Journal of Chromatography, 341 (1985) 432—436
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2566

Note

Simultaneous determination of cholestanol and cholesterol in human serum by high-performance liquid chromatography with fluorescence detection

CHIZUKO MATSUOKA, HITOSHI NOHTA, NAOTAKA KURODA and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(Received December 7th, 1984)

The concentration of cholestanol and the concentration ratio of cholestanol to cholesterol are increased in the sera of patients with cerebrotendinous xanthomatosis, obstructive jaundice and cholestatic hepatitis, whereas the cholesterol concentration in the serum of patients with cerebrotendinous xanthomatosis, compared with that for the other patients, remains within the normal range [1]. Therefore, the determination of cholesterol and cholestanol in serum gives important information, especially for the biological diagnosis of cerebrotendinous xanthomatosis [1].

However, the simultaneous determination of cholesterol and cholestanol in serum is difficult, because cholesterol and cholestanol are harldy separated by gas chromatography [2] or thin-layer chromatography [3] and the concentration of cholesterol in normal serum is about 500—1000 times higher than that of cholestanol. Only one method has been reported so far for the simultaneous determination of cholesterol and cholestanol: gas chromatography—mass fragmentography [4]. The method requires a large amount of serum (1.0 ml) and expensive equipment.

We have shown that 3-chloroformyl-7-methoxycoumarin (3CMC) [5] is a useful fluorescent derivatization reagent for alcoholic compounds in high-performance liquid chromatography (HPLC) [6]. We have recently found that epoxidized cholesterol (cholesterol-5α,6α-epoxide) and cholestanol can be completely separated by reversed-phase HPLC on a TSK gel ODS-120A column and thus developed a sensitive method for the simultaneous determination of cholesterol and cholestanol in a minute amount of human serum (50 μl) by

0378-4347/85/$03.30

HPLC with pre-column fluorescent derivatization with 3CMC after epoxidation of cholesterol.

EXPERIMENTAL

Materials and apparatus

All chemicals were of analytical-reagent grade unless indicated otherwise. Deionized, distilled water was used. Organic solvents were distilled and dried in the usual manner. Cholesterol was obtained from Nakarai Chemicals (Kyoto, Japan; standard reagent, purity 99%), Katayama Chemicals (Osaka, Japan; guaranteed reagent) and Sigma (St. Louis, MO, U.S.A.; standard reagent for chromatography, purity above 99%, and that from porcine liver, purity 99%). Cholestanol was purchased from Nakarai Chemicals and cholesterol-5α,6α-epoxide from Sigma. 3CMC was synthesized by the method of Baker and Collis [5]. Human sera were obtained from healthy volunteers in our laboratory and kept at −20°C until used.

Uncorrected fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorometer using 1 × 1 cm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. A Jasco TWINCLE chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (20-μl loop) and a Hitachi 650-10S spectrofluorometer fitted with an 18-μl flow cell as a fluorescence detector, operating at 400 nm emission and 355 nm excitation. A stainless-steel column (150 × 4.0 mm I.D.) was packed with TSK gel ODS-120A (particle size 5 μm) (Toyo Soda, Tokyo, Japan) using a slurry technique with chloroform as solvent.

Procedure

A mixture of 50 μl of serum and 1.0 ml of 0.2 *M* sodium hydroxide in 95% ethanol was placed in a PTFE screw-capped Pyrex culture tube (100 × 16 mm I.D.) (Iwaki Glass, Tokyo, Japan) and heated at 100°C for 10 min to hydrolyse esters of cholesterol and cholestanol. To the resulting mixture, 0.5 ml of water and 5.0 ml of *n*-hexane were added. The mixture was shaken on a vortex-type mixer for 10 min and centrifuged at 1000 *g* for 5 min. The organic layer (3.0 ml) was transferred into a glass-stoppered test-tube (10 ml) and the solvent was evaporated to dryness in vacuo at 30°C. The residue was dissolved in 1.0 ml of 0.3% (w/v) *m*-chloroperbenzoic acid solution in *n*-hexane by shaking on a vortex-type mixer for 1 min and incubated at 37°C for 30 min to epoxidize cholesterol. The mixture, after adding 4.0 ml of *n*-hexane, was washed with 2 ml of 0.2 *M* sodium hydroxide solution. The organic layer (3.0 ml) was transferred into another test-tube and the solvent was evaporated to dryness in vacuo at 30°C. The residue was dissolved in 0.2 ml of 20 m*M* 3CMC solution in acetone by shaking on a vortex-type mixer for 1 min. A 100-μl aliquot of the mixture was heated in a screw-capped reaction vial (3.5 ml) (Gasukuro Kogyo, Tokyo, Japan) at 100°C for 20 min. A 20-μl aliquot of the reaction mixture was injected into the chromatograph after the dilution with 0.9 ml of acetone and eluted with a mixture of methanol and tetrahydrofuran (13:1, v/v) containing 2% (v/v) of acetic acid at a flow-rate of 1.0 ml/min. The spectral

bandwidths in both the excitation and emission sides of the monochromator in the fluorescence detector were 2 nm for the first 8 min of the retention time and were then changed to 10 nm to obtain a high detector response. For the establishment of calibration graphs, series of cholestanol standard solutions in acetone (2—100 μg/ml) and cholesterol standard solutions in acetone (0.5—4.0 mg/ml) were prepared, and the standards (50 μl each) were carried through the procedure, alkali hydrolysis being omitted. The peak heights in the chromatograms were used for the quantification of cholestanol and cholesterol.

