Transesterification of Cholesteryl Esters[1]

M.P. Zubillaga and **G. Maerker**
Eastern Regional Research Center, ARS/USDA, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19118

The transesterification of cholesteryl stearate, oleate, linoleate, linolenate and arachidonate to the fatty acid methyl ester and free cholesterol under mild conditions is described. In adaptation of a published procedure the transesterification was carried out for two hr at room temperature in 1N NaOH in methanol:benzene (60:40, v/v). After addition of saturated sodium chloride solution the reaction mixture was extracted with ethyl acetate. The product mixture was checked for cholesterol oxides by HPLC fractionation followed by measurement of the oxides by direct on-column capillary GC. Although transesterification of cholesteryl stearate and oleate was complete (> 99%) in 30 min, a uniform reaction period of two hr, required by the polyunsaturated esters, was used for all cholesteryl esters. 7-Ketocholesterol, a principal oxidation product, was unaffected by the reaction conditions.

Autoxidation of cholesterol is known to generate numerous oxidation products (1) which have been grouped under the collective, though somewhat misleading, name of "cholesterol oxides." Some of the more prominent, and hence more easily detectable and measurable, of these cholesterol oxides have been reported to have adverse biological activity (2–4) and have been demonstrated to occur in animal-derived foods that had a known history of oxidative stress (5–11). In animal muscle tissue most of the total cholesterol occurs as the free sterol, but approximately 6% (12) is found as the ester of fatty acids. The relative cholesteryl ester content of organ tissues can be significantly higher.

Most recent investigators concerned with the determination of cholesterol and its oxidation products in foods and other substrates have used procedures in which saponification with hot alkali was an essential step in freeing the sterol and its oxides from the bulk of the accompanying lipids. This was followed by the isolation of the cholesterol oxides from the unsaponifiable residue. Oxidation that had occurred in the free sterol as well as in the sterol esters could thus be determined.

Because treatment with hot alkali is known to destroy some cholesterol oxides, especially 7-ketocholesterol (9,13–15), and has been reported to give rise to the formation of artifacts of other cholesterol derivatives (9,14) attempts have been made to avoid treatment with hot alkali. Oxidation products of free cholesterol have been determined by separating them from the bulk of the lipids by semipreparative HPLC (14,16,17), but cholesteryl esters and their oxides are not hydrolyzed in this procedure and are excluded from determination. Treatment with alkali at 25 C for 16 hr rather than at 81 C for two hr was successful in avoiding destruction of 7-ketocholesterol (5,18), but the effectiveness of these milder conditions in the saponification of cholesteryl esters was not reported.

During the course of some earlier studies of the oxidation of cholesterol in aqueous dispersions (19) it became apparent that cholesteryl stearate can be converted conveniently to methyl stearate and cholesterol by a mild transesterification procedure (20). Because transmethylation of glycerolipids is more rapid than that of cholesteryl palmitate (21), this procedure has the potential of serving the purpose of saponification: to liberate cholesterol from its esters and thereby allow the separation of total cholesterol from accompanying lipids. The current work was undertaken to investigate the applicability of the procedure to other cholesteryl esters and to test the effect of the transesterification conditions on cholesterol oxides.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol oxide standards were purchased from Sigma Chemical Company, (St. Louis, Missouri); Steraloids, Inc., (Wilton, New Hampshire); and Research Plus, Inc., (Bayonne, New Jersey). Cholesteryl esters and cholesterol, ash free and precipitated from alcohol, were purchased from Sigma Chemical Company. Cholesterol-5β,6β-epoxide was prepared from cholesterol via 3β,5α,6β-trihydroxycholestanol (22) and the corresponding triacetate (23) by the method of Chicoye et al. (13). The β-epoxide was purified by preparative TLC. All solvents used were "distilled in glass grade" and were degassed by vacuum filtration through a 0.2 μm filter. Water was double deionized, glass distilled and filtered via a Norganic (Millipore) filter. TLC plates, silica gel G and GHL (250 microns), were purchased from Analtech (Newark, Delaware).

Purification of cholesteryl esters. A solution of an impure cholesteryl ester (10 mg in 2 ml hexane:ethyl acetate, 100:2) was loaded into an ethyl acetate-washed and nitrogen-dried silica Sep-Pak (Waters Associates, Framingham, Massachusetts) and eluted with 8 ml of hexane:ethyl acetate, 100:2. The eluate was evaporated under nitrogen and the dry residue tested for purity by TLC (petroleum ether:ether:acetic acid, 80:20:1).

