Inhibitors of sterol synthesis. Reverse phase high performance liquid chromatography for the separation of cholesterol, 5α-cholest-8(14)-en-3β-ol-15-one, and their fatty acid esters

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Summary A relatively simple and rapid method was required for the separation of 5α-cholest-8(14)-en-3β-ol-15-one, a potent inhibitor of sterol synthesis, from its major metabolites. Conditions have been determined which permit the resolution of the 15-ketosterol and cholesterol and fatty acid esters of the two sterols by reverse phase high performance chromatography. This methodology also permits the resolution of the major esters of the 15-ketosterol and of cholesterol.—**Chu, A., and G. J. Schroepfer, Jr.** Inhibitors of sterol synthesis. Reverse phase high performance liquid chromatography for the separation of cholesterol, 5α-cholest-8(14)-en-3β-ol-15-one, and their fatty acid esters. *J. Lipid Res.* 1988. **29:** 235–239.

Supplementary key words 15-ketosterol • thin-layer chromatography

5α-Cholest-8(14)-en-3β-ol-15-one is a potent inhibitor of sterol synthesis in cultured mammalian cells (1–3) which has been shown to have significant hypocholesterolemic activity in rats (4, 5), mice (4), baboons (6), and rhesus monkeys (7). Delineation of the metabolism of the 15-ketosterol is a critical matter in an understanding of the overall action of this compound on cholesterol metabolism. The 15-ketosterol has been shown to serve as an efficient precursor of cholesterol and cholesteryl esters upon incubation with rat liver homogenate preparations (8) (Monger, D. J., and G. J. Schroepfer, Jr., unpublished results) and upon oral administration to rats (9) and baboons (Schroepfer, G. J., Jr. et al., unpublished results). The results of in vitro studies with rat liver preparations have also indicated the conversion of 5α-cholest-8(14)-en-3β-ol-15-one to fatty acid esters of the 15-ketosterol (Monger, D. J., and G. J. Schroepfer, Jr., unpublished results). Upon oral administration of the sterol to rats (9) and baboons (Schroepfer, G. J., Jr. et al., unpublished results), rapid metabolism of the compound to its fatty acid esters and to cholesterol and cholesteryl esters has been observed. These studies involved rather laborious and very time-consuming methodology, based largely on silicic acid column chromatography. Moreover, this methodology did not provide for the separation of individual fatty acid esters of the 15-ketosterol.

Described herein is a relatively simple and rapid reverse phase high performance liquid chromatographic (HPLC) system which permits the resolution of cholesterol, the 15-ketosterol, and the esters of the two sterols. Moreover, the major esters of the 15-ketosterol and of cholesterol are separable from each other using this method.

EXPERIMENTAL

All solvents for HPLC were of analytical grade (Burdick and Jackson, Inc., Muskegon, MI) and solvent mixtures were filtered through a 0.45-μm Nylon-66 membrane filter (Rainin Instrument Company, Inc., Woburn, MA) and degassed prior to use. HPLC analyses were carried out using pumps (Model 510), an ultraviolet detection unit (Model 481), and a U6K injector unit that were obtained from Waters Associates (Milford, MA). Unless stated otherwise, ultraviolet absorbance was monitored at 210 nm and eluted fractions were collected using an automatic fraction collector via a Teflon tubing connected to the outlet of the detector unit. HPLC separations were made on a 5-μm C_{18} Microsorb column (4.6 mm × 25

