Journal of Chromatography, 462 (1989) 467–470
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 047

Note

Chromatographic separation of cholesteryl acetate and its chloro analogues

HARI K. BHAT and G. A. S. ANSARI*

Department of Human Biological Chemistry and Genetics and Department of Pathology, University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)

(First received July 20th, 1988; revised manuscript received October 12th, 1988)

Cholesterol is esterified by long chain fatty acids for storage, transport and maintenance of cholesterol homeostasis in the body. Enzymes responsible for the formation of cholesterol esters are present both in the cell as well as in plasma[1–7].

Recently, it has been shown that xenobiotics containing free carboxylic functional groups or those which are converted to carboxylic acid during their metabolism result in the formation of cholesteryl esters[8–12]. These xenobiotic cholesteryl esters behave differently than the normally occurring long chain cholesteryl esters[10,11] and possess specific toxicity[13].

In order to study the formation and behavior of xenobiotic cholesteryl esters resulting from mono-, di- and tri-chloroacetic acids, we have synthesized and characterized mono-, di- and tri-chloroacetates of cholesterol. For the purpose of analysis of these cholesteryl chloroacetates in various tissues and fluids, we have developed chromatographic procedures which are described in this communication.

MATERIALS AND METHODS

Reagents

Cholesterol was purchased from MCB (Cincinnati, OH, U.S.A.). Cholesteryl acetate was obtained from Sigma (St. Louis, MO, U.S.A.). Chloroacetyl chloride, dichloroacetyl chloride and trichloroacetyl chloride were bought from Aldrich (Milwaukee, WI, U.S.A.).

Thin-layer chromatography (TLC) plates coated with silica gel were purchased from Analtech (Newark, DE, U.S.A.). Silica gel was purchased from E. Merck (Cherry Hill, NJ, U.S.A.). Solvents (HPLC-grade) were purchased from Fisher Scientific (Houston, TX, U.S.A.).

Purification of cholesterol and cholesteryl acetate

Cholesterol was purified by column chromatography over silica gel 60 (70–230 mesh) and eluted with hexane–ethylacetate (19:1, v/v). Fractions of 5 ml were collected and monitored by TLC as described later. Fractions corresponding to cholesterol were pooled and crystallized from methanol. Cholesteryl acetate was purified by crystallization from a hexane–acetone (1:5, v/v) mixture.

0021-9673/89/$03.50

Synthesis of mono-, di- and tri-chloroacetates of cholesterol

Cholesteryl monochloroacetate was prepared by dissolving 10 g of purified cholesterol in 14 ml of dry pyridine, to which 2.72 g of monochloroacetyl chloride were added. The reaction mixture was heated at 60°C for 4 h, cooled and poured into ice-cold water. The solid mass thereby formed was filtered, dried over anhydrous calcium chloride and recrystallized from hexane–acetone (1:1, v/v). Cholesteryl dichloro- and trichloroacetates were similarly prepared by using 3.67 g of dichloroacetyl chloride and 5.60 g of trichloroacetyl chloride, respectively. The structures of these compounds were confirmed by NMR and mass spectrometry (data not shown).

High-performance liquid chromatography (HPLC)

The chromatographic analysis was conducted on a Beckman Model 334 liquid chromatograph connected with an Ultrasphere ODS column (5 μm particle size, 25 cm × 4.6 mm I.D.) Altech Assoc. (Deerfield, IL, U.S.A.); a Beckman 165 variable-wavelength detector and a Perkin-Elmer 023 recorder.

Thin-layer chromatography

TLC was performed on silica gel G (250 μm) glass plates in a presaturated chromatographic chamber. The plates were air dried, sprayed with 50% sulfuric acid and heated at 120°C for 5 min, to detect cholesteryl esters.

Stock solutions

Stock solutions of standards were prepared by dissolving 20 mg in 10 ml of chloroform and stored at 4°C. A stock solution of the mixture was prepared by dissolving cholesteryl acetate (58.0 μmol), cholesteryl monochloroacetate (54.0

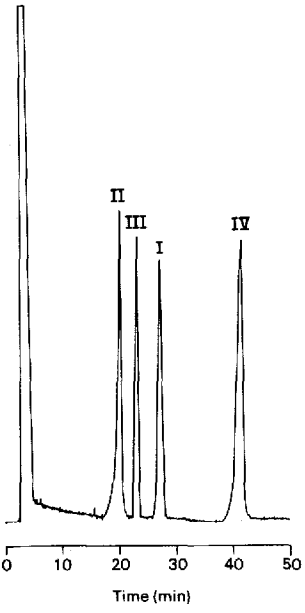

Fig. 1. Separation of cholesteryl acetate and its chloro analogues by HPLC. Injection volume 100 μl; detection at 210 nm; 0.300 a.u.f.s.

