Journal of Chromatography, 398 (1987) 372–374
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 530

Note

Determination of cholesterol and its fermentation products by high-performance liquid chromatography

H. S. KIM*, C. K. CHOI and Y. H. PARK
Biochemical Process Laboratory, Genetic Engineering Center, The Korea Advanced Institute of Science and Technology, P.O. Box 131, Seoul (Korea)
(Received February 16th, 1987)

As natural sources of steroids such as diosgenin became more scarce and expensive, selective degradation of sterols has become more attractive for the preparation of various steroid compounds[1,2]. We have been studying the microbial conversion of cholesterol to produce 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) which are key intermediates for the syntheses of steroids.

Although many steroid compounds have been reported to be analyzed by chromatographic methods[3–5], the establishment of proper analytical conditions was often quite difficult and time-consuming. In the course of our research, we have developed a rapid method for quantitative and simultaneous determination of cholesterol, AD and ADD, and this is now described.

EXPERIMENTAL

Reagents

Steroid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and all other reagents of analytical grade were obtained from Merck (Darmstadt, F.R.G.).

Preparation of standard steroid solutions

Standard solutions of cholesterol, AD and ADD were prepared by dissolving 10 mg of cholesterol, AD and ADD in 10 ml of methylene chloriode. An aliquot of each standard solution was diluted in methylene chloride to produce a final solution of the desired concentration.

Sample preparation from fermentation broth

A 200-ml volume of culture medium in a 1-l erlenmeyer flask was inoculated with a 48-h culture of *Mycobacterium* sp. NRRLB-3805 or *Mycobacterium* sp. NRRL B-3683 and cultivated on a reciprocal shaker (200 strokes per min; Kukje Sci. Co., Seoul, Korea) at 30°C. After 36 h, 200 mg of cholesterol dissolved in 4 ml ethanol were added and incubated for 72 h.

The fermentation broth was extracted with two volumes of methylene chloride, and the organic phase was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) before injection into the chromatograph.

0021-9673/87/$03.50

Chromatographic conditions

The chromatograph used was equipped with a programmable, variable-wavelength detector (Model 655A-12; Hitachi, Tokyo, Japan). The column (30 cm × 4.6 mm I.D.) was packed with nominal 10-μm silica gel (μPorasil; Waters Assoc., Milford, MA, U.S.A.). The mobile phase was *n*-hexane–isopropanol (85:15, v/v) and the flow-rate was 1 ml/min.

The column effluent was monitored at 208 nm for cholesterol, and at 250 nm for both AD and ADD. This was possible by using the time-programming function for the detection wavelength. Peak areas were calculated with an integrator (Model D-2000; Hitachi).

A 10-μl volume of the sample solution was injected by a microsyringe (Hamilton, Reno, NV, U.S.A.). All analyses were conducted at room temperature.

RESULTS AND DISCUSSION

In the normal-phase high-performance liquid chromatography (HPLC), nonpolar solvents were used as the mobile phase for cholesterol, AD and ADD because the steroid compounds were well solubilized in such solvents. Among various solvents tested, *n*-hexane and isopropanol were selected since their UV absorbances do not overlap with those of cholesterol ($UV_{max.}$ 208 nm), AD and ADD ($UV_{max.}$ 250 nm).

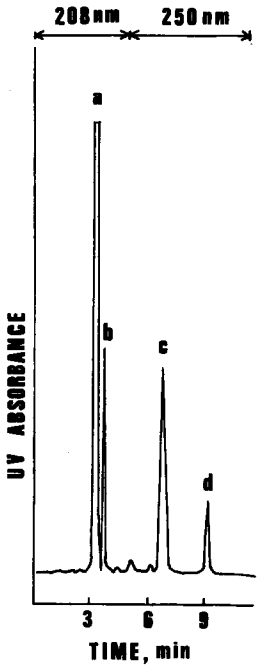

Fig. 1. Chromatogram of a sample extracted from fermentation broth with methylene chloride. The column eluate was initially monitored at 208 nm for 5 min, and then at 250 nm 5 min using the time programming function for the detection wavelength. Peaks: a = methylene chloride; b = cholesterol; c = AD; d = ADD.

The ratio of *n*-hexane to isopropanol which gives the best separation was determined experimentally to be 85:15 (v/v).

The elution occurred in the order of cholesterol, AD and ADD, *i.e.*, the retention time increased with increasing polarity of the steroid compound. The three compounds could be analyzed simultaneously within 10 min.

A typical elution pattern of the fermentation broth after extraction with methylene chloride is shown in Fig. 1. Extraction of culture broth with other solvents such as ethyl acetate, chloroform and diethyl ether was also tested, but they were found to be less efficient than methylene chloride. Ethyl acetate showed good extraction ability for the steroid compounds, but cholesterol could not be separated at any polarity of the mobile phase. In reversed-phase chromatography, an interference from the extraction solvent was often observed. To avoid this problem, it was previously reported that the extraction solvent was evaporated, and the dried sample was dissolved in a polar solvent[6,7]. However, in the present system, the extraction solvent was directly injected for HPLC so that the analysis could be performed rapidly.

Calculated peak areas and concentrations of standard steroid compounds were linearly correlated in the range of 1–10 μg. The lowest limits for the analysis of cholesterol, AD and ADD were observed to be 20, 2 and 1 ng, respectively.

This analytical method could be applied to determine cholesterol and its fermentation products when methylene chloride was used as the extraction solvent.

REFERENCES

1 T. L. Miller, in M. Moo-Young (Editor), *Comprehensive Biotechnology*, Vol. 3, Pergamon, New York, 1st ed., 1985, p. 297.
2 M. G. Wovcha and C. B. Biggs, *U.S. Pat.*, 4,293,644 (1981).
3 I. W. Duncan, P. H. Culbreth and C. A. Burtis, *J. Chromatogr.*, 162 (1979) 281.
4 C. Matsuoka, H. Nohta, N. Kuroda and Y. Ohkura, *J. Chromatogr.*, 341 (1985) 432.
5 D. D. Stump, E. F. Roth, Jr. and H. S. Gilbert, *J. Chromatogr.*, 306 (1984) 371.
6 I. R. Hunter, M. K. Walden and E. Heftmann, *J. Chromatogr.*, 153 (1978) 57.
7 D. G. Walters, P. M. D. Foster and R. C. Cottrell, *J. Chromatogr.*, 219 (1981) 152.