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

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cm; Rainin Instrument Company, Inc.) coupled to an RP-18 NewGuard cartridge (3.2 mm × 1.5 cm; Brownlee Labs, Inc., Santa Clara, CA) which was eluted in gradient manner with two solvent mixtures. Solvent mixture A was composed of isopropanol–methanol–water 5:4:1 and solvent mixture B was composed of isopropanol–methanol 4:1. Initially, a mixture of solvent A and solvent B (3:2) was pumped under isocratic conditions for 15 min. The mobile phase was then linearly programmed to a ratio of A to B of 1:3 using a Waters Model 680 automated gradient controller for the second 15-min period and then maintained at this ratio thereafter. The flow rate was increased in a linear fashion in the first 5 min from 1.0 ml per min to 1.25 ml per min and, in the next 10 min, from 1.25 ml per min to 1.45 ml per min. Thereafter, the flow rate was maintained at 1.45 ml per min (see Figs. 1 and 2). Esters of the 15-ketosterol were also separated on a 5-μm C_6 Spherisorb column (4.6 mm × 25 cm; Phase Separations, Norwalk, CT) using isocratic elution with a mixture of isopropanol–methanol–water 12.5:77.5:10 at a flow rate of 1.25 ml per min (see Fig. 3). Cholesteryl esters were also separated on the C_6 Spherisorb column using isocratic elution with a mixture of isopropanol–methanol–water 22.5:67.5:10 at a flow rate of 1.75 ml per min (see Fig. 4). Injections onto the HPLC columns were made using $CHCl_3$ (10 μl). All HPLC analyses were made at 20°C. Thin-layer chromatography (TLC) was carried out using silica gel G plates (Analtech, Newark, DE). Components on the plate were visualized after spraying with molybdic acid (10). Radio-TLC analyses were performed as described previously (11).

5α-Cholest-8(14)-en-3β-ol-15-one was prepared as described previously (1, 12). Cholesterol was purified by way of its dibromide derivative (13). Triolein and the palmitate, stearate, oleate, linoleate, and arachidonate esters of cholesterol were purchased from Sigma Chemical Company (St. Louis, MO). The palmitate, stearate, oleate, elaidate, linoleate, linolenate, and arachidonate esters of 5α-cholest-8(14)-en-3β-ol-15-one were prepared and characterized as described elsewhere (14). [2,4-^{3}H]5α-Cholest-8(14)-en-3β-ol-15-one (87.4 mCi per mmol) was prepared as described previously (15). The radiopurity was in excess of 99% as judged by the results of radio-TLC analysis (solvent system, hexane–ethyl acetate 7:3). [4-^{14}C]Cholesterol (55 mCi per mmol) was purchased from Amersham Corporation (Arlington Heights, IL) and, after purification by silicic acid column chromatography, showed a radiopurity in excess of 99% as judged by radio-TLC (solvent system, toluene–ethyl acetate 9:1). The oleate ester of [7n-^{3}H]cholesterol (2.0 mCi per mmol) was prepared by Dr. K.-S. Wang using a minor modification of the procedure utilized previously for the synthesis of the unlabeled ester (16) and showed a radiopurity in excess in 99% as judged by radio-TLC analyses in four solvent systems. The [1-^{14}C]palmitate ester of 5α-cholest-8(14)-en-3β-ol-15-one (~60 mCi per mmole) was also prepared by Dr. Wang. Radioactivity was assayed in a Model 4640 Packard Tri-Carb scintillation counter (Packard Instrument Company, Downers Grove, IL) using 0.4% 1,3-diphenyloxazole in toluene–ethanol (2:1) as the scintillation fluid.

RESULTS

Fig. 1 shows the separation of authentic samples (20–40 μg each) of 5α-cholest-8(14)-en-3β-ol-15-one cholesterol, the palmitate ester of the 15-ketosterol, triolein, and cholesteryl oleate by reverse phase HPLC on the C_{18} Microsorb column. Excellent resolution of each of the compounds was observed. Also shown in Fig. 1 is the elution of [2,4-^{3}H]5α-cholest-8(14)en-3β-ol-15-one, [4-^{14}C]cholesterol, the [1-^{14}C]palmitate ester of the 15-ketosterol, and the oleate ester of [7n-^{3}H]cholesterol. Essentially quantitative recovery of each of the labeled compounds was observed. The same column also provided separations not only of the 15-ketosterol, cholesterol, esters of the 15-ketosterol, and cholesteryl esters but also provided separations of the major esters of the 15-ketosterol and of cholesterol (**Fig. 2**). Excellent separations of the palmitate, stearate, oleate, linoleate, linolenate, and arachidonate esters of the 15-ketosterol were observed. In addition, separation of the oleate and elaidate esters of the 15-ketosterol was achieved. However, the palmitate and oleate esters of the 15-ketosterol were found to have the same retention times on this column. Fig. 2 also shows the separations of the stearate, oleate, linoleate, linolenate, and arachidonate esters of cholesterol. As with the case with the 15-ketosterol, the palmitate and oleate esters of cholesterol had the same retention times on this column. While resolutions of the oleate and palmitate esters were not achieved by this method or modifications of the reverse phase HPLC on the C_{18} Microsorb column, useful separations of these esters of the 15-ketosterol and of cholesterol were achieved by reverse phase HPLC on a C_6 Spherisorb column. **Fig. 3** shows the separation of the palmitate, stearate, oleate, linoleate, linolenate, arachidate, and arachidonate esters of the 15-ketosterol on the C_6-column (with monitoring of the ultraviolet absorbance at 259 nm). Using the same column, but with a less polar elution solvent mixture, useful separations of the palmitate, stearate, oleate, linoleate, linolenate, and arachidonate esters of cholesterol were also achieved (**Fig. 4**).

