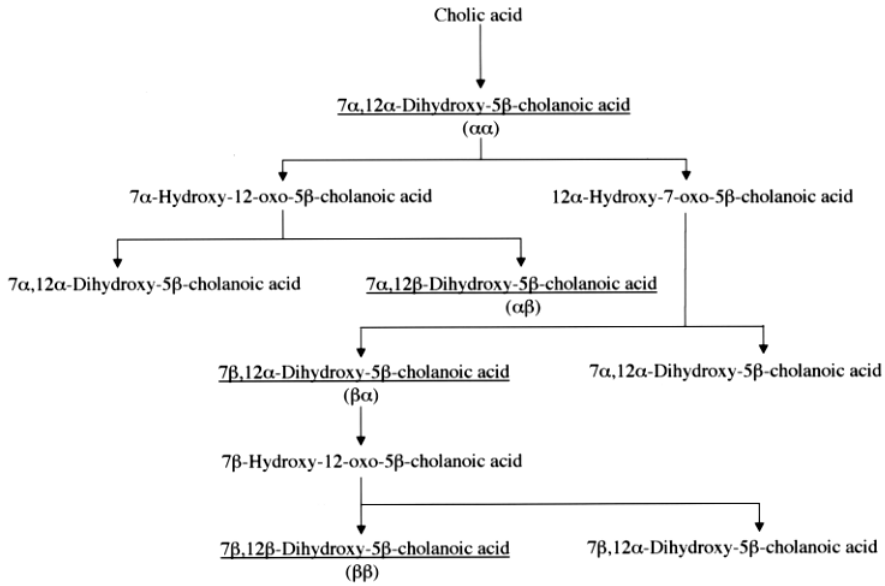

Fig. 3. Outline of chemical synthesis of four 7,12-dihydroxy-5β-cholanoic acid isomers from cholic acid.

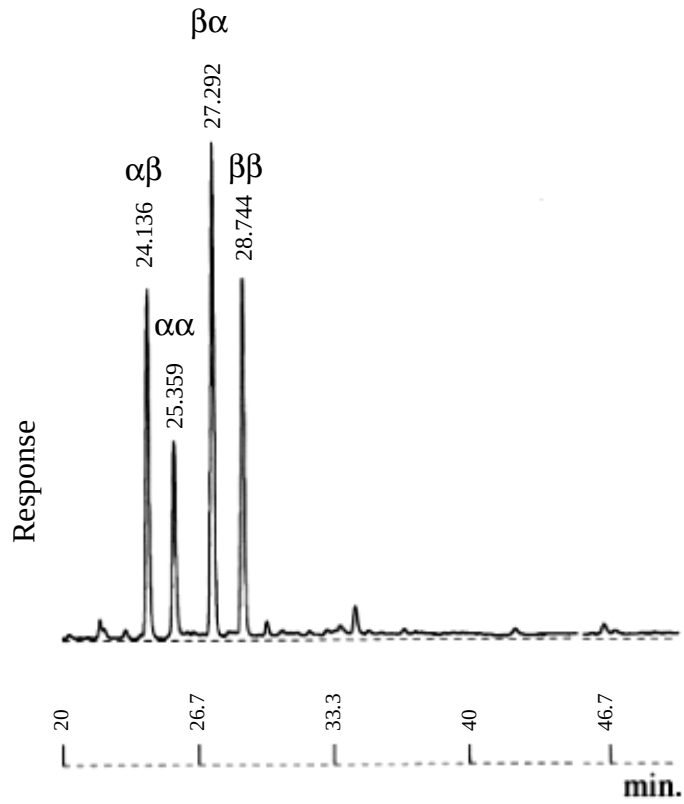

Fig. 4. Gas chromatogram of αα, αβ, βα and ββ (methyl ester DMES ether). (See Table 1 for gas chromatographic condition.)

Table 1. The relative retention times of internal standard compounds and authentic bile acids