Transesterification of cholesteryl esters. The procedure used was an adaptation of that described by Glass (20). To 10 mg of sample ester in a test tube was added 2 ml of a solution of NaOH (4.0 g) in a mixture of 60 ml absolute methanol and 40 ml benzene (100 ml). The mixture was warmed briefly to ensure homogeneity, air over the solution was displaced with nitrogen, and the tube was capped and allowed to stand in the dark at room temperature for two hr. At the end of this period, four ml saturated, aqueous NaCl solution was added, and the mixture was extracted four times with two ml ethyl acetate. The pooled extracts were dried over anhydrous Na_2SO_4, passed through a five-ml syringe fitted with a 0.45 μm Millipore filter, and stored at −20 C.

Liquid chromatography. Semipreparative HPLC was performed using the instrumentation and procedures described previously (14). The transesterified samples in ethyl acetate were treated by the procedure described (14) to isolate a cholesterol oxide fraction ready for gas chromatography.

[1]Presented in part at the AOCS meeting in New Orleans, LA, in May 1987.

Gas chromatography. The instrumentation used and the method of operation were as described previously. Cholesterol oxide fractions were reconstituted in heptane:isopropanol (90:10, v/v) prior to injection into the gas chromatograph.

RESULTS AND DISCUSSION

Purchased cholesteryl esters, particularly the more highly unsaturated linolenate and arachidonate, contained polar impurities that were readily removed by passage through a silica cartridge (Sep-Pak). Elution with hexane:ethyl acetate (100:2, v/v) caused retention of the impurities, presumably oxidation products, and resulted in an eluate which gave a single spot by thin layer chromatography (TLC). Previous experience in this laboratory (19) indicates that oxides of free (unesterified) cholesterol are likely to be retained on silica Sep-Paks when the eluting solvent is hexane:ethyl acetate, 100:2. However, TLC is insufficiently sensitive to provide direct evidence of the absence of such cholesterol oxides, since their concentration, if they are indeed present, would be below that detectable by TLC of the cholesteryl esters.

Several mild saponification or transesterification procedures were chosen from the published literature and subjected to preliminary exploration. The method reported by Glass (20) was selected as the most suitable and was modified to adapt it to the requirements of cholesteryl ester transesterification. The "sodium hydroxide" reagent was prepared as described by Glass, and the reaction was allowed to proceed in capped tubes under nitrogen and in the absence of light. At the end of a chosen reaction time, transesterification was stopped by addition of a saturated sodium chloride solution and extraction of the two-phase mixture with ethyl acetate.

TLC was used to estimate the time required to complete the reaction as illustrated in Figure 1. Experimentally, it was determined that 0.1 μg of cholesteryl ester was the least amount visible on a TLC plate. Therefore, when 10 μg was applied to the plate, absence of a spot in the cholesteryl ester region indicated that less than 1.0% of steryl ester remained in the sample. Completeness of the reaction was also judged by the weight of free cholesterol (plus oxides) recovered from the reaction mixture. The latter was loaded into a silica Sep-Pak cartridge which was then eluted with mixtures of hexane and ethyl acetate. Cholesterol was recovered by elution with hexane:ethyl acetate mixtures of 90:10 and 85:15 (v/v), while cholesterol oxides were recovered by elution with ethyl acetate or ethyl acetate:methanol (90:10, v/v).

Cholesteryl stearate and oleate were completely consumed in 30 min, but the more highly unsaturated steryl esters required up to two hr at room temperature. During the longer reaction periods fatty acid soaps began to appear as components of the reaction mixture, but this presented no problem, since the goal of this work was the liberation of free cholesterol and its derivatives, rather than generation and analysis of the fatty acid esters. If the latter were desired, it would be necessary to insert a step that assured complete reesterification of the fatty acids.

