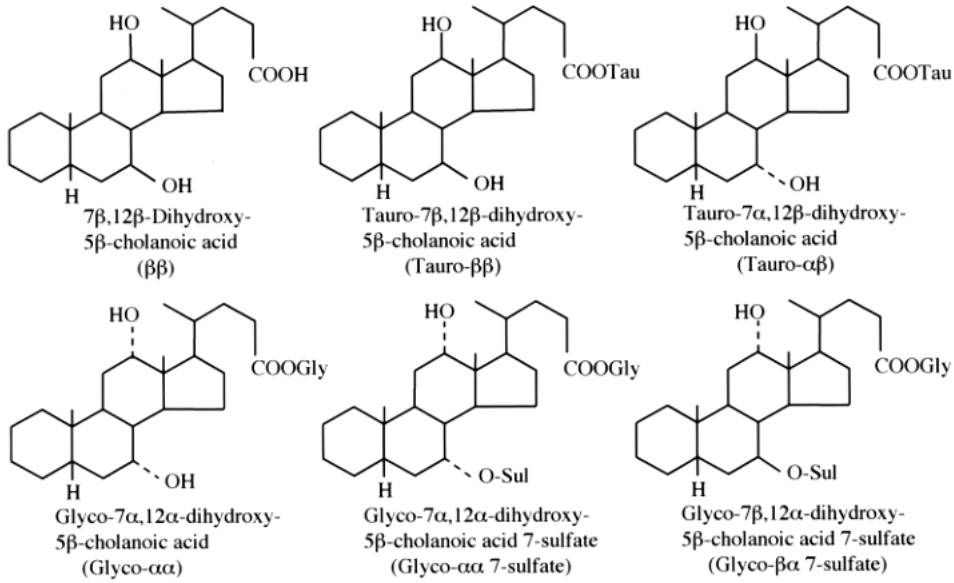

Fig. 7. Internal standard compounds chemically synthesized for gas chromatographic analysis after group separation.

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

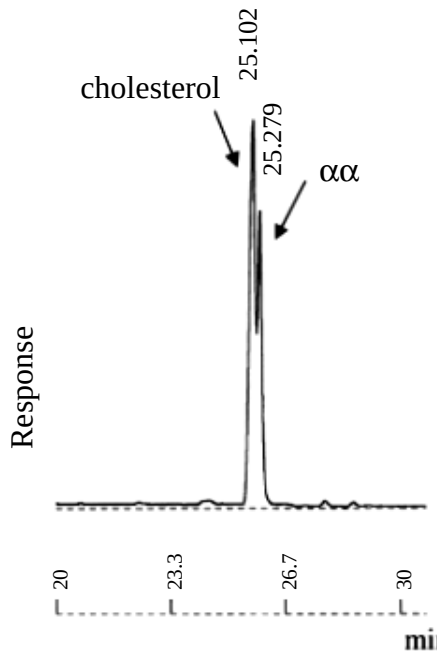

Fig. 8. Gas chromatogram of sulfate fraction from human urine with ββ, Glyco-αα, Tauro-ββ and Glyco-αα 7-sulfate. Cholesterol DMES ether and αα methyl ester DMES ether. (See Table 1 for gas chromatographic condition.)

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.