Internal standard compounds	Retention time	Relative retention time			
(1) 7α,12β-Dihydroxy-5β-cholanoic acid	24.110	**1.000**	0.954	0.888	0.840
(2) 7α,12α-Dihydroxy-5β-cholanoic acid	25.279	1.048	**1.000**	0.931	0.880
(3) 7β,12α-Dihydroxy-5β-cholanoic acid	27.162	1.127	1.074	**1.000**	0.946
(4) 7β,12β-Dihydroxy-5β-cholanoic acid	28.713	1.191	1.136	1.057	**1.000**
Authentic compounds					
(5) Cholesterol	25.102	1.041	0.993	0.924	0.874
(6) Lithocholic acid	24.016	0.996	0.950	0.884	0.834
(7) 3β-Hydroxy-5-cholenoic acid	28.102	1.166	1.112	1.035	0.979
(8) 3α,12β-Dihydroxy-5β-cholanoic acid	29.915	1.241	1.183	1.101	1.042
(9) Deoxycholic acid	30.972	1.285	1.225	1.140	1.079
(10) Chenodeoxycholic acid	32.678	1.355	1.293	1.203	1.138
(11) Norcholic acid	33.513	1.390	1.326	1.234	1.167
(12) Hyodeoxycholic acid	33.877	1.405	1.340	1.247	1.180
(13) Ursodeoxycholic acid	34.810	1.444	1.377	1.282	1.212
(14) 3β,7β-Dihydroxy-5β-cholanoic acid	35.579	1.476	1.408	1.310	1.239
(15) 3α,7α,12β-Trihydroxy-5β-cholanoic acid	37.193	1.543	1.471	1.369	1.295
(16) Cholic acid	39.672	1.645	1.569	1.461	1.382
(17) 3α,7β,12α-Trihydroxy-5β-cholanoic acid	40.570	1.683	1.605	1.494	1.413
(18) Hyocholic acid	42.879	1.778	1.696	1.579	1.493
(19) 3α,7α-Dihydroxy-12-oxo-5β-cholanoic acid	43.790	1.816	1.732	1.612	1.525

Gas chromatographic condition was programmed at 250℃ for 5 min, from 250℃ to 270℃ at 0.2℃/min, at 270℃ for 10 min, from 270℃ to 280℃ at 0.5℃/min, and then at 280℃ for 30 min.
Capillary column, Hicap CBP-1 capillary column(25m x 0.25mm I.D).
Bile acids were derivatized to methyl ester DMES ether.

both peaks of αβ and lithocholic acid practically pile up in the same gas chromatogram (Fig. 5).

Calibration curves for the quantitative determination of several bile acids have a linear relationship going through the origin between the weight ratio and the peak area ratio of each bile acid to the internal standard compound(s).

By way of example, the linear calibration curves for several bile acids are shown in Fig. 6 when ββ is used as an internal standard compound. Then, the absolute amount of each bile acid in a biological sample can be calculated by the following formula (Arimoto et al., 1982).

$$W = A \times B$$

W: Absolute amount (weight) of each bile acid in the sample assayed.

A: Weight ratio of each bile acid to ββ, obtained by multiplying the slope value of the calibration curve by the peak area ratio of each bile acid peak to ββ peak from the gas chromatogram.

B: Amount (weight) of internal standard compound added in the biological sample.

Total bile acid amounts in an analytical sample can be calculated by adding the amounts of all kinds of bile acids analyzed.

Occasionally confirming the calibration curve is recommended.

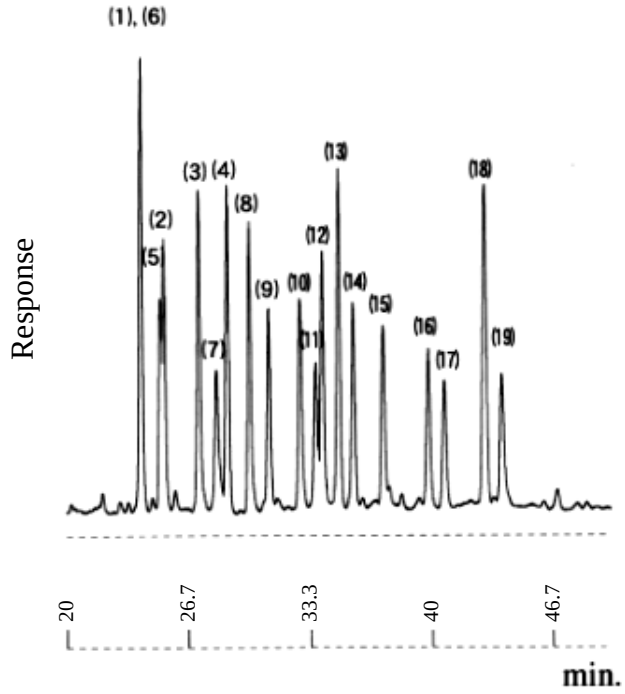

Fig. 5. Gas chromatogram of authentic bile acids and internal standard compounds (methyl ester DMES ether). (See Table 1 for the number of peaks and gas chromatographic condition.)

