Separation and Quantitation of Cholesterol Oxides by HPLC with an Evaporative Light Scattering Detector in a Model System

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ABSTRACT: A method to analyze cholesterol and 10 of its oxidation products, ranging from the weakly polar cholest-4-ene-3,6-dione to moderately polar cholest-5-ene-3β,7α-diol, in a single run is described. The separation was achieved by normal-phase gradient high-performance liquid chromatography with an evaporative light-scattering detector. This universal mass detector does not detect changes in solvent composition; this makes it possible to employ gradients, an essential technique whenever a wide range of compounds with diverse characteristics is to be separated. Standards at concentrations from 0.1–1.0 µg were separated within 37 min on an alumina/silica column with a gradient elution system that contained dichloromethane, acetonitrile, and water.
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KEY WORDS: Cholesterol, cholesterol oxidation products, evaporative light-scattering detection, HPLC, quantitation.

Cholesterol and cholesterol oxidation products (COP) have been investigated extensively; many methods for their determination exist because clinical and animal studies have confirmed that a number of COP are cytotoxic, mutagenic, and carcinogenic (1–3). Most of the earlier analytical procedures were performed by thin-layer chromatography (TLC) or gas–liquid chromatography (GC) or both. TLC is primarily a qualitative technique; its utility is somewhat limited due to loading capacity restrictions and the fact that the technique exposes these air-sensitive compounds to large surface areas, which increases their potential to autooxidize during the operation. In GC, high inlet and oven temperatures often result in the thermal destruction of heat-sensitive compounds or their decomposition into other oxidized components (4). Saponification by means of heating and/or the preparation of volatile derivatives may not be quantitative and can result in the formation of artifacts (5–7). Currently, high-performance liquid chromatography (HPLC) is the method of choice because COP analysis can be achieved without the need to form derivatives; hence, it is faster, needs less manipulation, and in theory, should be more accurate than TLC or GC.

This paper describes an HPLC method with a normal-phase column and an evaporative light-scattering detector (ELSD) for the separation of COP with a wide range of polarities, from the weakly polar A-ring oxidation products to the more polar B-ring cholesterol oxides. Many of the previously published procedures were limited to the separation of narrowly defined groups of COP. Advantages of employing an ELSD are it will detect all solutes as long as the analyte is less volatile than the mobile phase, detector response is based on mass, and it has greater sensitivity than refractive index (RI) and ultraviolet (UV) detectors at low wavelengths with no baseline shifts due to gradient changes or solvent fronts. Disadvantages include a limited range of linear response and the need for solutes to be less volatile than the solvent (8).

MATERIALS AND METHODS

Reagents. The cholesterol oxide standards were purchased from Research Plus, Inc. (Bayonne, NJ), Sigma Chemical Co. (St. Louis, MO), or Steraloids, Inc. (Wilton, NH). Solvents were "distilled in glass grade," and the water was double deionized, glass-distilled. HPLC solvents were degassed by helium sparge.

HPLC. HPLC was conducted with a system that was equipped with a Waters pump, model 510 (Waters Associates, Milford, MA), and controlled by an Autochrom CIM (Milford, MA) gradient programmer. A Rainin A1-1 autosampler (Woburn, MA) with a 20-µL loop was used to apply the samples. Separations were achieved with a normal-phase 5-µm Chromega column, packed with 16% alumina/84% silica (250 mm × 46 mm i.d.) (ES Industries, Berlin, NJ), and a guard column (20 mm × 2 mm), packed with 5-µm Lichrosorb Si60 (EM Science, Gibbstown, NJ). The eluent flow rate through the chromatographic column was 1.0 mL/min. The column was equilibrated with a mobile phase consisting of 94% A (A = dichloromethane) and 6% B (B = acetonitrile/water, 99.05:0.95, vol/vol). The gradient program was 0 to 6 min (94:6/A:B), then 79:21/A:B and maintained for the next 27 min. A linear gradient (3%/min) was then initiated for 5 min to return to the initial concentration of 94:6/A:B. A Varex Universal evaporative light-scattering detector, Model IIA (Rockville, MD) was operated at a temperature of 75°C with nitrogen (Air Products, grade B, 99.5%

