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Application News

No. M283

Gas Chromatography Mass Spectrometry

Analysis of Bile Acid by GC-MS

With active research on intestinal flora, analysis of the components produced by intestinal flora, which include bile acids, has attracted attention.

"Bile acid" is a generic term for compounds having a cholanic acid skeleton, which is a steroid derivative. In general, LC-MS/MS is frequently used in analyses of bile acid, but because many of the individual compounds have similar molecular structures, GC-MS is also used in many cases when more reliable separation is required. In order to secure volatility in GC-MS analyses of bile acid, derivatization of hydrophilic side chains such as the carboxyl group and hydroxy group is necessary.

This article introduces an example of quantitative analysis of bile acid by methylation of the carboxyl group and trimethylsilylation (TMS) of the hydroxy group. In this example, a dynamic range of 100 times or more and good linearity with a coefficient of determination $R^2 \geq 0.995$ were confirmed in all of 22 bile acid compounds.

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Bile Acid Structure and Derivatization

Bile acid compounds cannot be analyzed by GC-MS without pretreatment due to their comparatively large molecular weight and their low volatility owing to the presence of hydrophilic residues. Derivatization of these hydrophilic residues is necessary for GC-MS analysis. The important residues in bile acids are the hydroxy group and carboxyl group, and at least these groups must be derivatized by substituting different residues.

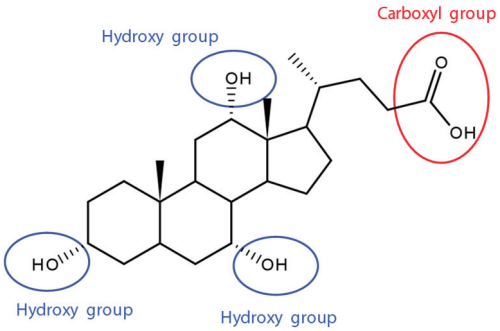

Fig. 1 Structure of Cholic Acid (Bile Acid Compound) Where Three Hydroxy Groups and One Carboxyl Group Exist in One Molecule.

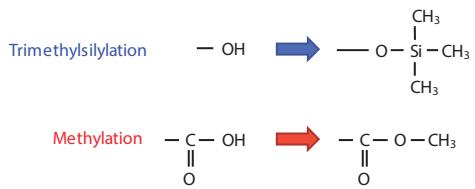

Fig. 2 Schematic Diagram of Derivatization with Trimethylsilylation of Hydroxy Groups, Methylation of Carboxyl Group.

Derivatization of both hydrophilic residues, that is, the hydroxy groups and the carboxyl group, is generally possible by trimethylsilylation, but the derivative compounds are unstable in bile acids. Therefore, in this paper, we adopted a derivatization method consisting of methylation of the carboxyl group, followed by trimethylsilylation of the hydroxy groups. Fig. 3 shows the details of the pretreatment protocol.

Take a fixed quantity of a bile acid standard solution of known concentration with a micropipette, and vaporize the solvent completely with a centrifugal concentrator.

↓

[Methylation]

After sufficiently mixing 20 μL of methanol, 80 μL of benzene, 50 μL of TMS diazomethane (approx. 10% hexane solution), vaporize completely by N_2 purge in a draft.

↓

[Trimethylsilylation]

Add 25 μL of pyridine and 5 μL of trimethylchlorosilane (TMCS) to 50 μL of N-trimethylsilylimidazole (TMSI), and treat at 60 °C for 10 min.

↓

Introduce into a vial and inject into the GC-MS.

Fig. 3 Derivatization Protocol for Bile Acid

Measurement of Derivatized Bile Acid

The derivatized bile acid described above was analyzed with a GC-MS. The analysis conditions are shown below.

Table 1 Measurement Conditions

GC-MS	: GCMS-QP™2020 NX
Column	: DB-5MS (30 m × 0.25 mm, 0.25 μm)
GC	
Injection mode	: Splitless
Vaporizing chamber temp.	: 310 °C
Column oven temp.	: 230 °C (2 min) → (8 °C -/min) → 310 °C (5 min)
Control mode	: Linear velocity (45.3 cm/s)
Purge flow rate	: 3.0 mL/min
MS	
Measurement mode	: Scan or SIM (Scan range: *m/z* 35-700)
Ion source temp.	: 200 °C
Interface temp.	: 280 °C
Event time	: 0.3 s

Confirmation of Quantitation Accuracy

The above-mentioned pretreatment to reference standards of 8 μg each of 22 types of bile acid was carried out, and a GC-MS analysis was conducted in the scan mode. Fig. 4 shows the cholic acid peaks.

After confirming the retention time and mass spectra of each bile acid, the same treatment as described above was carried out for 0.1, 0.5, 1.0, 5.0, 10, 50, 100 and 500 ng reference standard mixtures of the 22 bile acids. SIM analysis was carried out, and a calibration curve was prepared by the internal standard method using Cholic acid-d5 as the internal standard. Fig. 5 shows the calibration curve. The dynamic range and linearity of each bile acid were as shown in Table 2. For compounds from which multiple peaks were detected from one standard, the respective peaks are indicated by the numbers in parentheses. Table 2 shows the reproducibility at the minimum concentration in each dynamic range for n=3.