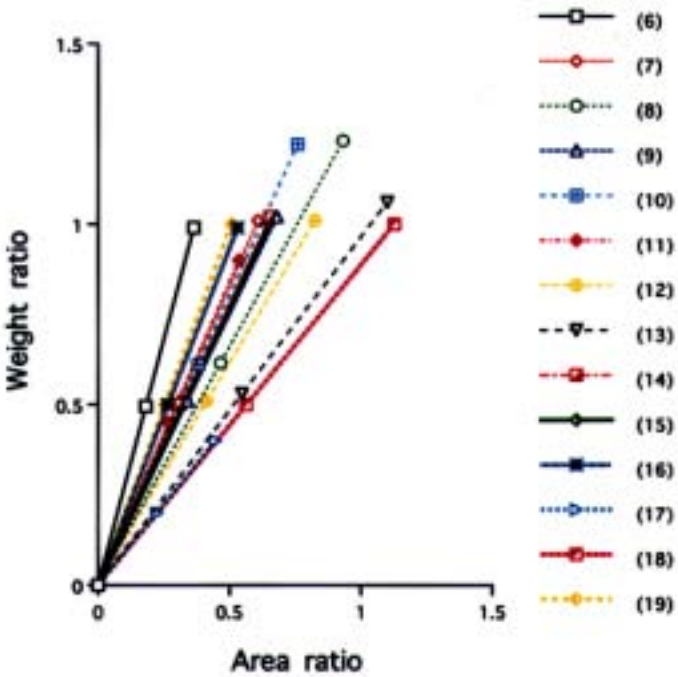

Fig. 6. Calibration curves of several natural bile acids to artificial ββ as internal standard compound for quantitative determination. (See Table 1 for the number of peaks.)

$$\text{Weight ratio: } \frac{\text{Weight of each bile acid}}{\text{Weight of } \beta\beta}$$

$$\text{Area ratio: } \frac{\text{Peak area of each bile acid}}{\text{Peak area of } \beta\beta}$$

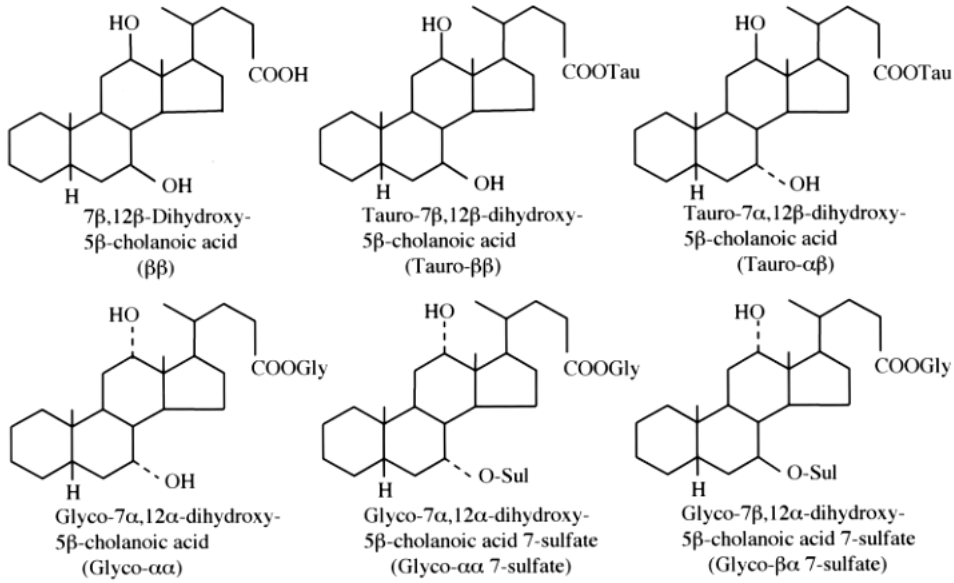

Fig. 7. Internal standard compounds chemically synthesized for gas chromatographic analysis after group separation.

Yamaga and coworkers (1983) adopted ββ as an internal standard compound for the quantitative determination of total bile acids in urine by gas chromatography. Other workers used tauro-7α,12α-dihydroxy-5β-cholanoic acid (Tauro-αα) as an internal standard compound for the same project (Ghoos et al., 1983). These synthesized compounds (αα, βα, ββ and Tauro-αα except αβ, because the peak of αβ piles up on that of lithocholic acid) are adequate internal standard compounds for the quantitative determination of bile acids by gas chromatography. However, as far as only one kind of internal standard compound is used, the analysis after group separation of bile acids can not attain accurate data in all fractions, since ββ transfers only into the nonamidate fraction after an ion exchange gel column chromatography. On the other hand, Tauro-αα transfers only into the taurine-conjugate fraction in a similar meaning as above. Accordingly, adequate internal standard compounds must be added into other fractions except the nonamidate fraction in the case of ββ and tauro-conjugate fraction in the case of Tauro-αα on half way just after group separation in systematic analysis.

