Journal of Chromatography, 171 (1979) 469–472
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CHROM. 11,586

Note

Quantitative determination of cholestanols and their derivatives by gas chromatography–mass fragmentography

MASAHIKO TOHMA, YUKO NAKATA and TAKAO KUROSAWA

Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University, Ishikari-Tobetsu, Hokkaido 061-02 (Japan)

(Received October 17th, 1978)

In the course of our investigation of models of the biological oxidation of steroids, we have reported on the stereoselective β-epoxidation of cholesterol with ferric acetylacetonate and hydrogen peroxide[1], and on the autoxidation of cholesterol giving 5α-cholestane-3β,5,6β,7α-tetrol[2].

In these investigations it was difficult to analyze quantitatively each component in the mixture of cholestanols and their derivatives by gas–liquid chromatography (GLC), because compounds with similar chemical structures were poorly resolved. The use of combined gas chromatography–mass spectrometry (GC–MS) gave a high specificity for the analysis by providing a physicochemical identification of steroids[3–5].

In this paper, we describe the simple analysis of cholesterol, cholestanols and their deuterated derivatives by gas chromatography–mass fragmentography.

EXPERIMENTAL

Materials

Cholesterol was purchased from Wako (Osaka, Japan). 4-Cholesten-3-one, 5β-cholestan-3-one, 5α-cholestan-3-one, 5β-cholestan-3α-ol, 5α-cholestan-3α-ol, 5α-cholestan-3β-ol and 5α-cholestane were prepared from cholesterol by conventional methods.

3β-Deutero-, 5β-deutero- and 3β,5β-dideutero-5β-cholestan-3α-ol were synthesized from 4-cholesten-3-one by reduction with deuterium–paladium on charcoal and/or lithium aluminium deuteride.

Gas chromatography–mass spectrometry

GC–MS was performed using a Shimadzu-LKB 9000 system, equipped with a multiple-ion detector and a data-processing system (Shimadzu GC-MS PAC 300M). The GC column (2 m × 3 mm I.D.) was filled with 1.5% SE-30 on Chromosorb W (60–80 mesh) and the column temperature was maintained at 255°. The flow-rate of helium carrier gas was 30 ml/min. The temperature of the flash heater was 290° and of the separator 270°; the mass spectra were recorded at 70 eV, with an ion source temperature of 290°.

Derivatization

Trimethylsilyl (TMS) ethers were formed by allowing the hydroxy steroids (40–200 ng) to react with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine (0.02 ml, TMS-HT from Tokyo Kasei Kogyo, Tokyo, Japan) for 15 min until the reactions were complete, but without the formation of the enol ethers of the oxo compounds.

RESULTS AND DISCUSSION

Gas chromatographic separations of steroids have been extensively studied by many workers[6] without achieving appreciable separation of cholestanols and their derivatives for quantitative analysis. We have also unsuccessfully tried to separate these compounds by GC with an SE-30 column. Although polysulphone and other polar phases improved the separations somewhat, the stereols were not resolved completely owing to leading and/or tailing.

Since these compounds gave appropriate fragments by electron impact mass spectrometry as summarized in Table I, mass fragmentography appeared the most suitable method to be developed. Fig. 1 shows a mass fragmentogram for a mixture of cholestanols and their derivatives when 5α-cholestane is used as internal standard. It suggests that these compounds can be analyzed quantitatively by the gas chromatography–mass fragmentography.

TABLE I

MASS FRAGMENTOGRAPHIC DATA OF CHOLESTANOLS AND THEIR DERIVATIVES

Retention times were obtained for the trimethylsilyl ethers of hydroxy steroids. Relative responses were calculated on the basis of the ion peak area of 5α-cholestane as internal standard.

Compound	*Retention time (min)*	*Monitoring ion m/e*	*Relative response*
5α-Cholestane	10.0	372	100
5β-Cholestan-3α-ol	16.5	370	241
3β-Deutero-5β-cholestan-3α-ol		371	241
5-Deutero-5β-cholestan-3α-ol		371	241
3β,5-Dideutero-5β-cholestan-3α-ol		372	241
5α-Cholestan-3β-ol	20.4	370	52
5α-Cholestan-3α-ol	16.2	370	114
Cholesterol	19.8	368	132
5β-Cholestan-3-one	17.3	386	61
5-Deutero-5β-cholestan-3-one		387	61
5α-Cholestan-3-one	18.9	386	60
4-Cholesten-3-one	21.6	384	21

Relative responses for each selected ion peak of these compounds in mass fragmentography are given in Table I. They were calculated on the basis of the area (100) of the molecular ion peak of 5α-cholestane at *m/e* 372. From these data, it appears that the quantitative precisions of the experimental results for the compounds having a small response, such as 5α-cholestan-3β-ol and the oxosteroids, can be increased by the gain control in the multiple ion detector. The trimethylsilyl ethers of