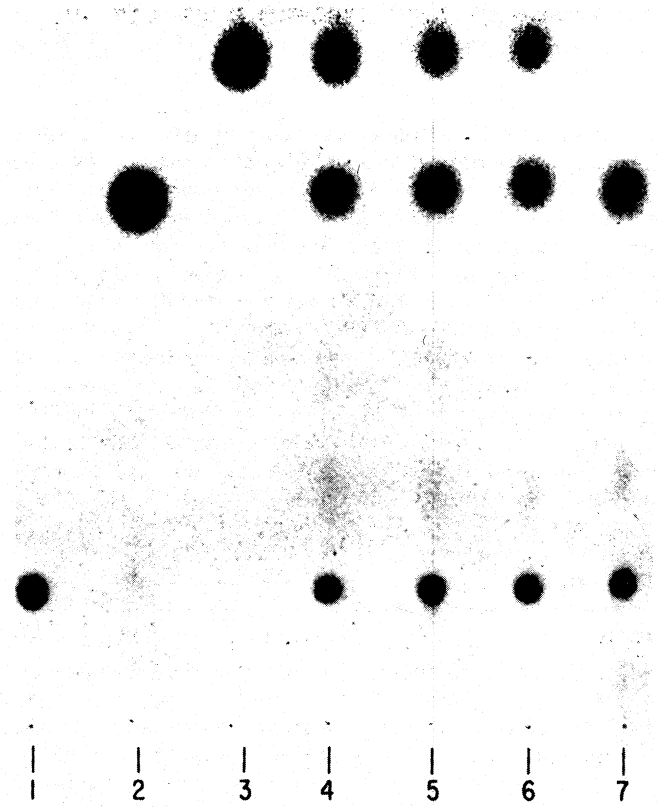

FIG. 1. Progress of transesterification of cholesteryl linoleate as followed by TLC on Silica Gel. Developing solvent: Petroleum ether:ether:acetic acid (80:20:1, v/v/v). Spots visualized by spray with 50% H_2SO_4 followed by charring. Numbered lanes are: 1, cholesterol; 2, methyl linoleate; 3, cholesteryl linoleate; 4,5,6 and 7, cholesteryl linoleate after 0.5 hr, 0.75 hr, 1.0 hr and 2 hr reaction, respectively.

During the course of the current study it was assumed that the esters of oxidized cholesterol, i.e., cholesteryl esters in which oxidation has occurred on the cholesterol portion of the molecule, are transesterified with no greater difficulty than those of unoxidized cholesteryl esters. This hypothesis has not been tested in the absence of appropriate model compounds but seems reasonable, since the reaction site is the carbonyl function of the acyl moiety some distance removed from the site of oxidation.

Because of the known instability of 7-ketocholesterol to alkali at elevated temperatures (9,13–15), it was subjected to the transesterification conditions of the current procedure. After two hr of treatment, 7-keto-3,5-cholestadiene, the predominant decomposition product of 7-ketocholesterol, could not be detected by GC or TLC, and recovery of the starting material was quantitative. Another common cholesterol oxidation product, 5α,6α-epoxycholestanol, which previously had been reported to be sensitive to alkaline media (9,24) was also tested by the current procedure. Its previously reported (14) stability was confirmed here, also.

A series of transesterifications was performed in which the substrate was a specific cholesteryl ester, e.g., cholesteryl linoleate, plus a three-fold molar excess of the corresponding methyl ester, e.g., methyl linoleate.

The purpose of this series of experiments was to test the question whether the excess lipid would influence the ease of transesterification or the amount of cholesterol oxide formation. The excess methyl ester did not affect the transesterification reaction or the amounts of cholesterol oxides formed.

It was desired to obtain an estimate of the extent to which cholesterol oxidizes during saponification and isolation, i.e., to assess the amount of artifact formation of cholesterol oxides. Our previous method (14) to enrich the cholesterol oxide portion of the sample by semipreparative HPLC and measurement of the oxides by GC was again applied. Our previous work had indicated (14) that a certain amount of unavoidable oxidation occurs during handling when pure cholesterol is treated by this procedure. In the current work it was difficult to distinguish between cholesterol oxidation products present in the starting material (at the 1 ppm level) and those formed during implementation of the procedure. At present there is no available methodology that distinguishes the pure cholesteryl esters from those oxidized either on the cholesterol moiety or the acyl portion. Our approach to solving this problem was to run a control reaction simultaneously with the transesterification of samples. The control differed from the samples only in that in the control the reaction mixture contained no sodium hydroxide. The amounts of cholesterol oxides formed in the samples, as judged from GC retention times, did not differ materially from those formed in the controls in the reaction of cholesteryl stearate, oleate and linoleate, but were slightly higher for cholesteryl linolenate and arachidonate. Even for these two highly unsaturated cholesteryl esters there was some doubt whether the peaks identified as cholesterol oxides by retention time were not due to other impurities. In the reactions in which a three-molar excess of the corresponding methyl ester was present during the transesterification of the cholesteryl ester, the trend toward greater formation of cholesterol oxides in the most highly unsaturated esters was also observed.