RESULTS AND DISCUSSION

The 3CMC esters of cholesterol and cholestanol were hardly separated by reversed-phase HPLC on a TSK gel ODS-120A column [6]. In the determination of cholestanol in serum by gas chromatography, a large amount of interfering cholesterol was removed by epoxidizing it with *m*-chloroperbenzoic acid in chloroform (product, cholesterol-5α,6α-epoxide) [7, 8]. The epoxidation can be carried out in *n*-hexane with warming for 30 min at 37°C. The esterification of cholesterol-5α,6α-epoxide and cholestanol with 3CMC in acetone is completed within 20 min at 100°C. The separation of 3CMC esters of cholesterol-5α,6α-epoxide and cholestanol can be achieved by HPLC with methanol containing small amounts of tetrahydrofuran and acetic acid as the mobile phase; tetrahydrofuran provides a rapid separation of peaks and acetic acid serves to sharpen the peaks.

Fig. 1 shows the chromatograms obtained from cholesterol and cholestanol standards according to the procedure without alkali hydrolysis. The detector response is adjusted by changing the spectral bandwidths in both the excitation and emission monochromators of the fluorescence detector as described in the procedure, because there is a very large difference in concentration between the 3CMC esters of cholesterol-5α,6α-epoxide and cholestanol. This could not be achieved by changing the sensitivity range of the fluorescence detector. The 3CMC ester of cholestanol is eluted at a retention time of 13.6 min (Fig. 1B). In the chromatogram from cholesterol, however, two peaks are observed at retention times of 4.8 and 13.6 min (Fig. 1A). The eluates from peaks 1 and 2 in Fig. 1A and B have fluorescence excitation maxima at 353 and 359 nm, respectively, and both emission maxima at 402 nm. The retention times and the fluorescence excitation and emission spectra of the eluates from peaks 1 and 2 in Fig. 1A are identical with those obtained with authentic samples of cholesterol-5α,6α-epoxide and cholestanol, respectively. These observations indicate that the commercial preparations of cholesterol contain a relatively large amount of cholestanol; the concentration ratio of cholestanol to cholesterol was in the range 0.0014—0.0023.

Esters of cholesterol and cholestanol in serum are hydrolysed at 100°C in aqueous ethanolic sodium hydroxide solution and the resulting free cholesterol and cholestanol are extracted with *n*-hexane. The extract is subjected to epoxidation and then to fluorescent derivatization, followed by HPLC.

Fig. 2 shows typical chromatograms obtained from the serum of a healthy man and the same serum fortified with cholestanol. The fluorescence excitation and emission spectra of peaks 1 and 2 in Fig. 2A and B are identical with those in Fig. 1A and B, respectively.

