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Utility of 19-Hydroxycholesterol as an Internal Standard Compound for the Quantitative Determination of Sterols Using Capillary Gas Chromatograph

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Gas chromatography is able to detect many kinds of sterols simultaneously. In order to identify and estimate accurately many kinds of sterols in biological samples by gas chromatography, it is indispensable to add an internal standard compound into the analytical samples prior to the analysis. However, there is no report on a suitable internal standard compound for the determination of sterols. In this study, 19-hydroxycholesterol was proved to be a suitable internal standard compound for simultaneous and quantitative determination of several sterols in various analytical samples by capillary gas chromatography, including the quantitative determination of β-cholestanol in the human plasma. Moreover, the excellent condition of gas chromatographic analysis for quantitative determination of several sterols in various analytical samples was found.

Key words: capillary gas chromatography; 19-hydroxycholesterol; internal standard compound; quantitative determination; sterols

There are many reports on the action of intestinal flora on several compounds such as sterols and bile acids, in both *in vivo* and *in vitro* experiments (Björkhem and Gustafsson, 1971; Eyssen et al., 1973; Uchida et al., 1977; Macdonald et al.,1983). However, the kinds of intestinal flora concerned with the metabolism of sterols in their host, and so on, have not yet been sufficiently solved. In order to investigate a number of unsolved problems, the quantitative determination of various sterols in feeds, feces, blood and intestinal contents of experimental animals needs to be carried out precisely in various experiments using intestinal flora. Moreover, the concentration of 5α-cholestan- 3β-ol (β-cholestanol), a kind of sterol, in the serum of patients with cerebrotendinous xanthomatosis (CTX), a genetic disorder of the cholesterol metabolism, increases abnormally (Salen, 1971; Seyama et al., 1976). Therefore, analysis of β-cholestanol in the serum of patients with CTX is useful from the viewpoint of biochemical diagnosis (Seyama, 1999).

For the simultaneous determination of various sterols and bile acids, gas chromatography, gas chromatography mass spectrometry (GC-MS) and/or high performance liquid chromatography (HPLC) are available means. In comparison with the above mentioned instruments, gas chromatography is a better means for qualitative and quantitative determination of sterols and bile acids, because a gas chromatograph is very sensitive, is in widespread use in many laboratories and is simple in its operation.

Determination of sterols by gas chromatography has been carried out by procedures such as extraction, hydrolysis, derivatization and injection into the gas chromatograph. It is impossible to obtain 100% recovery of the sterols in the sample. Moreover, recovery may differ from

Abbreviations: β-cholestanol, 5α-cholestan-3β-ol; CTX, cerebrotendinous xanthomatosis; GC-MS, gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; TMS, trimethylsilyl

assay to assay. Therefore, it is indispensable to add an internal standard compound into the analytical samples for correcting the loss of sterols during the analysis.

We have established a systematic method for gas chromatographic analysis of bile acids in biological samples using some internal standard compounds (Yamaga et al., 2001). However, a suitable internal standard compound for gas chromatographic analysis of sterols has not yet been reported.

This paper describes the internal standard compound and the excellent conditions of gas chromatography for the qualitative and quantitative determination of several sterols simultaneously using a capillary gas chromatograph.

Experimental Procedures

Materials

Cholesterol, 19-hydroxycholesterol (an internal standard compound), lanosterol, stigmastanol and 5β-cholestan-3α-ol were purchased from Sigma, St. Louis, MO. Coprostanol was purchased from Gasukuro Kogyo Inc., Tokyo, Japan. β-Cholestanol and *N*-trimethylsilylimidazole were purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan. Campesterol, β-sitosterol and stigmasterol were purchased from Tama Biochemical Co., LTD, Tokyo, Japan. Other chemicals were of analytical reagent grade, and all organic solvents were distilled before use.

Extraction of sterols from analytical samples

Biological samples (20–50 mg for feces and feed, and 10–100 μL for human plasma) with and without 5–10 μg of 19-hydroxycholesterol as an internal standard compound were hydrolyzed with 1 mL of 1 M ethanolic sodium hydroxide for 30 min at 80˚C. After addition of 0.5 mL of distilled water into the hydrolysate, nonsaponifiable materials (sterols) were extracted with *n*-hexane, and then the solvent was evaporated to dryness.