Internal standard compounds for the determination of group separated bile acids

Yamaga and coworkers (1987) have chemically synthesized four internal standard compounds exactly transferred into each conjugate fraction by group separation. They are four different compounds using 7,12-dihydroxy-5β-cholanoic acid isomers; 7β,12β-dihydroxy-5β-cholanoic acid (ββ) for the nonamidate fraction, glyco-7α,12α-dihydroxy-5β-cholanoic acid (Glyco-αα) for the glycine-conjugate fraction, tauro-7β,12β-dihydroxy-5β-cholanoic acid (Tauro-ββ) for the taurine-conjugate fraction and glyco-7α, 12α-dihydroxy-5β-cholanoic acid 7-sulfate (Glyco-αα 7-sulfate) for the sulfate fraction (Fig. 7). An artificial sample composed of these four compounds is fractionated into each fraction with PHP-LH-20 gel column, and then each internal standard compound appears as a peak in each corresponding fraction coinciding with the internal standard compound by gas chromatographic analysis. However, when

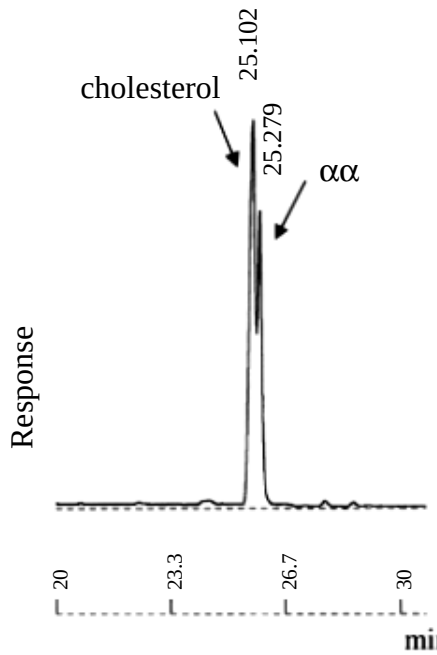

Fig. 8. Gas chromatogram of sulfate fraction from human urine with ββ, Glyco-αα, Tauro-ββ and Glyco-αα 7-sulfate. Cholesterol DMES ether and αα methyl ester DMES ether. (See Table 1 for gas chromatographic condition.)

human urine with Glyco-αα 7-sulfate added is analyzed in the same way, αα and cholesterol appear as peaks on the same gas chromatogram having almost the same retention time in sulfate fraction in some biological samples. This evidence indicates that cholesterol sulfate in urine is fractionated into the sulfate fraction when the extracts from urine using Amberlite XAD-2 or Sepak C_{18} are fractionated with PHP-LH-20 gel column, and then cholesterol is detected in sulfate fraction (Fig. 8). In fact, cholesterol sulfate is present in biological samples, especially in urine (Winter and Bongioanni, 1968: Muskiet et al., 1983). Therefore, it becomes necessary either to remove cholesterol from the solvolysate by extraction with hexane after solvolysis of the sulfate fraction or to select another sulfated candidate from 7,12-dihydroxy-5β-cholanoic acid isomers except αα. Finally, the combination of ββ, Glyco-αα, Tauro-αβ and Glyco-βα 7-sulfate (Fig. 7) is most suitable as an internal standard compound in gas chromatographic analysis after group separation.

Figure 9 shows the result of an artificial sample containing ββ, Glyco-αα, Tauro-αβ and Glyco-βα 7-sulfate as four internal standard compounds being analyzed by the systematic analysis shown in Fig. 1. Four internal standard compounds are well fractionated into individual corresponding fraction. Besides, Fig. 10 shows the results that bile acids in human urine with and without the four internal standard compounds were analyzed by the same method described above. When both profiles from the analysis of a urinary sample with and without four internal standard compounds were compared, neither peaks of bile acid nor of non-bile acid compound piling up on the peak of the internal standard compound are detected on the gas chromatogram in any fraction. Moreover, the profile from the analysis of the urinary sample with the above combination of internal standard compounds lends a helpful suggestion. It enables us to judge from the gas chromatogram whether the fractionation with PHP-LH-20 gel column is perfect or not, as the different four internal standard compounds were used. For example, the peaks of different internal standard compounds more than two are detected in one fraction when the fractionation is incomplete. Furthermore, the peak of an internal standard compound in each fraction acts as a peculiar indicator for the quantitative determination of each bile acid in its fraction and for the identification of bile acid peaks by agreement with the relative retention time of individual bile acids to the internal standard compound appearing in its fraction (Table 1).

The amount of each internal standard compound, 1–5 μg in 1 mL of serum and in 5 mL of urine, added in a biological sample should be enough in the analysis, though it depends on the concentration of bile acids in the biological sample.