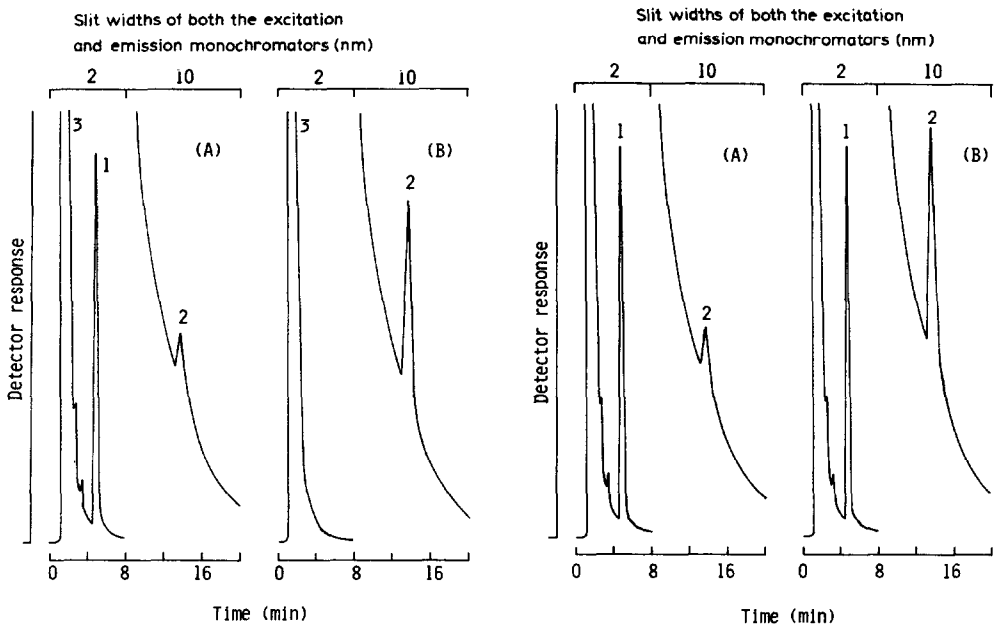

Fig. 1. Chromatograms of 3CMC esters of (A) cholesterol and (B) cholestanol. Portions (50 μl) of commercial preparations of cholesterol and cholestanol in acetone (2.0 mg/ml and 10 μg/ml, respectively) were treated according to the procedure without alkali hydrolysis. Peaks: 1 = cholesterol-5α,6α-epoxide; 2 = cholestanol (in A, cholestanol contained in the commercial preparation of cholesterol as impurity; in B, the commercial preparation of cholestanol); 3 = components of the reagent blank.

Fig. 2. Chromatograms from (A) normal serum and (B) the serum fortified with cholestanol (10 μg/ml). Peaks: 1 = cholesterol-5α,6α-epoxide; 2 = cholestanol.

The calibration graphs for cholesterol and cholestanol were linear up to at least 4.0 mg/ml and 100 μg/ml, respectively, and passed through the origin. The limits of detection for cholesterol and cholestanol were 2.72 and 0.15 pmol in a 20-μl injection volume, corresponding to 5.8 and 0.34 μg/ml in serum, respectively, at a signal-to-noise ratio of 2. The sensitivity may permit the assay of cholesterol and cholestanol in 0.1 and 5.7 μl of normal serum, respectively.

The recoveries of cholesterol and cholestanol (1.0 mg/ml and 5 μg/ml, respectively) from serum were 99.9 ± 5.8% and 98.8 ± 5.6% (mean ± S.D., n = 10), respectively. The precision was established by repeated assays (n = 10) using serum containing cholesterol and cholestanol at 1.76 mg/ml and 3.13 μg/ml, respectively. The coefficients of variation were 4.9% and 4.4%, respectively.

Comparison with a fluorimetric method for the assay of cholesterol in serum based on the enzymatic reaction (cholesterol ester hydrolase—cholesterol oxidase—peroxidase system) with fluorogenic substrate (tyramine) [9] showed a correlation coefficient of 0.955 (n = 15), and the regression equation for the present method (x) against the fluorimetric enzymatic method (y) was $y = 0.912\,x + 0.16$. The amounts of cholestanol and cholesterol in the serum of

TABLE I

CONCENTRATIONS OF CHOLESTANOL AND CHOLESTEROL AND THEIR RATIO IN SERA FROM NORMAL PERSONS

Age (years)	Sex*	Cholestanol (μg/ml)	Cholesterol (mg/ml)	Ratio of cholestanol to cholesterol × 100
22	M	2.44	1.94	0.13
24	M	2.83	1.93	0.15
24	M	4.13	1.89	0.22
25	M	1.10	1.35	0.08
25	M	1.70	1.33	0.13
26	M	2.63	1.40	0.19
26	M	1.80	1.21	0.15
26	M	2.19	1.94	0.11
26	M	1.20	0.90	0.13
30	M	2.84	2.11	0.13
33	M	3.13	1.92	0.16
38	M	3.03	1.50	0.20
54	M	3.95	1.90	0.21
22	F	2.95	1.94	0.15
33	F	4.44	2.20	0.20
Mean ± S.D.		1.70 ± 0.38	2.69 ± 1.00	0.16 ± 0.04

*M = Male; F = female.

fifteen healthy subjects (22—54 years) determined by this method and their concentration ratios are shown in Table I. The values are in good agreement with published data [1, 4, 8].

This study provides the first HPLC method for the determination of cholestanol along with cholesterol. The method is sensitive, rapid and simple and should be useful for biomedical investigations of cholesterol and cholestanol.

REFERENCES

1 S. Kamei, Y. Seyama, K. Ichikawa, T. Kasama, A. Okubo and T. Yamakawa, Jap. J. Clin. Chem., 7 (1978) 151.
2 T.T. Ishikawa, J.B. Brazier, L.E. Stewart, R.W. Fallat and C.J. Gluech, J. Lab. Clin. Med., 87 (1976) 345.
3 G. Salen, Ann. Intern. Med., 75 (1971) 843.
4 Y. Seyama, K. Ichikawa and T. Yamakawa, J. Biochem., 80 (1976) 223.
5 W. Baker and C.B. Collis, J. Chem. Soc., s12 (1949).
6 C. Hamada, M. Iwasaki, N. Kuroda and Y. Ohkura, J. Chromatogr., 341 (1985) 426.
7 L.F. Fieser and M. Fieser, Reagents for Organic Synthesis, Vol. 1, Wiley, New York, 1967, p. 135.
8 S. Serizawa, Y. Seyama, H. Otsuka, T. Kasama and T. Yamakawa, J. Biochem., 90 (1981) 17.
9 C. Hamada, M. Iwasaki, K. Zaitsu and Y. Ohkura, Chem. Pharm. Bull., 28 (1980) 3131.