HPLC Separation and Quantitation of Cholesterol Oxidation Products with Flame Ionization Detection[1]

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Several products of cholesterol oxidation have been found in and isolated from foods and have been reported to have adverse biological activity. The successful separation and quantitation by isocratic HPLC of a wide polarity range of cholesterol oxidation products has eluded chromatographers. This study reports the development of an effective HPLC method for the separation and quantitation of a complex mixture of cholesterol and nine oxidation products by use of a binary solvent system of hexane and 2-propanol in a three-part gradient with detection by an HPLC flame ionization detector. Response factors relative to cholesterol were determined, and coefficients of variation were calculated. Each product gave a linear response ($R > 0.99$) over a concentration range of 100–0.39 μg/100 μL.

The well-known sensitivity of cholesterol to oxidation has given increasing concern to food scientists for two reasons: (a) Certain prominent oxidation products of cholesterol have been implicated in adverse human health effects (Smith, 1981; Addis et al., 1983; Peng and Taylor, 1984; Peng et al., 1985). (b) These oxidation products repeatedly have been demonstrated to be present in a variety of foods (Tsai et al., 1980; Missler et al., 1985; Finocchiaro et al., 1984; Bascoul et al., 1986) that had been processed or stored under oxidizing conditions.

Cholesterol oxides, a term commonly applied to mixtures of cholesterol oxidation products, if present in foods occur in rather low concentration. Their isolation and determination have presented a difficult analytical problem. High-performance liquid chromatographic (HPLC) methods, both normal phase and reversed phase, have been applied to the separation of cholesterol oxides by several investigators (Bascoul et al., 1983; Shen and Sheppard, 1983; Ansari and Smith, 1979; Tsai and Hudson, 1981). In general, normal-phase, i.e. adsorption, liquid chromatographic systems were more effective than reversed phase, but full resolution over the entire polarity range of oxidation products was not achieved with the use of isocratic solvent systems. In some cases separation and quantitation were achieved for narrowly defined groups of oxidation products such as C-7-substituted cholesterol (Park and Addis, 1985) or the isomeric cholesterol 5,6-epoxides (Sugino et al., 1986; Tsai et al., 1980; Tsai and Hudson, 1985). A limited number of cholesterol oxides were separated with the use of a gradient solvent system and UV detection (Csiky, 1982).

Significant improvements in the separation and quantitation of lipids have been achieved by the use of HPLC with gradient elution systems and with flame ionization detection (FID) (Phillips et al., 1984; Smith et al., 1985; Norman and St. John, 1986).

The purpose of this work was to study the separation and quantitation of mixtures of cholesterol oxides by use of HPLC with FID detection.

EXPERIMENTAL SECTION

Materials. Cholesterol oxide standards were purchased from Sigma Chemical Co. (St. Louis, MO), Steraloids, Inc. (Wilton, NH), and Research Plus, Inc. (Bayonne, NJ).

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Table I

peak no.	compound	rel ret volume (K')	rel resp factor	coeff of variation, % ($n = 5$)
1	cholesta-3,5-dien-7-one	0.15	1.08	7.2
2	cholest-5-en-3β-ol	1.17	1.00	4.4
3	cholest-5-ene-3β,25-diol	3.17	1.05	2.3
4	cholesterol 5α,6α-epoxide	5.34	1.33	3.7
5	cholesterol 5β,6β-epoxide	6.17	1.17	3.3
6	5α-cholestan-6-one	6.81	1.46	2.9
7	3β-hydroxycholest-5-en-7-one	8.13	1.31	5.0
8	cholest-5-ene-3β,7β-diol	12.45	0.94	2.9
9	cholest-5-ene-3β,7α-diol	12.79	1.00	3.5
10	5α-cholestane-3β,5,6β-triol	14.75	1.48	2.6

Cholesterol 5β,6β-epoxide was prepared from cholesterol via 5α-cholestane-3β,5,6β-triol (Fieser and Rajagopalan, 1963) and the corresponding triacetate (Davis and Petrow, 1949) by the method of Chicoye et al. (1968). The β-epoxide was purified by preparatory thin-layer chromatography (TLC). All purchased cholesterol oxide standards had a purity of 96% or greater by HPLC and gas chromatography (GC) and were used as received, except cholesta-3,5-dien-7-one which was upgraded to 99% purity by preparatory TLC. All solvents used were distilled in glass grade and were degassed by vacuum filtration through a 0.2-μm filter. TLC plates, silica gel G and GHL (250 μm) were purchased from Analtech (Newark, DE).