DISCUSSION

5α-Cholest-8(14)-en-3β-ol-15-one, a potent inhibitor of sterol synthesis, has been shown to serve as a precursor of

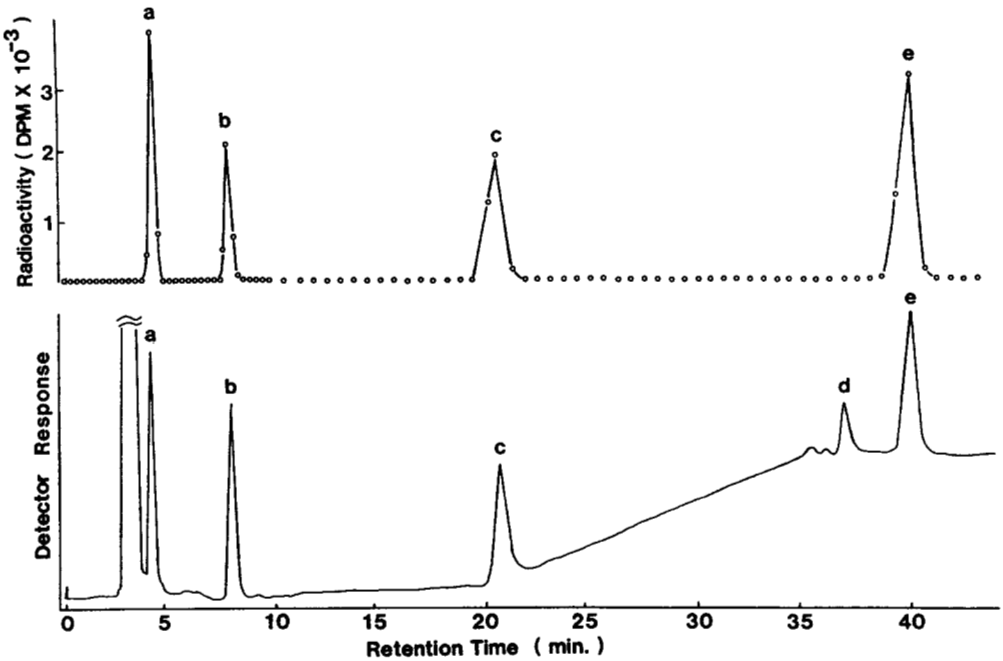

Fig. 1. Reverse phase HPLC separation (below) of: (a), 5α-cholest-8(14)-en-3β-ol-15-one (20 μg); (b), cholesterol (20 μg); (c), the palmitate ester of 5α-cholest-8(14)-en-3β-ol-15-one (40 μg); (d), triolein (40 μg); and (e), cholesteryl oleate (40 μg) on a C_{18} Microsorb column. Also shown (above) is separation of: (a), [2,4-^{3}H]5α-cholest-8(14)-en-3β-ol-15-one (4,500 dpm); (b), [4-^{14}C]cholesterol (3,000 dpm); (c), the [1-^{14}C]palmitate ester of 5α-cholest-8(14)-en-3β-ol-15-one (3,000 dpm); and (d), the oleate ester of [7n-^{3}H]cholesterol. The development conditions were as follows. Initially, a 3:2 mixture of solvent A (isopropanol–methanol–water 5:4:1) and solvent B (isopropanol–methanol 4:1) was pumped under isocratic conditions for 15 min. The mobile phase was then linearly programmed to a ratio of A to B of 1:3 for the second 15 min, and then maintained at this ratio thereafter.