Verification of Sample Stability

The above-mentioned pretreatment to reference standards of 8 μg each of the 22 bile acids was carried out, and a second analysis was conducted 20 h after the initial analysis. The relative intensity of the peaks at that time (the ratio to the intensity of the peaks of a first analysis) is shown in the column "Relative peak area after 20 h" in Table 2. All of the bile acid peaks showed satisfactory reproducibility of 70% to 120%. When a similar analysis was also carried out after 38 h, the peak areas of several bile acids showed a decreasing tendency, but with the exception of those samples, stable measurement of the majority of bile acids was possible.

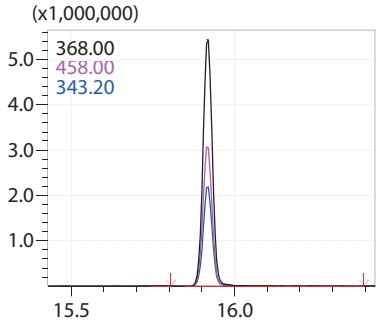

Fig. 4 Mass Chromatogram of 8 μg Sample of Cholic Acid

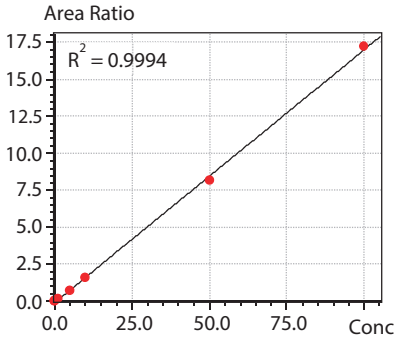

Fig. 5 Calibration Curve of Cholic Acid

Table 2 Measurement Results of 22 Bile Acids

Bile acid	CAS No.	Quantitative analysis *m/z*	Validation *m/z*	Dynamic range (ng)	Linearity (R^2)	Reproducibility of n=3 (%RSD)	Relative peak area after 20 h	Relative peak area after 38 h
Cholic acid	81-25-4	368.0	458.0	0.1 – 100	0.999	6.65	0.984	1.011
Chenodeoxycholic acid	474-25-9	370.0	355.0	0.5 – 100	0.999	4.71	0.949	0.920
Ursodeoxycholic acid	128-13-2	460.0	370.0	0.5 - 500	0.999	2.24	1.206	0.924
Deoxycholic acid	302-95-4	255.0	370.0	0.5 - 100	0.999	6.11	0.980	0.714
Lithocholic acid	434-13-9	372.0	357.0	1 - 500	0.999	7.66	0.991	0.750
Hyocholic acid	547-75-1	458.0	369.0	0.1 - 100	0.999	5.51	1.036	1.035
3-oxocholic acid_(1)	2304-89-4	546.0	341.0	1 - 500	0.999	7.95	0.848	0.575
3-oxocholic acid_(2)	2304-89-4	269.0	384.0	0.5 - 500	0.999	2.36	0.914	0.714
7-oxodeoxycholic acid	911-40-0	341.0	359.0	1 - 500	0.999	5.51	1.029	1.053
7-oxolithocholic acid	4651-67-6	293.2	353.25	5 - 500	0.998	8.89	0.817	0.920
Ursocholic acid	2955-27-3	253.0	343.0	0.5 - 100	0.999	5.52	1.019	0.840
3-oxodeoxycholic acid_(1)	4185-01-7	548.0	343.0	5 - 500	0.999	4.27	0.959	0.645
3-oxodeoxycholic acid_(2)	4185-01-7	548.0	343.0	10 - 500	0.999	12.39	0.930	0.633
3-oxodeoxycholic acid_(3)	4185-01-7	271.0	253.0	5 – 500	0.999	8.20	1.043	0.867
3-oxolithocholic acid_(1)	1553-56-6	460.0	431.0	5 - 500	0.999	5.43	0.959	0.924
3-oxolithocholic acid_(2)	1553-56-6	460.0	431.0	10 - 500	0.997	7.97	0.720	0.481
3-oxolithocholic acid_(3)	1553-56-6	273.0	318.0	5 – 500	0.999	3.08	0.885	0.809
Hyodeoxycholic acid	83-49-8	370.0	255.0	0.5 - 500	0.999	6.44	1.139	0.860
3β-hydroxy-5-cholenoic acid	5255-17-4	331.0	370.0	5 - 500	0.999	3.95	0.993	0.927
α-muricholic acid	2393-58-0	458.0	443.0	5 - 500	0.999	5.09	1.027	0.834
β-muricholic acid	2393-59-1	285.0	458.0	0.5 - 500	0.998	7.21	0.866	1.033
ω-muricholic acid	6830-03-1	285.0	369.0	0.5 - 500	0.999	4.49	0.842	0.882
Phoenodeoxycholic acid	566-17-6	255.0	370.0	0.5 - 500	0.999	5.48	0.977	0.962
Isolithocholic acid	1534-35-6	372.0	215.0	1 - 500	0.999	2.38	1.051	0.920
Alloisolthocholic acid	2276-93-9	372.0	447.0	5 - 500	0.999	4.35	1.080	1.033
12-oxolithocholic acid	5130-29-0	476.0	386.0	5 - 500	0.998	8.51	0.769	0.937
isodeoxycholic acid	570-63-8	255.0	345.0	0.5 - 500	0.999	0.68	0.952	0.623

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