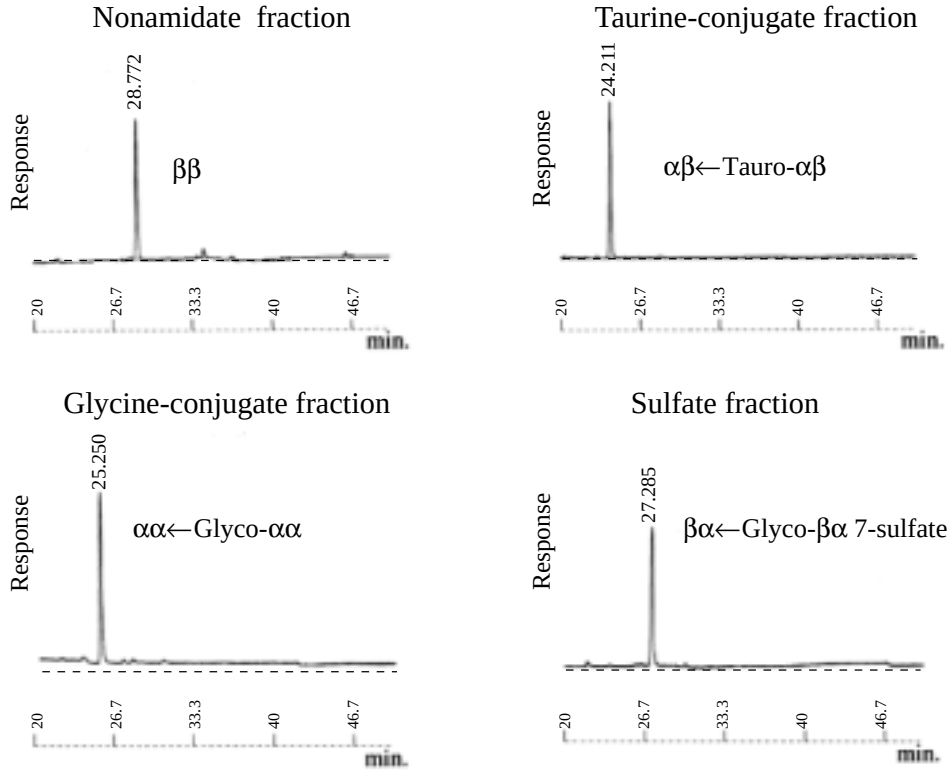

Fig. 9. Gas chromatograms of nonamidate, glycine-conjugate, taurine-conjugate and sulfate fractions from artificial sample added ββ, Glyco-αα, Tauro-αβ, and Glyco-βα 7-sulfate. (See Table 1 for gas chromatographic condition.)

Views on the future

It is inevitable that in analytical experiment a number of complicated procedures result in the loss of the compound analyzed as mentioned above. Therefore, the use of internal standard compound(s) is indispensable for the correction of the data obtained by quantitative analysis. Very few investigators have described what compound(s) have been adopted as internal standard compounds and many investigators are apt to omit the description of whether internal standard compound(s) were used or not in gas chromatographic analysis. The cause may be basically that there have been very few investigations on internal standard compounds in the past; but on the other hand there seems to be an increasing trend that acquisition of experimental data is too far reaching for consideration in the experimental method. If so, the authors themselves imply no reliability in the data they have obtained.

It is important to obtain data by appropriate experimental methods, and then, discussion is to be established based on reliable analytical data.

This article comprehensively described the necessity of suitable internal standard compounds and their importance in gas chromatography at the present time as a general review. At the present time, in the quantitative determination of bile acids using gas chromatography, either ba or bb is the most suitable internal standard compound for the determination of total bile acid amounts in the biological sample.