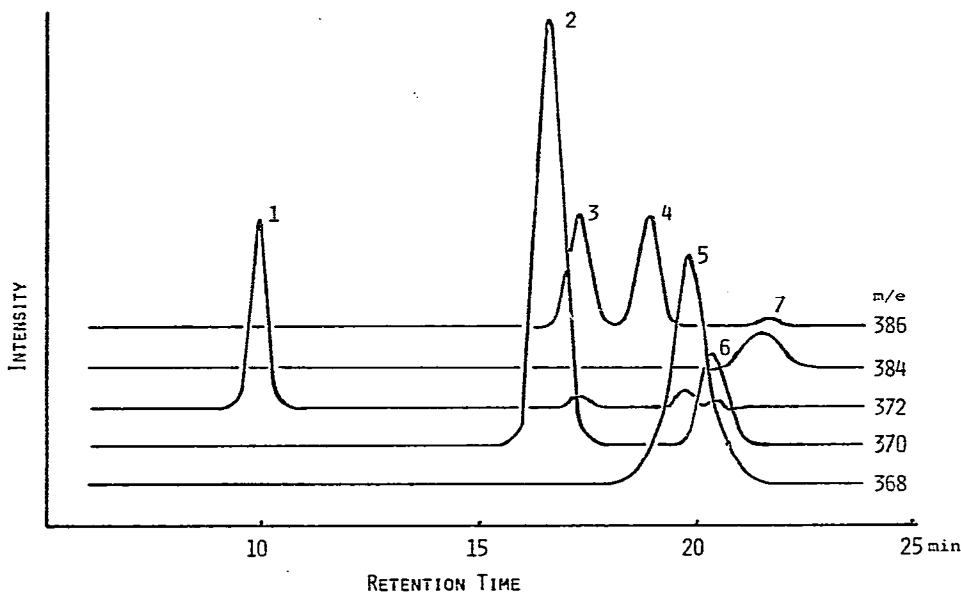

Fig. 1. Mass fragmentogram for a mixture of cholestanols and their derivatives. Peaks: 1 = 5α-cholestane; 2 = 5β-cholestan-3α-ol; 3 = 5β-cholestan-3-one; 4 = 5α-cholestan-3-one; 5 = cholesterol; 6 = 5α-cholestan-3β-ol; 7 = 4-cholesten-3-one.

5α- and 5β-cholestan-3α-ol could not determined by mass fragmentography, because they have similar retentions and the same characteristic fragment ion at *m/e* 370. Improved GC separation of these cholestanols may be obtained by conversion into 5α- and 5β-cholestan-3-one respectively. The typical calibration curves for the mass fragmentographic determination of the standard compounds were obtained by plotting the peak area ratios of the focused ion *versus* the amounts of each compound,

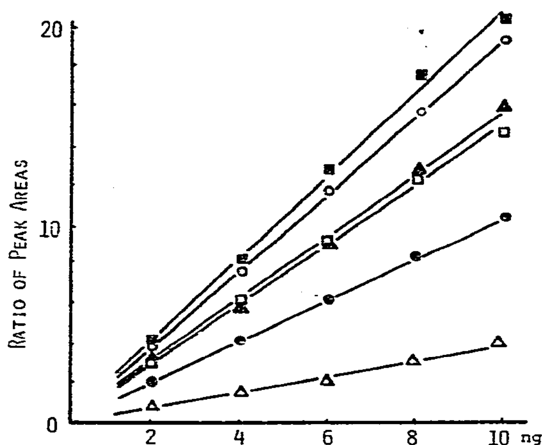

Fig. 2. Calibration curves obtained from measuring peak area ratios of the monitoring ions in a mixture of various amounts of cholestanol derivatives and a fixed amount (1.0-ng) of 5α-cholestane. Lines: ■, cholesterol (*m/e* 368); ○, 5β-cholestan-3α-ol (*m/e* 370); ▲, 5β-cholestan-3-one (*m/e* 386); □, 5α-cholestan-3-one (*m/e* 386); ●, 4-cholesten-3-one (*m/e* 384); △, 5α-cholestan-3β-ol (*m/e* 370).

as shown in Fig. 2. They gave a linear relationship over a range from 2 to 10 ng or perhaps even larger amounts, the precision of the measurements being *ca.* 2% under selected conditions.

Subsequently, the determination of the deuterium incorporation into 5β-cholestan-3α-ol was studied, and the calibration curve (Fig. 3) was obtained by determining the ratios of the peak area of the deuterated compound (*m/e* 371) to the total area of 5β-cholestan-3α-ol (*m/e* 370 + *m/e* 371) at various concentrations. This method, applied to other deuterated compounds, gave similar results. It appears to be useful for the investigation of the reductive metabolism of 4-cholesten-3-one with 3α-hydroxy steroid dehydrogenase and 5β-reductase[7-9].

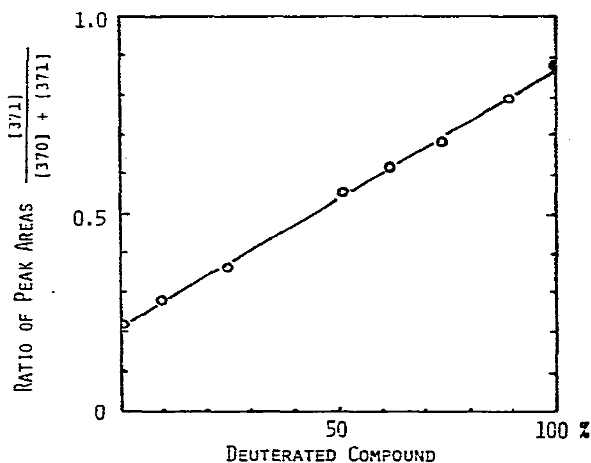

Fig. 3. Calibration curve for percent 3β-deutero-5β-cholestan-3α-ol in 5β-cholestan-3α-ol.

ACKNOWLEDGEMENT

The authors are grateful to Professor M. Kimura and Dr. I. Makino, Hokkaido University, for their valuable advice, and Mr. A. Sakushima of this faculty for his kind help in the GC–MS operation.

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