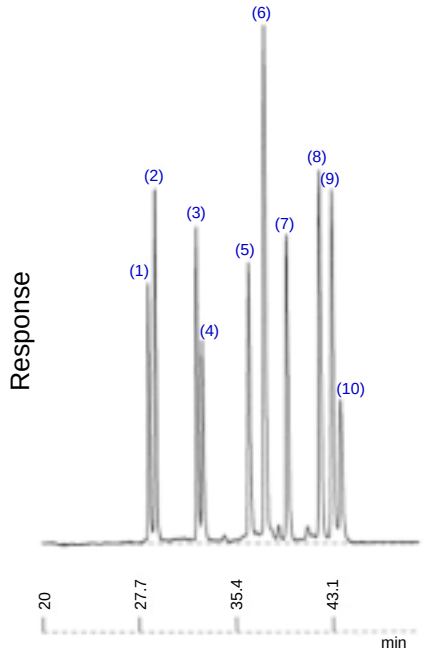

Fig. 1. Capillary gas chromatogram of several authentic sterols. The peaks of sterols are as follows:
(1) Coprostanol,
(2) 5β-Cholestan-3α-ol,
(3) Cholesterol,
(4) β-Cholestanol,
(5) 19-Hydroxycholesterol (internal standard compound),
(6) Campesterol,
(7) Stigmasterol,
(8) Lanosterol,
(9) β-Sitosterol,
(10) Stigmastanol.
See the text on derivatization and gas chromatography of sterols.

Derivatization

Extracted sterols were converted into trimethylsilyl (TMS) derivatives with *N*-trimethylsilylimidazole at room temperature overnight or at 80˚C for 30 min. The sterol-TMS derivatives were extracted with *n*-hexane and evaporated to dryness after washing with distilled water. An aliquot of the sterol-TMS derivatives dissolved in *n*-hexane was injected into the gas chromatograph.

Gas chromatography

The gas chromatograph (Model GC-14A, Shimadzu, Kyoto, Japan) was equipped with HiCap CBP-1 capillary column (25 m × 0.25 mm I.D., Shimadzu) and with a solventless injector (Shimadzu). The capillary column was programmed at 240˚C for 20 min, from 240˚C to 260˚C at a rate of 2˚C/min, and then at 260˚C for 20 min. Helium was used as the carrier gas. Capillary gas chromatograms and analytical data of the sterols were obtained using a computerized data system (Model C-R4A Chromatopac, Shimadzu) connected with the gas chromatograph.

Qualitative and quantitative determination of sterols by gas chromatography

Identification of peaks on the gas chromatogram from the specimens was carried out in agreement with the found and calculated relative retention time between them and the authentic sterols to the internal standard compound (the retention time of 19-hydroxycholesterol = 1.00). The relative factors for quantitative analysis in the gas chromatography between the peak area ratio and the weight ratio of each sterol to 19-hydroxycholesterol were estimated automatically by injecting a known amount of authentic sterols and the internal standard compound into the gas chromatograph.

Results and Discussion

In Fig.1, many kinds of authentic sterols, which are found in specimens of animals and plants, have been detected separately as peaks of sterol-TMS derivatives under the excellent conditions of capillary gas chromatography described in experimental procedures. Figure 2 shows capillary gas chromatograms of nonsaponifiable sterol-TMS derivatives extracted from feed and feces of mice and rats as well as plasma of humans under the same conditions of capillary gas chromatography for analyzing the authentic sterols. These results suggest that it is possible to detect separately many kinds of sterols from