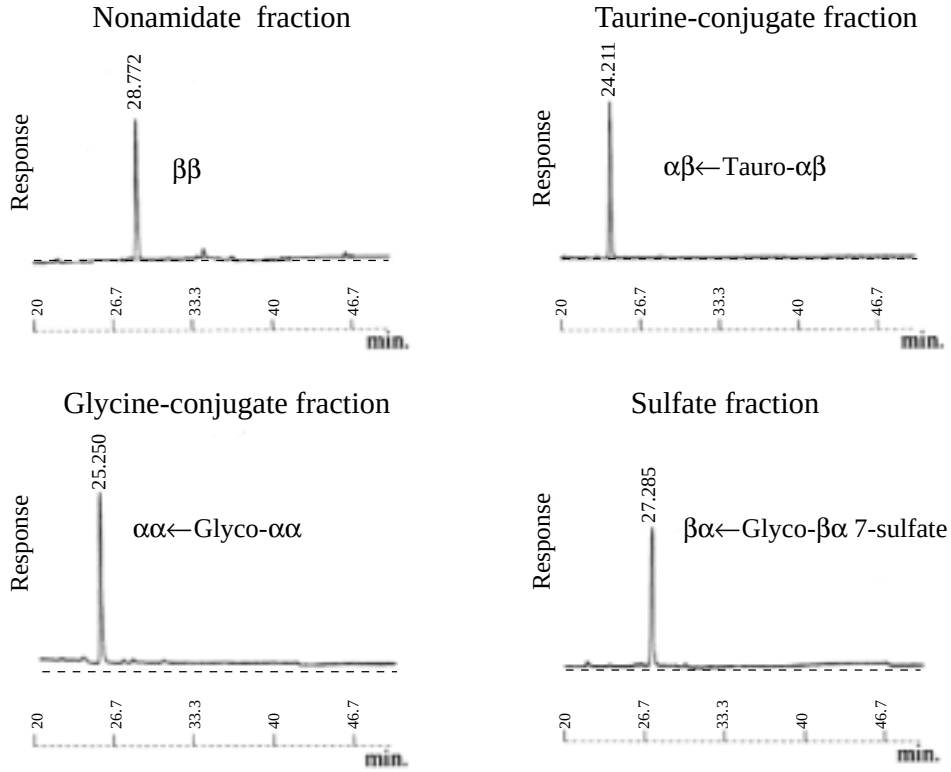

Fig. 9. Gas chromatograms of nonamidate, glycine-conjugate, taurine-conjugate and sulfate fractions from artificial sample added ββ, Glyco-αα, Tauro-αβ, and Glyco-βα 7-sulfate. (See Table 1 for gas chromatographic condition.)

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.

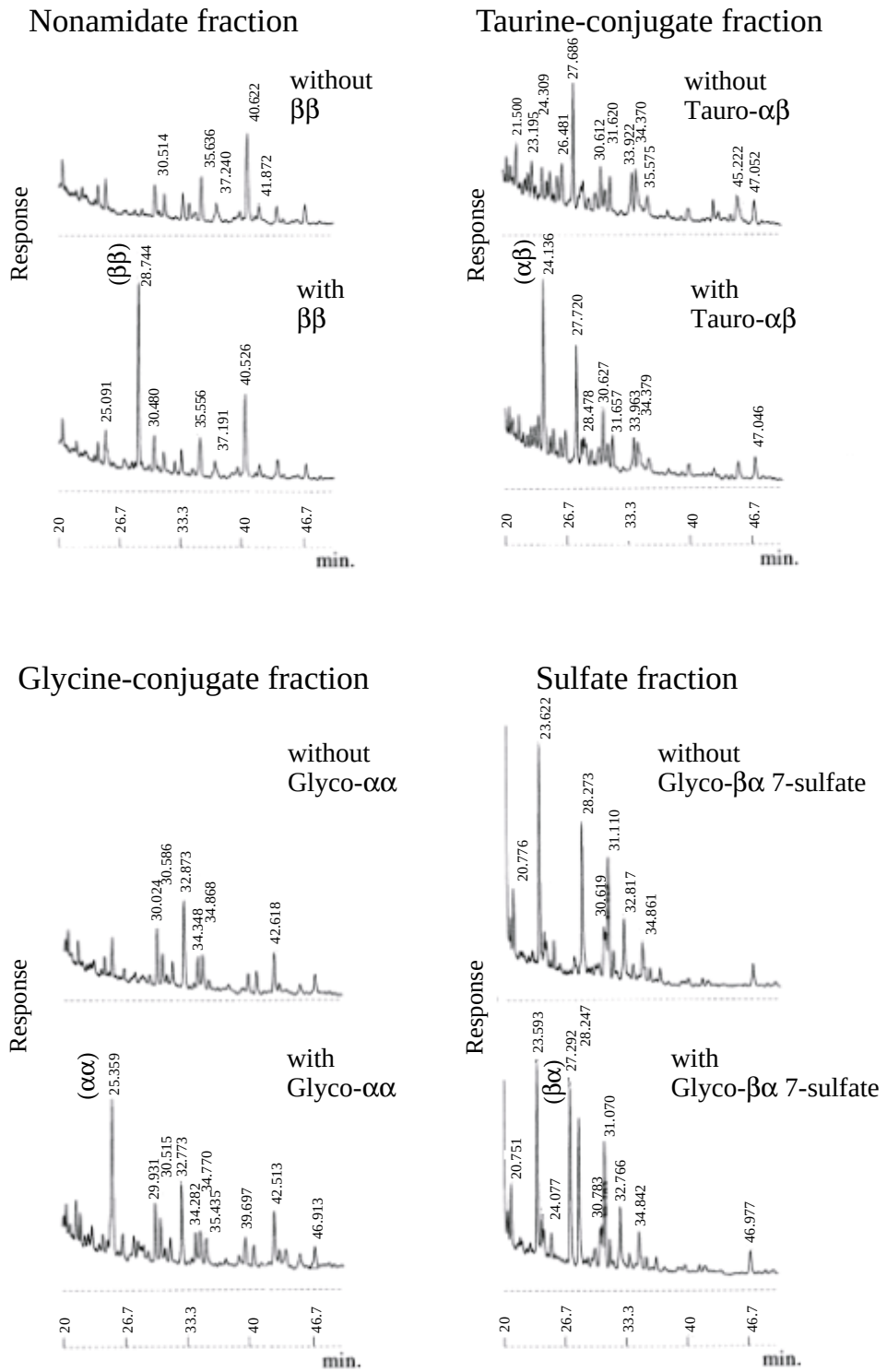

Fig. 10. Gas chromatograms of nonamidate, glycine-conjugate, taurine-conjugate and sulfate fractions from human urine with and without four internal standard compounds. (See Table 1 for gas chromatographic condition.)

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).

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