cholesterol, fatty acid esters of cholesterol, and fatty acid esters of the 15-ketosterol (8, 9) (Monger, D. J., and G. J. Schroepfer; and Schroepfer, G. J., et al., unpublished results). Separation of the 15-ketosterol from these metabolites can be achieved by silicic acid column chromatography (8, 9) (Monger, D. J., and G. J. Schroepfer; and Schroepfer, G. J., et al., unpublished results). However, this approach is very time-consuming and labor-

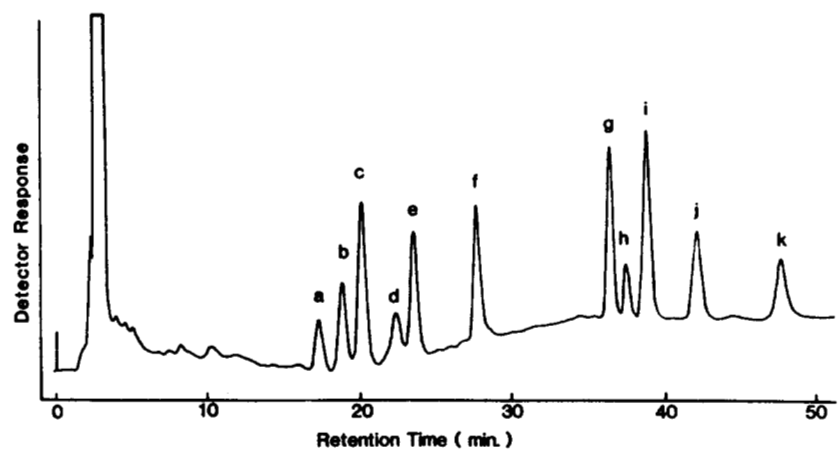

Fig. 2. Reverse phase HPLC of: (a), linolenate (10 μg); (b), arachidonate (10 μg); (c), linoleate (20 μg); (d), elaidate (6 μg); (e), oleate (40 μg); (f), stearate (40 μg) esters of 5α-cholest-8(14)-en-3β-ol-15-one; and (g), linolenate (20 μg); (h), arachidonate (10 μg); (i), linoleate (20 μg); (j), oleate (40 μg); and (k), stearate (40 μg) esters of cholesterol on a C_{18} Microsorb column. The development conditions were as described in the legend to Fig. 1.

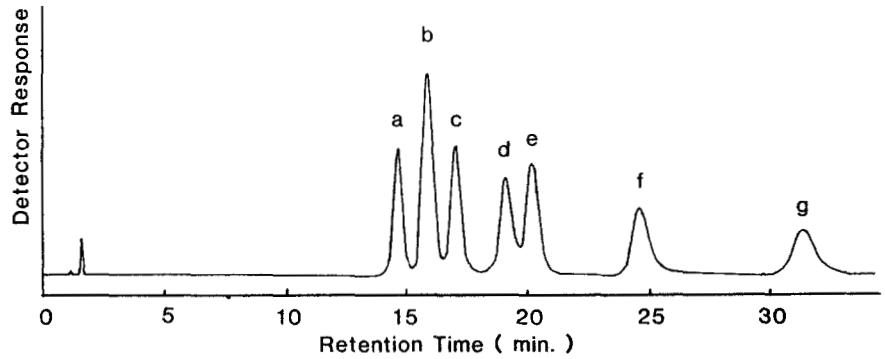

Fig. 3. Reverse phase HPLC of (a), linolenate (1.5 μg); (b), arachidonate (3 μg); (c), linoleate (1.5 μg); (d), palmitate (1.5 μg); (e), oleate (1.5 μg); (f), stearate (1.5 μg); and (g), arachidate (1.5 μg) esters of 5α-cholest-8(14)-en-3β-ol-15-one on a C_6 Spherisorb column. The development conditions were isocratic elution with a mixture of isopropanol–methanol–water 12.5:77.5:10. In this experiment, the ultraviolet absorbance was monitored at 259 nm.