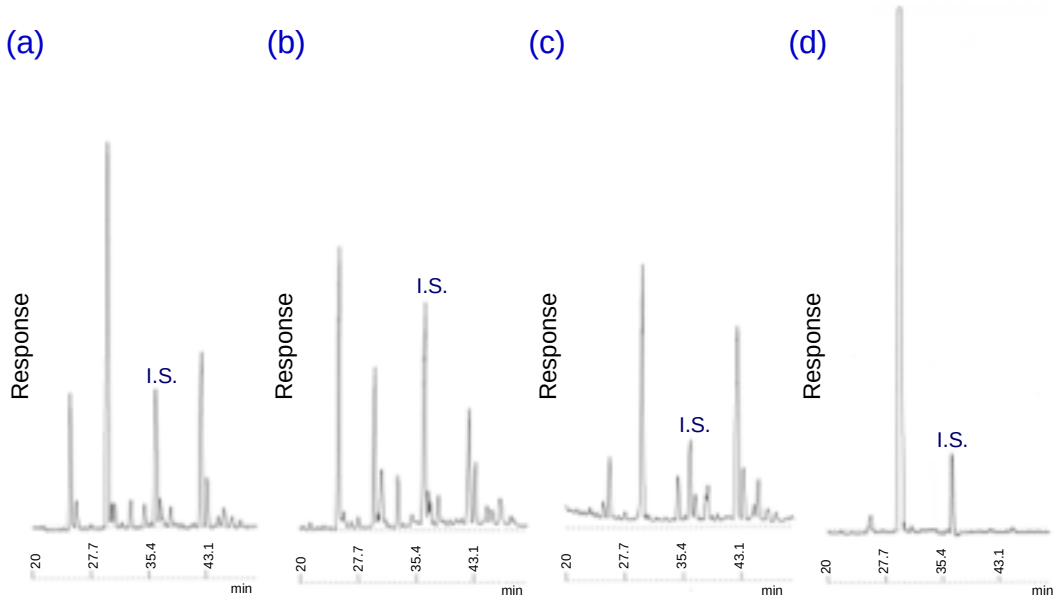

Fig. 2. Capillary gas chromatograms of nonsaponifiable fractions with 19-hydroxycholesterol. (**a**) mouse feces, (**b**) rat feces, (**c**) mouse and rat feed and (**d**) human plasma. I.S. indicates the peak of 19-hydroxycholesterol as internal standard compound. See the text on gas chromatography.

Table 1. Retention times and relative retention times of internal standard compounds and several authentic sterols

	Sterols	Retention time (min)	Relative retention time
(1)	Coprostanol	28.547	0.782
(2)	5β-Cholestan-3α-ol	29.083	0.797
(3)	Cholesterol	32.367	0.887
(4)	β-Cholestanol	32.795	0.898
(5)	19-Hydroxycholesterol	36.505	1.000
(6)	Campesterol	37.768	1.035
(7)	Stigmasterol	39.517	1.083
(8)	Lanosterol	42.199	1.156
(9)	β-Sitosterol	43.197	1.183
(10)	Stigmastanol	43.834	1.201

Gas chromatographic condition is described in experimental procedures.
Capillary column is HiCap CBP-1 capillary column (25 m × 0.25 mm I.D.).
Sterols are derivatized to TMS-ether.
Relative retention times are expressed relative to 19-hydroxycholesterol.

analytical samples as peaks on a capillary gas chromatogram, as shown in Fig. 2, under the same experimental conditions as gas chromatography.

However, in order to accurately identify and estimate these detectable peaks of sterols from samples on a gas chromatogram, it is indispensable to add an internal standard compound into the analytical samples before the extraction of the sterols.

The fundamental requirements for selecting a suitable internal standard compound for the determination of sterols by gas chromatography are as follows —

i) an internal standard compound must be added to the analytical samples before the extraction of sterols, and then the peak of an internal standard compound must appear together with peaks of sterol derivatives in the analytical samples on the same gas chromatogram;
ii) the internal standard compound must be stable. During the analysis, it must not decompose into other compounds;
iii) the peak of the internal standard compound must not pile up on the peaks of sterol derivatives and other mixed compounds in the biological sample;
iv) the retention time of the internal standard compound must not be largely distant from those of sterols which are being assayed; and
v) a linear relationship should be maintained between the measured peak height or area and the weight of the sterol to the internal standard compound.

Previously, among 5α-cholestane (Setoguchi et al., 1974; Ishikawa et al., 1976), 5β-cholestan-3β-ol (Kasama et al., 1987), 5β-cholestan-3α-ol (Byun et al.,1988) and 5α-cholestan-3α-ol (Serizawa et al., 1981), each one has been used as an internal standard compound for only quantitative determination of β-cholestanol in human serum by gas chromatography, GC-MS and/or HPLC for the biochemical diagnosis of CTX patients. But these internal standard compounds are homologous in structure and appear earlier than many other kinds of sterols such as campesterol, stigmasterol and lanosterol, etc., in gas chromatographic analysis which strongly differs from the above mentioned 1st and 4th criteria for internal standard compounds.