Liquid Chromatography. Analytical HPLC was performed with a Waters Associates system consisting of two Model 6000A pumps, a Model 721 system controller, and a Model 730 data module. Injections were made via an Altex Model 210 injector equipped with a 100-μL injection loop. The detector was a Tracor Model 945 flame ionization HPLC detector (Tracor Instruments, Austin, TX). Chromatograms were recorded and integrated on an HP 3390A integrator (Hewlett-Packard, Avondale, PA). Normal-phase separations were performed on a 3.9 mm × 30 cm, 10-μm μ-Porasil column (Waters Associates) at ambient temperature.

Separation of the 10 compounds of interest was achieved with a gradient solvent system at a total flow rate of 1.5 mL/min. The mobile phase consisted of solvent A, which was hexane-2-propanol (100:3, v/v), and solvent B, which was 2-propanol. At injection the solvent was 100% A. The solvent composition changed concavely (Waters curve 8) over a period of 15 min to 98.5% A and 1.5% B. At 15 min the solvent composition changed linearly over a period of 10 min to 97.0% A and 3% B. During the following 15 min the solvent composition changed linearly to 50% A and 50% B.

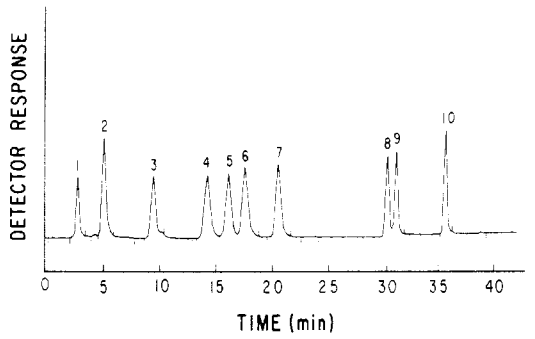

Figure 1. HPLC of cholesterol and nine oxidation products, each at 12.5 μg/100 μL. Peaks numbered as in Table I.

Thin-Layer Chromatography. Analytical and preparative TLC were performed as described previously (Maerker and Bunick, 1986b).

RESULTS AND DISCUSSION

The cholesterol oxidation products of interest in this study are listed in Table I together with their retention volumes and response factors. Not all of these oxidation products are seen often in the same mixture when cholesterol in foods is subjected to oxidation. When cholesterol is oxidized in solution or dispersion, the most prominent products are 3β-hydroxycholest-5-en-7-one (peak 7), the two isomeric cholest-5-ene-3β,7-diols (peaks 8 and 9), and the two isomeric cholesterol 5,6-epoxides (peaks 4 and 5) (Maerker, 1987). If oxidation occurs in neutral or acid medium, or if the oxidation products are put in contact with aqueous media of pH 7 or less, the 5α-cholestane-3β,5,6β-triol (peak 10), which is a hydration product of the two epoxides, is likely to be present. The first compound, cholesta-3,5-dien-7-one (peak 1) is likely to be present when the substrate containing 3β-hydroxycholest-5-en-7-one has been exposed to pH 7 or greater, for instance when the cholesterol oxides have been isolated from the unsaponifiable residue after the saponification of lipids. The compound represented by peak 3 (cholest-5-ene-3β,25-diol) is usually seen only when cholesterol is oxidized in the solid state. The compounds of greatest concern from the standpoint of biological activity are those represented by peaks 3, 4, and 10.