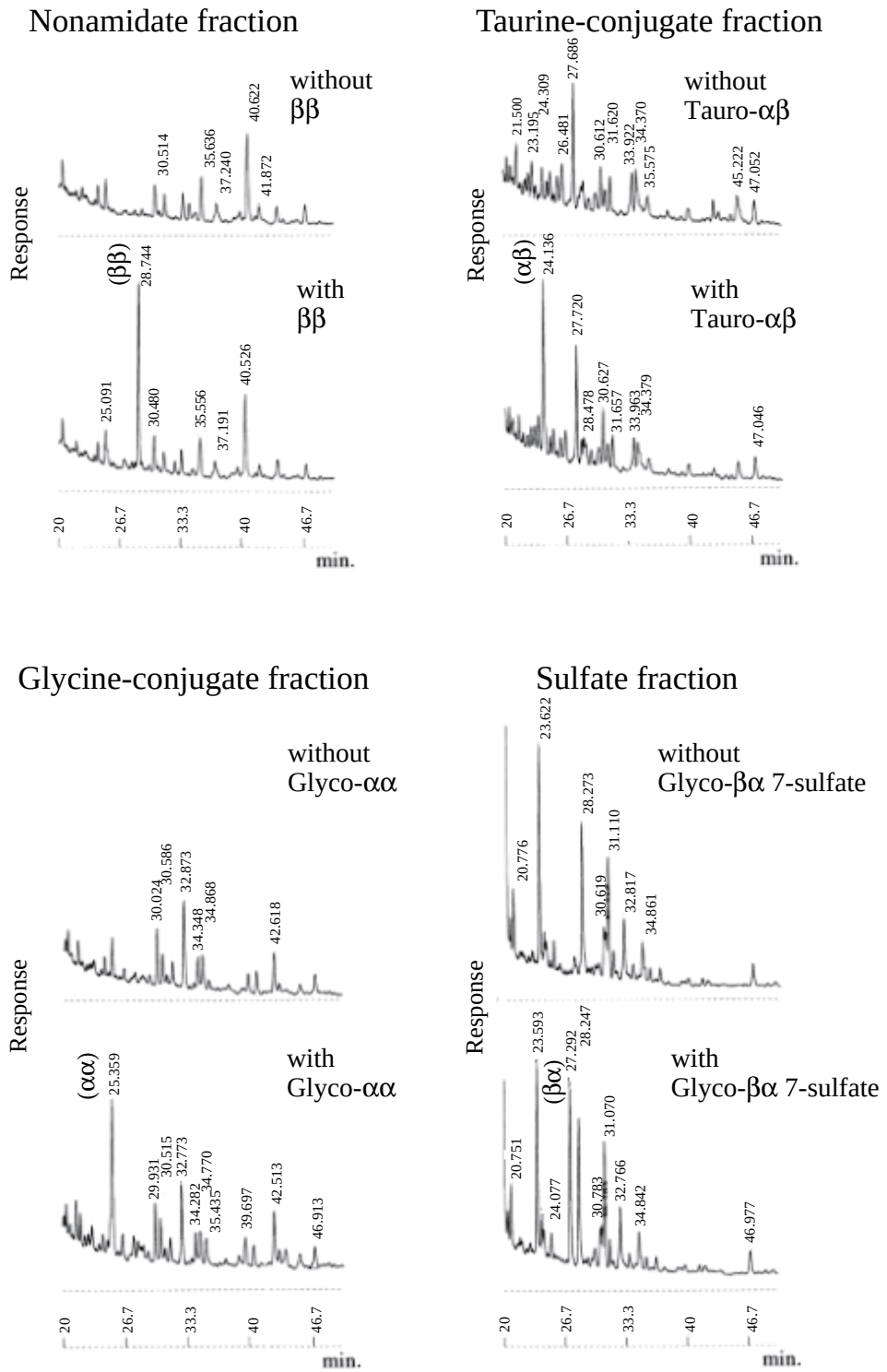

Fig. 10. Gas chromatograms of nonamidate, glycine-conjugate, taurine-conjugate and sulfate fractions from human urine with and without four internal standard compounds. (See Table 1 for gas chromatographic condition.)

On the other hand, the combination of bb, Glyco-aa, Tauro-ab and Glyco-ba 7-sulfate is the the most suitable internal standard compound for the accurate determination of bile acid amounts in each fraction after group separation of bile acids in the biological sample using the ion exchange gel column. However, it is not because all problems in the accurate determination of bile acids using gas chromatography were completely solved. When unknown bile acid(s) and new bile acid form(s) conjugating with other substance(s) are discovered, the development of new internal standard compound(s) and new systematic analyses may become necessary. It has already been requested that new systematic analyses for glucoside, glucuronide and *N*-acetylglucosaminide of bile acids should be developed without delay including new internal standard compound(s).

Acknowledgments: We sincerely thank Ms. Yumiko Uyama of the Department of Biochemistry, Tottori University Faculty of Medicine for her help in clerical works.