During metabolic oxidation of cholesterol, 19-hydroxycholesterol (a particular oxysterol) is formed (Verhagen et al., 1996). So far as we know, there is no report on the detection of 19-hydroxycholesterol in biological samples. We

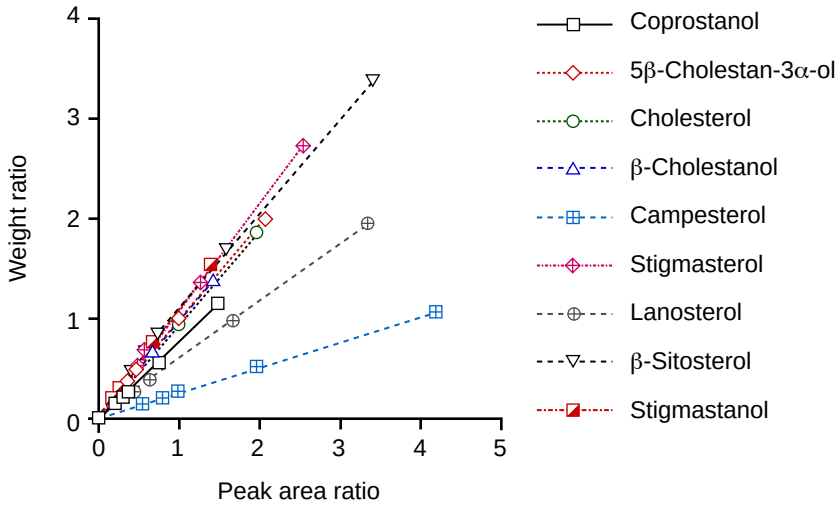

Fig. 3. Calibration curves of several sterols to 19-hydroxycholesterol for quantitative determination.

$$\text{Weight ratio} = \frac{\text{Weight of each sterol}}{\text{Weight of 19-hydroxycholesterol}} \qquad \text{Area ratio} = \frac{\text{Peak area of each sterol}}{\text{Peak area of 19-hydroxycholesterol}}$$

were interested in investigating this particular sterol, 19-hydroxycholesterol, as an internal standard compound for the quantitative determination of sterols using a capillary gas chromatograph. The peak of 19-hydroxycholesterol was in the middle domain between those of the 1st and the last sterols among many sterol peaks on the gas chromatogram (Fig. 1), and the retention time of 19-hydroxycholesterol was also different from those of many authentic sterols (Table 1). Besides, there is no peak which overlaps at the position of the 19-hydroxycholesterol peak in all analytical samples. Calibration curves for the quantitative determination of several sterols maintained a linear relationship going through the origin between the weight ratio and the peak area ratio of each sterol to 19-hydroxycholesterol in the range of the measurement (Fig. 3). From our present experimental findings we can conclude that 19-hydroxycholesterol fullfills all the requirements of an internal standard compound. Using both HiCap CBP-1 capillary column made with methyl silicone and major authentic sterols found in specimens of animals and plants, excellent condition for gas chromatography was established as described in experimental procedures.

The amount of β-cholestanol in human serum is usually determined for the biochemical diagnosis of CTX. The level of β-cholestanol is less than 0.2 mg/100 mL on an average in healthy subjects, and in the serum of CTX patients the level of β-cholestanol increases about 20–30 times compared with that of healthy humans (Seyama, 1999). The cholesterol level in the serum of CTX patients is usually within the normal range (130–260 mg/mL) (Salen, 1971). As we have had no chance to collect blood samples from CTX patients, different amounts (1.1, 2.2, 3.1, 5.2, 6.1 and 8.6 μg) of authentic β-cholestanol were added into 100 μL of healthy subject plasma, (cholesterol concentration, 219 mg/100 mL in plasma) to prepare artificial plasma samples of CTX patients, and then these samples were analyzed. Although the retention times of β-cholestanol and cholesterol from artificial plasma samples were adjacent in the gas chromatogram, the larger cholesterol peak