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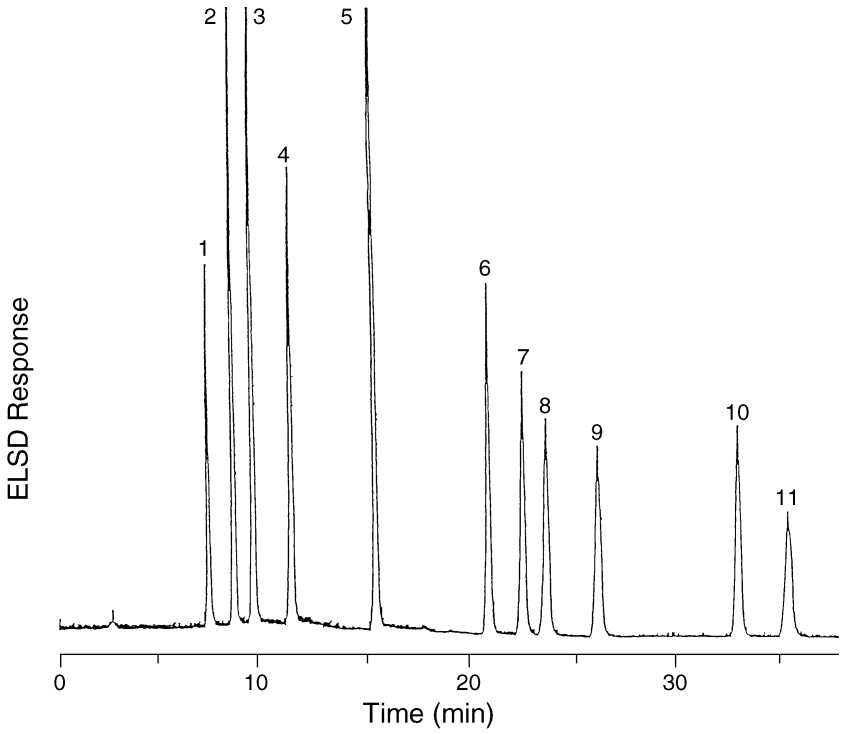

FIG. 1. High-performance liquid chromatography chromatogram of a mixture of cholesterol and cholesterol oxide standards with an evaporative light-scattering detector, an Al/Si column, and a binary gradient of dichloromethane/acetonitrile–water. Peak identification is given in Table 1.

pure) as the nebulizing gas at 1.5 L/min. A PE Nelson 1020 (Perkin Elmer, Norwalk, CN) integrator was used to collect and process the data.

RESULTS AND DISCUSSION

The complete separation of cholesterol and 10 sterol standards (Table 1) on an alumina/silica column by a step-gradient elution system, composed of dichloromethane and a mixture of acetonitrile/water, is shown in Figure 1. A silica column (5 µm, 250 mm × 46 mm) was insufficiently polar to resolve the A-ring COP (peak no. 1–3).

The minimum detectable limit (MDL) at a 3:1 signal-to-noise ratio was determined to be 75 ng/injection. The sensitivity of the ELSD is controlled by a number of factors (9). To determine the optimal conditions needed to maximize detector response while still maintaining a high degree of reproducibility and a stable baseline, different temperatures and carrier gas flow rates were examined in a sequential manner as suggested by the manufacturer. Cholesterol and cholest-4-en-3-one were injected onto the alumina/silica HPLC column, and the effects of different evaporator tube temperatures (70–95°C) and nebulizer gas flows (1.5–2.35 L/min) on detector response were recorded (Table 2). The detector was most sensitive to changes in nebulizer gas flow rate. Increasing the flow rate results in decreasing droplet size with a con-

TABLE 1
Peak Identification

Peak no.	Compound	Trivial name
1	Cholest-4-ene-3,6-dione	4-Ene-3,6-dione
2	Cholest-4-en-3-one	4-En-3-one
3	Cholest-4,6-dien-3-one	4,6-Dien-3-one
4	Cholest-5-en-3β-ol	Cholesterol
5	20α-Hydroxycholest-5-en-3β-ol (I.S.)[a]	20α-Hydroxycholesterol
6	3β-Hydroxycholest-5-en-7-one	7-Ketocholesterol
7	5,6β-Epoxy-5α-cholestan-3β-ol	β-Epoxide
8	5,6α-Epoxy-5α-cholestan-3β-ol	α-Epoxide
9	3β-Hydroxy-5α-cholestan-6-one	6-Ketocholestanol
10	Cholest-5-ene-3β,7β-diol	7β-Hydroxycholesterol
11	Cholest-5ene-3β,7α-diol	7α-Hydroxycholesterol

[a]I.S.= Internal standard.

TABLE 2
Effect of Drift Tube Temperature and Nebulizer Gas Flow Rate on ELSD Response

		4-En-3-one[c]			Cholesterol			
Temperature (°C)	Flow[a]	Area	Ht.	S/N[b]	Area	Ht.	S/N[b]	Noise
70	1.88	8557	0.999	58.74	6832	0.588	34.57	0.017
75	1.50	19734	2.159	58.35	16277	1.401	37.87	0.037
75	1.88	9375	1.040	57.79	7588	0.629	34.99	0.018
80	1.50	18106	2.063	30.34	14257	1.242	18.26	0.068
80	1.88	9980	1.130	32.29	7803	0.677	19.34	0.035
80	2.35	5636	0.659	50.75	4460	0.393	30.19	0.013
85	1.50	18084	1.875	23.15	14238	1.234	15.23	0.081
85	1.88	9880	1.111	39.67	7857	0.673	24.05	0.028
90	1.88	10106	1.142	29.29	8536	0.697	17.87	0.039
95	1.88	10359	1.183	18.78	8096	0.697	11.06	0.063

[a]Nitrogen (L/min).
[b]Signal-to-noise ratio.
[c]4-Cholesten-3-one.

comitant effect on detector response, according to Mourey and Oppenheimer (10). Increasing the temperature resulted in relatively minor changes in detector response when compared to the effect of the flow rates. Lower temperatures and higher flow rates generally increased the signal-to-noise ratio. As a result, the heated detector drift tube was operated at 75°C with a nitrogen flow rate of 1.5 L/min.