TABLE I

STANDARD CURVE OF CHOLESTERYL ESTERS

The data presented are averages of three experiments ± standard deviation.

Concentration (μg)	*Peak area (mm^2)*			
	I	*II*	*III*	*IV*
2.0	1.51 ± 0.31	1.44 ± 0.17	1.76 ± 0.13	1.83 ± 0.09
4.0	2.72 ± 0.49	2.48 ± 0.40	3.71 ± 0.54	3.54 ± 0.06
6.0	3.38 ± 0.47	3.20 ± 0.39	5.51 ± 0.34	5.83 ± 0.72
8.0	4.08 ± 0.24	4.90 ± 0.91	5.90 ± 0.44	5.94 ± 0.09
10.0	5.86 ± 0.11	6.29 ± 0.28	8.14 ± 0.74	7.61 ± 1.25
Correlation coefficient	0.990	0.984	0.983	0.976

μmol), cholesteryl dichloroacetate (50.30 μmol), and cholesteryl trichloroacetate (47.0 μmol) in 10 ml chloroform. Dilutions were made from the stock solutions of standards and mixture to estimate the detection limit.

RESULTS AND DISCUSSION

Cholesteryl acetate (I), cholesteryl monochloroacetate (II), cholesteryl dichloroacetate (III), and cholesteryl trichloroacetate (IV) can be resolved by HPLC using hexane–methanol (1:32, v/v) at a flow-rate of 1 ml/min in 42 min (Fig. 1). Under these conditions cholesteryl monochloroacetate eluted first (19.8 min) and cholesteryl trichloroacetate last (41.6 min). Cholesteryl acetate had a retention time higher than

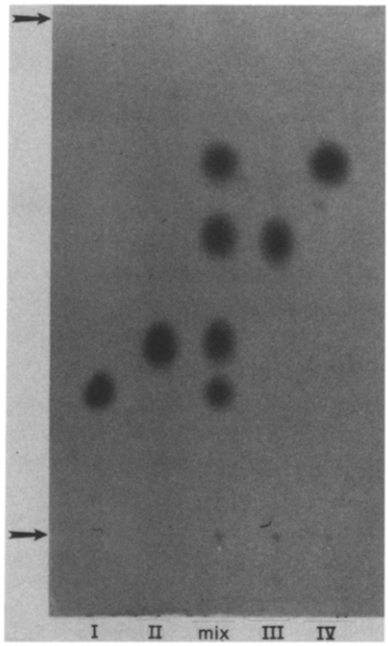

Fig. 2. Separation of cholesteryl acetate and its chloro analogues by TLC.

TABLE II

R_F VALUES RELATIVE TO CHOLESTERYL ACETATE, USING DIFFERENT RATIOS OF HEXANE AND ETHYL ACETATE

R_F values of cholesteryl acetate were: 0.66, 0.55, 0.37, 0.35 and 0.29 using hexane–ethyl acetate 4:1, 9:1, 19:1, 24:1 and 66:1 (v/v), respectively.

Solvent system: hexane–ethyl acetate (v/v)	*Relative R_F*		
	II	*III*	*IV*
4:1	1.06	1.14	1.18
9:1	1.13	1.27	1.36
19:1	1.24	1.73	2.02
24:1	1.26	1.77	2.14
66:1	1.29	1.95	2.41

cholesteryl dichloroacetate and lower than cholesteryl trichloroacetate. Similar resolution was achieved by using hexane–methanol (1:19, v/v) in 35 min (I, 23.24; II, 17.80; III, 20.12; IV, 34.8 min). The standard curve for each ester was determined (Table I) and was found to be linear between 2–10 μg at 0.05 a.u.f.s. Under these conditions, cholesteryl palmitate, cholesteryl stearate and cholesteryl oleate have a much longer retention time (>90 min).

Fig. 2 shows that compounds I–IV could be resolved on a silica gel TLC plate, using hexane–ethyl acetate (66:1, v/v) as the mobile phase. Table II summarizes the relative R_F values relative to compound I, using different solvent systems. As evident from Table II, the solvent system for the best separation of these halogenated esters of cholesterol is hexane–ethyl acetate (66:1, v/v). Cholesterol does not move in this solvent system. If we use only hexane as a solvent, compound I does not move and compound II barely moves (R_F 0.02). If hexane and ethyl acetate are used in the ratio of 4:1 (v/v) a poor resolution of compound III and IV is obtained.

ACKNOWLEDGEMENTS

This research was supported by grants ES 04815 awarded by the National Institute of Environmental Health Sciences and DK 27135 awarded by the National Institute of Diabetes, Digestive and Kidney Diseases.

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