References

1 Ali SS, Javitt NB. Quantitative estimation of bile salts in serum. Can J Biochem 1970;48:1054–1057.
2 Alme B, Bremmelgaard A, Sjövall J, Thomassen P. Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas-liquid chromatography-mass spectrometry. J Lipid Res 1977;18:339–362.
3 Alme B, Norden A, Sjövall J. Glucuronides of unconjugated 6-hydroxylated bile acids in urine of a patient with malabsorption. Clin Chim Acta 1978;86:251–259.
4 Alme B, Sjövall J. Analysis of bile acid glucuronides in urine. Identification of 3α,6α,12α-trihydroxy-5β-cholanoic acid. J Steroid Biochem 1980;13:907–916.
5 Amuro Y, Hayashi E, Endo T, Higashino K, Kishimoto S. Unusual trihydroxylated bile acids in urine of patients with live cirrhosis. Clin Chim Acta 1983;127:61–67.
6 Aries V, Hill MJ. Degradation of steroids by intestinal bacteria I. Deconjugation of bile salts. Biochem Biophys Acta 1970;202:526–534.
7 Arimoto K, Adachi K, Yamaga N. 7β,12β-Dihydroxy-5β-cholan-24-oic acid as an internal standard for quantitative determination of bile acids by gas chromatography. Steroids 1982;39:631–641.
8 Back P. Identification and quantitative determination of urinary bile acids excreted in cholestasis. Chin Chim Acta 1937;44:199–207.
9 Back P, Spaczynski K, Gerok W. Bile salt glucuronides in urine. Hoppe-Syler's Z Physiol Chem 1974;355:749–752.
10 Back P, Walter K. Developmental patterns of bile acid metabolism as revealed by acid analysis of meconium. Gastroenterology 1980;78:671–676.
11 Beppu T, Seyama Y, Kasama T, Serizawa S, Yamakawa T. Serum bile acid profiles in cerebrotendinous xanthomatosis. Clin Chim Acta 1982; 118:167–75.
12 Campbell CG, Mcguffie C, Powell LW. The measurement of sulphated and non-sulphated bile acids in serum using gas-liquid chromatography. Clin Chim Acta 1975;63:249–262.
13 Cantafora A, Angelico M, Attili AF, Ercoli L, Capocaccia L. An improved gas-chromatographic method for the determination of sulfated and unsulfated bile acids in serum. Clin Chim Acta 1979;95:501–508.
14 Danielsson H. Mechanisms of bile acid biosynthesis. In: Nair PP, Kritchhevsky D, eds. The bile acids. vol 2. New York: Plenum Press; 1973. p. 1–32.
15 Dietschy JM. Mechanisms for the intestinal absorption of bile acids. J Lipid Res 1968;9:297–309.
16 Dowling RH. The enterohepatic circulation. Gastroenterology 1972;62:122–140.
17 Endo T, Uchida K, Amuro Y, Higashino K, Yamamuro Y. The bile acid metabolism in benign recurrent intrahepatic cholestasis. Comparative studies on the icteric and aniicteric phases of a single case. Gastroenterology 1979;76: 1002–1006.
18 Eneroth P, Gordon B, Rhyage R, Sjövall J. Characterization of trisubstituted cholanoic acids in human feces. J Lipid Res 1966;7:511–530.
19 Garbutt J, Heaton KW, Lack L, Tyor MP. Increased ratio of glicine- to taurin-conjugated bile salts in patients with ileal disorders. Gastroenterology 1969;56:711–720.
20 Ghoos Y, Rutgeerts P, Vanterappen G. Tauro-7α,12α-dihydroxy-5β-cholanoic acid as internal standard in the gas-liquid chromatographic analysis of bile acid methyl ester acetate. J Lipid Res 1983;24:1376–1379.
21 Goto J, Hasegawa M, Kato H, Nambara T. A new method for simultaneous determination of bile acids in human bile without hydrolysis. Clin Chim Acta 1978;87:141–147.
22 Heaton KW. Bile salts on health and disease. Edinburgh: Churchill Livingstone; 1972.
23 Hill MJ, Drasar BS. Degradation of bile salts by human intestinal bacteria. Gut 1968;9:22–27.
24 Imai K, Tamura Z, Mashige F, Osuge T. Gas chromatography of bile acids as their hexafluoro-