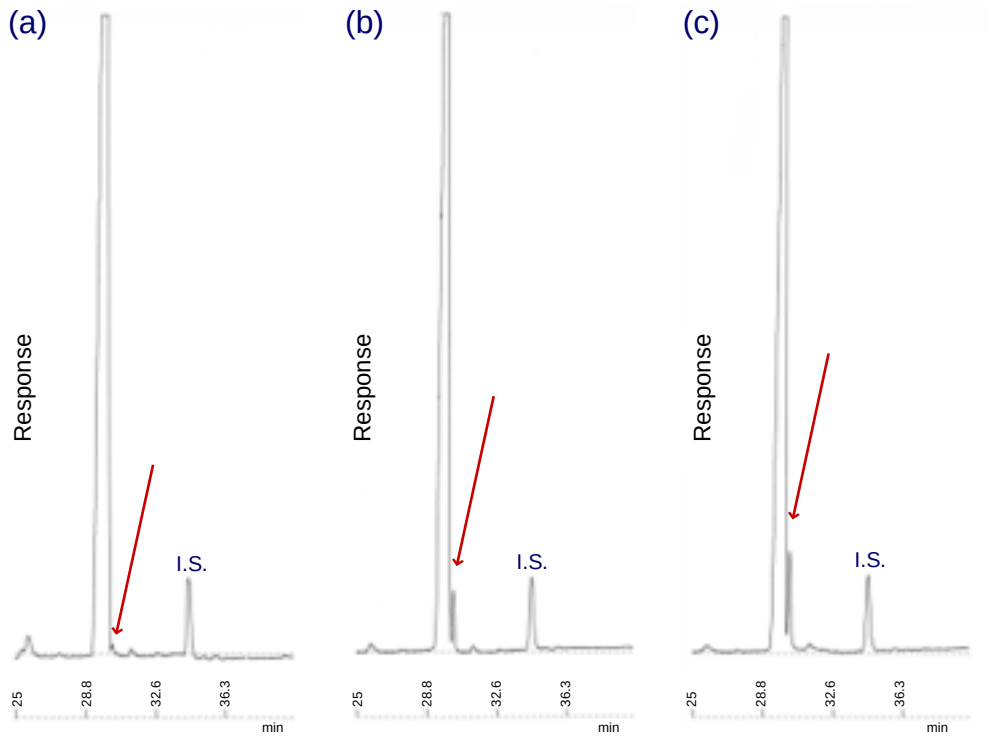

Fig. 4. Gas chromatograms of nonsaponifiable fractions from healthy subject plasma and artificial plasma samples. (**a**) Healthy subject plasma (100 μL), (**b**) Artificial plasma sample (100 μL) with 3.1 μg of authentic β-cholestanol and (**c**) Artificial plasma sample (100 μL) with 6.1 μg of authentic β-cholestanol. The arrow point indicates the peak of β-cholestanol. I.S. means the peak of 19-hydroxycholesterol. See the text on gas chromatography.

hardly ever overlapped onto the smaller β-cholestanol one in the same gas chromatogram (Fig. 4). Besides, the amount of β-cholestanol in each artificial plasma sample of CTX patients was as expected (Fig. 5). Cholesterol amount in the serum of healthy subjects is 130–260 mg/100 mL on the average. Cholesterol level in the serum of CTX patients is usually within this normal range (Salen, 1971). But in these analytical conditions it is uncertain whether or not the cholesterol peak may pile up on that of β-cholestanol if the cholesterol level increases above the normal level. For this reason, the relation between the amount of cholesterol and β-cholestanol was investigated. A sharp peak of β-cholestanol appeared as a discernible shoulder at the end side of the larger cholesterol peak. When the level of cholesterol was above 300 mg/100 mL, the level of β-cholestanol was determined differently. In these cases, the level of β-cholestanol could not be determined accurately, but it could be judged that the level of measured β-cholestanol in artificial samples was more than that of β-cholestanol in the plasma of normal subjects.

Due to the large difference between the level of cholesterol and β-cholestanol in the human plasma of both normal individuals and patients with CTX and CTX-like disease, 19-hydroxycholesterol should not be considered as an internal standard compound only in the case of the simultaneous determination of cholesterol and β-cholestanol. So, it is better to be determined the cholesterol level by the enzymatic method.

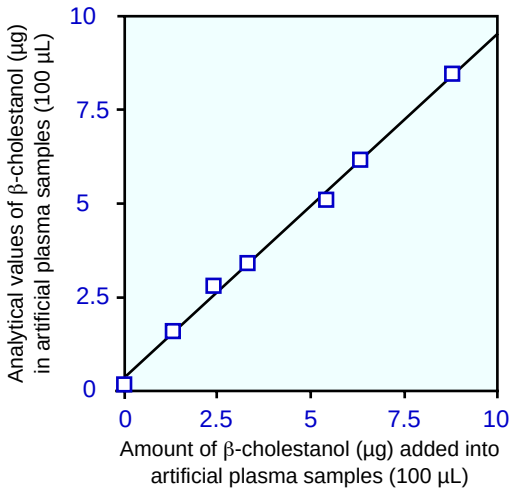

Fig. 5. Recovery experiments of β-cholestanol (μg) in artificial plasma samples (100 μL) prepared from healthy subject plasma by adding different amount of authentic β-cholestanol.

As a result, we conclude from our experimental findings that 19-hydroxycholesterol becomes an appropriate internal standard compound for the simultaneous determination of several sterols, including β-cholestanol in the serum of CTX patients.

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