Linearity. ELSD response has been reported to be sigmoidal, exponential, and linear within limited concentration ranges (11,12). For cholesterol, a linear concentration range between 11–36 µg was reported by Spanos and Schwartz (13), and Moreau (8) reported a linear range for lipids between 10–200 µg but parabolic behavior below 10 µg. The quantity of COP examined in this study ranged between 100–1000 ng. Ten dilutions for each of the 11 compounds were prepared. All were spiked with the 20α-hydroxycholesterol, the internal standard (I.S.), and each dilution was chromatographed twice to result in 220 separate determinations. A plot of the data, area as a function of concentration, resulted in a parabolic-shaped curve. The data were subjected to analysis with "SigmaPlot," a curve-fitting program (Jandel Scientific, Corte Madera, CA). The equation that resulted in the best fit for the data points was a power function of the form:

$$\text{Area} = k(\text{conc})^m$$
$$\ln(\text{area}) = \ln K + m \ln(\text{conc}) \qquad [1]$$

where m = the coefficient of the curvature in the concentration/peak area curves. The ln(area) vs. ln(conc) plots (Fig. 2) of the data resulted in average slopes of $m = 1.634 \pm 0.024$ SD with correlation coefficients > 0.991 (Table 3). Although the area was curvilinear with concentration, the curvature m was the same for all compounds.

Repeatability. To determine the precision of the method, and in particular the consistency of response of the ELSD, a solution containing each sterol (500 ng) plus the I.S. (500 ng) in 95:5 CH_2Cl_2/CH_3CN was injected five times. The repeatability of the procedure can be gauged by the standard error of the means, which is listed for the areas (0.91–3.10%), for the retention times (0.08–1.21%), and for the normalized areas (0.88–3.34%). To facilitate comparisons of peak areas, the results were normalized to the peak area of the I.S. ($\text{Area}_{\text{sample}}/\text{Area}_{\text{I.S.}}$). The deviation in relative retention times, $R_{t,\text{sample}}/R_{t,\text{I.S.}}$, of 1% or less indicates that the chromatography is reproducible.

This study describes a method that is capable of separating cholesterol oxides with a wide variety of polarities.

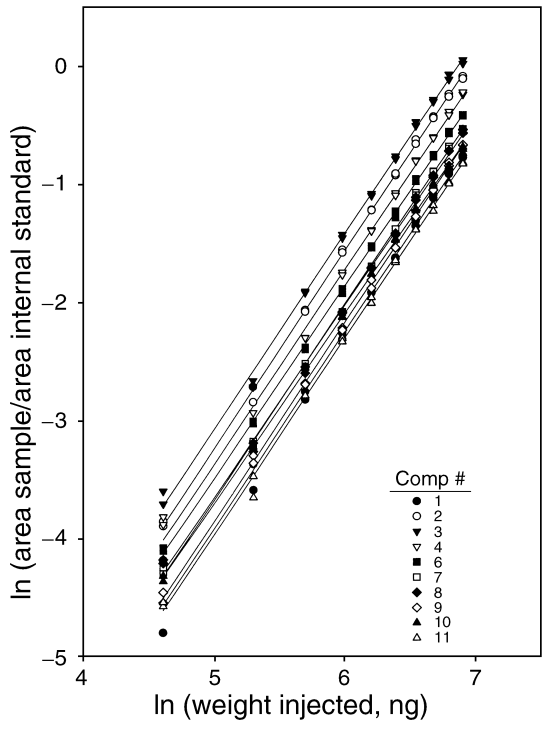

FIG. 2. Logarithmic (ln) response curves for cholesterol and cholesterol oxides with an evaporative light-scattering detector. Normalized peak areas versus concentration (0.1–1.0 g). Peak identification is given in Table 1.

TABLE 3
Linear Regression Equations for Cholesterol and Cholesterol Oxides

Compound	Slope	Intercept	R^2
1	1.65	–12.17	0.991
2	1.66	–11.52	0.999
3	1.64	–11.28	0.998
4	1.63	–11.52	0.993
6	1.61	–11.55	1.000
7	1.64	–11.88	0.999
8	1.61	–11.69	0.998
9	1.66	–12.17	0.999
10	1.59	–11.64	1.000
11	1.65	–12.19	0.998

ELSD, a universal detector, permits the use of numerous combinations of volatile solvents to form gradients that make it possible to achieve complete separation without the need to employ other separation techniques. The data indicate that this detector is stable, and the results are reproducible and appropriate when analyzing for COP by HPLC.

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