isopropyl ester trifluoroacetyl derivatives. J Chromatogr 1976;120:181–186.
25 Karlangnis G, Paumgartner G. Analysis of bile acids in serum and bile by capillary gas-liquid chromatography. J Lipid Res 1978;19:771–774.
26 Karlangnis G, Paumgartner G. Determination of bile acids in serum by capillary gas-liquid chromatography. Clin Chim Acta 1979;92:19–26.
27 Klaassen CD. Gas-liquid-chromatographic determination of bile acids in bile. Clin Chim Acta 1971;35:225–229.
28 Lack L, Weiner IM. Role of the intestine during the enterohepatic circulation of bile salts. Gastroenterology 1967;52:282–287.
29 Lepase G, Fontaine A, Roy CC. Vulnerability of bile acid to alkaline hydrolysis. J Lipid Res 1978; 19:505–509.
30 Makino I, Shinozaki K, Nakagawa S. Sulfated bile acid in urine of patients with hepatobiliary diseases. Lipids 1973;8:47–49.
31 Makita M, Wells W. Quantitative analysis of fecal bile acids by gas-liquid chromatography. Anal Biochem 1963;5:523–530.
32 Marschall HU, Green G, Egestad B, Sjövall J. Isolation of bile acid glucosides and *N*-acetylglucosaminides from human urine by ion exchange chromatography and reversed phase high performance liquid chromatography. J Chromatogr 1988; 452;459–468.
33 Marschall HU, Egestad B, Matern H, Matern S, Sjövall J. *N*-Acetylglucosaminides. A new type of bile acid conjugate in man. J Biol chem 1989; 246:12989–12993.
34 Miyazaki H, Ishibashi M, Itoh M, Nambara T. Use of new silylating agents for identification of hydroxylated steroids by gas chromatography and gas chromatography-mass spectrometry. Biomed Mass Spectrom 1977;4:23–35.
35 Muskiet AF, Jansen G, Wolther BG, Marinkovic Ilsen A, van Voorst Vader PC. Gas chromatographic determination of cholesterol sulfate in plasma and erythrocytes, for the diagnosis of recessive X-linked ichthyosis. Clin Chem 1983; 29:1404–1407.
36 Nair PP, Gordon M, Reback J. The enzymic cleavage of the carbon-nitrogen bond in 3α,7α,12α-trihydroxy-5β-cholan-24-oyl glycine. J Biol Chem 1967;242:7–11.
37 Nair PP, Garcia C. A modified gas liquid chromatographic procedure the rapid determination of bile acids in biological fluids. Anal Biochem 1969;29:164–166.
38 Neale G, Lewis B, Weaver V, Panveliiwala D. Serum bile acids in liver disease. Gut 1971;12: 145–152.
39 Palmer RH. The formation of bile acid sulfates: a new pathway of bile acid metabolism in humans. Proc Nat Acad Sci USA 1967;58:1047–1050.
40 Roda E, Aliini R, Mazzella G, Roda A, Sama C, Festi D, Barbara L. Enterohepatic circulation of bile acids after cholecytectomy. Gut 1978;19: 640–649.
41 Roovers E, Evrard E, Vanderhaeghe H. Analysis of bile acid glucuronides in urine. Identification of 3α,6α,12α-trihydroxy-5β-cholanoic acid. Clin Chim Acta 1968;19:449–457.
42 Sawada K. Studies on the bile acids in meconium. Yonago Igaku Zasshi 1981;32:53–67 (in Japanese with English abstract).
43 Shimada K, Bricknell KS, Finegold SM. Deconjugation of bile acids by intestinal bacteria: review of literature and additional studies. J Infec Dis 1969;119:73–81.
44 Stiehl A. Bile salt sulphates in cholestasis. Eur J Clin Invest 1974;4:59–63.
45 Subbiah MTR. Hyocholic acid as internal standard for quantitation of human fecal bile acids. J Lipid Res 1973;14:692–694.
46 Takikawa H, Otsuka H, Beppu T, Seyama Y, Yamakawa T. Quantitative determination of bile acid glucuronides in serum by mass fragmentography. J Biochem 1982;92:985–998.
47 Takikawa H, Beppu T, Seyama Y, Wada T. Quantitative determination of nonsulfated bile acids in the serum of patients with hepatobiliary diseases by mass fragmentography. Gastroenterol Jpn 1983a;18:246–251.
48 Takikawa H, Otsuka H, Beppu T, Seyama Y, Yamakawa T. Serum concentrations of bile acid glucuronides in hepatobiliary diseases. Digestion 1983b;27:189–195.
49 Tyor M, Garbutt JT, Lack L. Metabolism and transport of bile salts in the intestine. Am J Med 1971;51:614–625.
50 van Berge-Henegouwen GP, Ruben A, Brandt KH. Quantitative analysis of bile acids in serum and bile, using gas liquid chromatography. Clin Chim Acta 1974;54:249–261.
51 van Berge-Henegouwen GP, Hofmann AF, Ruben A. A simple batch adsorption procedure for the isolation of sulfated and non-sulfated bile acids from serum. Clin Chim Acta 1976;73:469–474.
52 van Berge-Henegouwen GP, Allan RN, Hofmann AF, Yu PYS. A falile hydrolysis-solvolysis procedure for conjugated bile acid sulfate. J Lipid Res 1977;18:118–122.
53 Vanden Heuvel WJA, Sweeley CC, Horning EC. Microanalytical separations by gas chromatography in the sex hormone and bile acid series. Biochem Biophys Res Commun 1960;3:33–36.
54 Winter JS, Bongiovanni AM. Identification of cholesterol sulfate in urine and plasma of normal and hypercholesterolemic subjects. J Clin Endocrinol Metab 1968;28:927–930.
55 Yamaga N, Adachi K, Arimoto K, Miyake S, Shimizu K. Reexamination on quantitative determination of urinary bile acids by gas chromatography. Igaku No Ayumi 1983;125:87–89 (in Japa-

nese).
56 Yamaga N, Adachi K, Kohara H, Shimizu K. Internal standards for quantitative gas chromatography of individual bile acids after group separation of bile acids in urine. J Chromatogr Biomed Appl 1987;422:25–32.
57 Yamaga N, Kohara H. Conjugation types of 7β-hydroxylated bile acids detected in healthy human urine. J Biochem 1994a;116:1123–1126.
58 Yamaga N, Kohara H, Sagayama A. Uncommon urinary excretion pattern of bile acids in healthy human: identification and determination of 7β-hydroxylated bile acid series. Yonago Acta Med 1994b;37:17–23.
59 Yamaga N, Ikebuchi J, Kohara H, Ogura Y, Yamada K. Analysis of bile acids in urine specimens from healthy humans; determination of several bile acids with β-hydroxyl and carbonyl groups. J Biochem 1996;119:725–730.
60 Yamaga N, Nagano Y, Yamada K. An examination of alkaline hydrolyzing condition of conjugated bile acids with carbonyl groups. Yonago Acta Med 1997;40:73–77.

Received April 2, 2001; accepted May 10, 2001

Corresponding author: Nobuo Yamaga