Isocratic adsorption HPLC with hexane–2-propanol (97:3) gives poor resolution of some of the cholesterol oxidation products and fails to elute the most polar of these, 5α-cholestane-3β,5,6β-triol (Tsai and Hudson, 1981; Shen and Sheppard, 1983). The gradient solvent system developed for the current investigation starts with essentially the same solvent system used by others, hexane–2-propanol (100:3) and overcomes the deficiencies of the isocratic procedure, as shown in the chromatogram of Figure 1. Excellent resolution between peaks and symmetrical peak shapes (peak asymmetry factor 1.0–1.25) were observed with the use of the solvent gradient. The FID detector used in this system, while it has its obvious limitations regarding solvent volatility (Smith et al., 1985), is well suited for the quantitation of cholesterol oxides and makes an important contribution to the effectiveness of this procedure.

The relative retention volumes (K') calculated for the individual cholesterol oxides (Tsai and Hudson, 1981) appear in Table I. It is interesting to compare the order of elution of components in this HPLC system with that obtained by capillary GC (Maerker and Unruh, 1986). The order of elution of cholesterol and 3β-hydroxycholest-5-en-7-one, of the isomeric cholesterol 5,6-epoxides, and of the isomeric cholest-5-ene-3β,7-diols is reversed in the two

Table II. Linearity of Response in the Range 100–0.39 μg[a]

peak no.	compound	slope[b]	y int	rms error
1	cholesta-3,5-dien-7-one	0.169	0.251	0.318
2	cholest-5-en-3β-ol	0.157	0.0191	0.184
3	cholest-5-ene-3β,25-diol	0.165	−0.301	0.243
4	cholesterol 5α,6α-epoxide	0.209	−0.294	0.255
5	cholesterol 5β,6β-epoxide	0.184	−0.177	0.201
6	5α-cholestan-6-one	0.230	−0.314	0.277
7	3β-hydroxycholest-5-en-7-one	0.206	−0.182	0.229
8	cholest-5-ene-3β,7β-diol	0.147	−0.249	0.214
9	cholest-5-ene-3β,7α-diol	0.156	−0.332	0.440
10	5α-cholestane-3β,5,6β-triol	0.232	−0.939	1.017

[a] All calculations based on eight concentration points, except peaks 3 and 7 which were based on seven points. [b] Correlation coefficient for all slopes was >0.99.

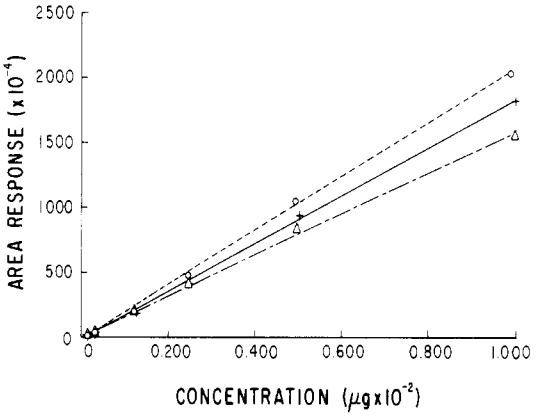

Figure 2. Concentration vs detector response of three of the components of the 10-component mixture: (Δ) cholest-5-en-3β-ol, (+) cholesterol 5β,6β-epoxide, and (O) 3β-hydroxycholest-5-en-7-one.

chromatographic procedures. Furthermore, cholest-5-ene-3β,25-diol, which is not well resolved from the cholest-5-ene-3β,7-diols by GC, except after silylation of the mixture, elutes well ahead of the cholest-5-ene-3β,7-diols in the HPLC procedure.

In experiments conducted to define the parameters of the system, solutions were employed containing equal weights of each of the 10 components, and 100 μL of each solution was injected. For studies of linearity of response, a stock solution of all of the components was prepared in hexane–2-propanol (100:3) and then serially diluted as needed. Concentrations employed in this study ranged from 100 μg/component per injection to 0.39 μg/component per injection. At the lowest concentration (highest dilution) level (0.39 μg/component) all components still gave substantial area counts (20 000–100 000). Linearity of response data are shown in Table II and illustrated in Figure 2. The data indicate that linearity of response over the concentration range is excellent for all components except for the more polar triol (peak 10, 5α-cholestane-3β,5,6β-triol) where a somewhat greater scatter of points is seen.