Two other procedures for the transesterification or saponification of cholesteryl esters under mild conditions were evaluated. In the method reported by Christie (21) a mixture of dry ether and methyl acetate is the solvent and sodium methoxide in methanol is the methylating agent. In our hands, the methanolysis of cholesteryl stearate and oleate was complete after one hr at room temperature as reported, but 7-ketocholesterol gave indications of decomposition. In a modification of the method of Lepage (25), a partly aqueous system was tested, but 1N NaOH was used instead of the recommended acetyl chloride, because the hydrogen chloride generated is known to decompose epoxides. The solvent was methanol/benzene/water (71:19:10, v/v/v) and the reaction temperature was 40 C. Cholesteryl esters required 24 hr for complete saponification, but 7-ketocholesterol appeared to be stable. Both of these alternative procedures had obvious disadvantages compared to the modified Glass method.

REFERENCES

1. Smith, L.L., in *Cholesterol Autoxidation*, Plenum Press, New York, 1981 , pp. 49–62.
2. Addis, P.B., A.S. Csallany and S.E. Kindom, in *ACS Symposium Series No. 234*, edited by J.W. Finley and D.E. Schwass, American Chemical Society, Washington, DC, 1983, pp. 85–98.
3. Peng, S.-K., and C.B. Taylor, in *Dietary Fats and Health*, edited by E.G. Perkins and W.J. Visek, American Oil Chemists' Society, Champaign, IL, 1983, pp. 919–933.
4. Sevanian, A., and A.R. Peterson, *Proc. Nat. Acad. Sci. USA 81*:4198 (1984).
5. Park, S.W., and P.B. Addis, *J. Agric. Food Chem. 34*:653 (1986).
6. Bascoul, J., N. Domergue, M. Olle and A. Crastes de Paulet, *Lipids 21*:383 (1986).
7. Fischer, K.-H., G. Laskawy and W. Grosch, *Z. Lebensm. Unters. Forsch. 181*:14 (1985).
8. Luby, J.M., J.I. Gray, B.R. Harte and T.C. Ryan, *J. Food Sci. 51*:904 (1986).
9. Finocchiaro, E.T., and T. Richardson, *J. Food Prot. 46*:917 (1983).
10. Tsai, L.S., and C.A. Hudson, *J. Food Sci. 50*:229 (1985).
11. Missler, S.R., B.A. Wasilchuk and C. Merritt Jr., *Ibid. 50*:595. (1985).
12. Tu, C., W.D. Powrie and O. Fennema, *Ibid. 32*:30 (1967).
13. Chicoye, E., W.D. Powrie and O. Fennema, *Lipids 3*:335 (1968).
14. Maerker, G., and J. Unruh Jr., *J. Am. Oil Chem. Soc. 63*:767 (1986).
15. Maerker, G., *Ibid. 64*:388 (1987).
16. Csiky, I., *J. Chromatogr. 241*:381 (1982).
17. Park, S.W., and P.B. Addis, *J. Food Sci. 50*:1437 (1985).
18. Chicoye, E., W.D. Powrie and O. Fennema, *Ibid. 33*:581 (1968).
19. Maerker, G., and F.J. Bunick, *J. Am. Oil Chem. Soc. 63*:771 (1986).
20. Glass, R.L., *Lipids 6*:919 (1971).
21. Christie, W.W., *J. Lipid Res. 23*:1072 (1982).
22. Fieser, L.F., and S. Rajagopalan, *J. Am. Chem. Soc. 71*:3938 (1963).
23. Davis, M., and V.A. Petrow, *J. Chem. Soc.* 2536 (1949).
24. Tsai, L.S., K. Ijichi, C.A. Hudson and J.J. Meehan, *Lipids 15*:124 (1980).
25. Lepage, G., and C.C. Roy, *J. Lipid Res. 27*:114 (1986).

[Received June 29, 1987;
accepted October 22, 1987]