intensive. Described above is a relatively simple and rapid method which, in one run, permits the resolution of the 15-ketosterol from cholesterol and from fatty acid esters of the 15-ketosterol and of cholesterol. In addition, the latter method provides separations of the major fatty acid esters of the 15-ketosterol and of cholesterol. This single column reverse phase HPLC analysis is relatively rapid (development time, ~1 hr) in comparison with silicic acid column chromatographic analysis for the same purpose (development time, ~100 hr). For analyses requiring the separations of the oleate and palmitate esters of the 15-ketosterol, reverse phase HPLC on C_6 Microsorb columns can be employed. The oleate and palmitate esters of cholesterol can also be resolved on the C_6 Microsorb column or on other reverse phase HPLC systems described by others (17–19). It should be noted that, with the solvent systems employed, the elution of the sterols and their esters can be followed by monitoring their absorbance at 210 nm. Addi-

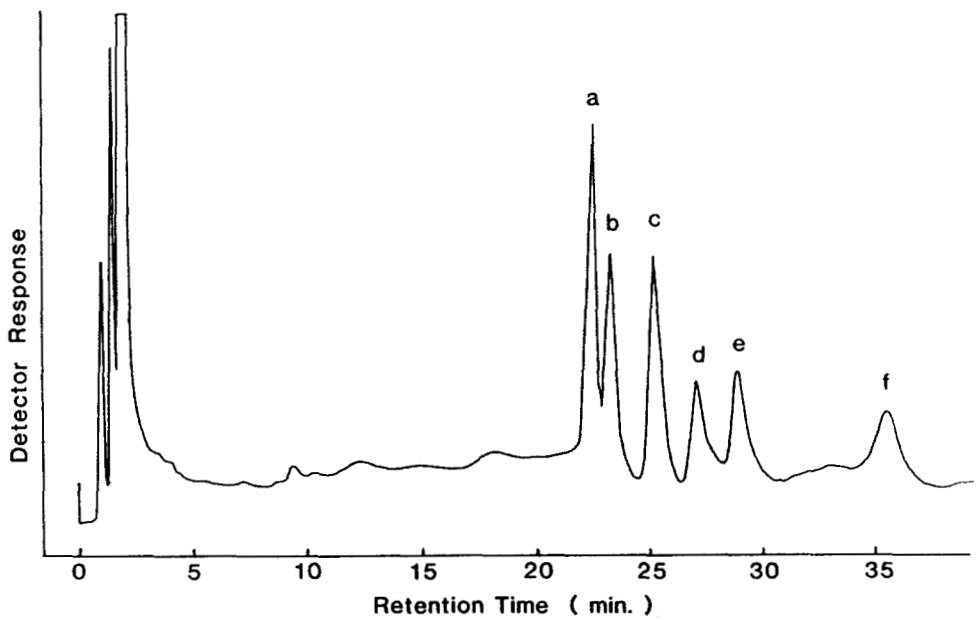

Fig. 4. Reverse phase HPLC of (a), linolenate (50 μg); (b), arachidonate (20 μg); (c), linoleate (25 μg); (d), palmitate (50 μg); (e), oleate (25 μg); and (f), stearate (50 μg) esters of cholesterol on a C_6 Spherisorb column. The development conditions were isocratic elution with a mixture of isopropanol–methanol–water 22.5:67.5:10.

tionally, the specific absorbance of the α,β-unsaturated keto function ($\Delta^{8(14)}$-15-one) of the 15-ketosterol and its esters can be monitored at 259 nm.

This method should be applicable, with minimal or no modification, to the study of the metabolism of other C_{27} 3β-hydroxysterols with a ketone function.

This work was supported in part by a grant (HL-22532) from the National Institutes of Health. The support of the Ralph and Dorothy Looney Endowment Fund and the American Cyanamid Company is also acknowledged.

Manuscript received 8 June 1987 and in revised form 6 August 1987.

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