Reproducibility of response was tested by replicate (n = 5) injection of the mixtures of 10 standards, each at 12.5 μg/injection. Coefficients of variation were calculated [(standard deviation/mean) × 100] and are given in Table I. Relative response factors (Table I) were calculated from the slopes (Table II) of the concentration vs detector response lines. The slope of cholesterol was taken as unity.

The results of this study indicate that normal-phase gradient HPLC with FID detection is an effective procedure for the separation and quantitation of individual members of complex mixtures of cholesterol oxidation

products. Though the lower detection limits of this HPLC methodology do not compare favorably with the more sensitive capillary GC analysis (Maerker and Unruh, 1986), the HPLC method is particularly effective when the need arises to separate and measure cholesterol oxides that are sensitive to the elevated temperatures of GC (Maerker, 1987) and that are unresolvable by TLC.

ACKNOWLEDGMENT

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Registry No. Cholestra-3,5-dien-7-one, 567-72-6; cholesterol, 57-88-5; cholest-5-ene-3β,25-diol, 2140-46-7; cholesterol 5α,6α-epoxide, 1250-95-9; cholesterol 5β,6β-epoxide, 4025-59-6; 5α-cholestan-6-one, 570-46-7; 3β-hydroxycholest-5-en-7-one, 566-28-9; cholest-5-ene-3β,7β-diol, 566-27-8; cholest-5-ene-3β,7α-diol, 566-26-7; 5α-cholestane-3β,5,6β-triol, 1253-84-5.

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Analysis of Chlorimuron Ethyl in Crops by High-Performance Liquid Chromatography

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Methods are presented for the residual determination of chlorimuron ethyl in soybeans and some soybean rotational crops (wheat, corn kernels, potatoes, turnips). Chlorimuron ethyl was extracted from the matrix and cleaned up by one or two of four available methods (a doublet Bond Elut Si column, Sep Paks, a LiChroprep Si60 medium-pressure liquid chromatography column, or aqueous–organic solvent partitioning). The compound was then quantitated in a normal-phase HPLC employing a photoconductivity detector. The minimum detection level was 0.01 ppm as established with fortification recovery experiments, and recoveries averaged above 90% for 0.01 to 0.1 ppm fortifications.

Chlorimuron ethyl (Figure 1) is the active ingredient of a new selective soybean herbicide sold by Du Pont under the trade name of CLASSIC herbicide. This molecule belongs to a class of compounds called sufonylureas. These compounds in general are thermally unstable, rendering them difficult to detect by gas chromatography procedures; however, several investigators have made sulfonylurea derivatives detectable by gas chromatography (Braselton et al., 1975, 1976, 1977; Midha et al., 1976; Kleber et al., 1977; Prescott and Redman, 1972; Sabih and Sabih, 1970; Simmons et al., 1972; Hartvig et al., 1980). Other investigators used liquid chromatography with ultraviolet absorbance detectors to analyze for these compounds (Beyer, 1972; Harzer, 1980; Molins et al., 1975; Raghow and Meyer, 1981; Reinaure et al., 1980; Robertson et al., 1979; Sved et al., 1976; Tsugi and Binns, 1982; Uihlein and Sistovaris, 1982; Waahlin-Boll and Melander, 1979; Weber, 1976). Kimura et al. (1980) compared gas chromatography and liquid chromatography methods and found similar sensitivities and reproducibilities.

Some other approaches used for sulfonylurea analyses include radioimmunoassay (Kajinuma et al., 1982; Kelley et al., 1985), bioassay (Hsiao and Smith, 1983; Bond and Roberts, 1976), hydrolysis followed by fluorescence detection of the dansyl derivative (Huck, 1978), and precolumn derivatization followed by fluormetric detection (Besenfelder, 1981).

Recent approaches at analyzing pesticides, including sulfonylureas, have made use of the liquid chromatography, photoconductivity detector. This detector is sensitive and selective for sulfur, halogens, nitrogen, and phosphorus atoms. Studies using this detector for pesticides have been reported by Buttler and Hormann (1981), Walters (1983),

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