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Comparative high-performance liquid chromatographic analyses of cholesterol and its oxidation products using diode-array ultraviolet and laser light-scattering detection

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Abstract

Comparative high-performance liquid chromatographic (HPLC) analyses of cholesterol and its oxidization products (COPS) showed that cholesterol, cholestane, 7-ketocholesterol and 7α, 7β-, 20- and 25-hydroxycholesterol were detected by both ultraviolet (UV) and laser light-scattering detection (LLSD). In addition, the use of LLSD allowed the detection of cholestanetriol and α- and β-epoxides. The limits of detection of COPS varied from 0.5 to 25 μg/ml, depending on both the compound and the detector. The HPLC analyses demonstrated a linear correlation between the UV response and concentrations of products in the range 0–500 μg/ml whereas a linear correlationship with LLSD was obtained by plotting logarithmic coordinates in the same range.

1. Introduction

Cholesterol oxidation products (COPS) are found in many common foods and have been shown to be atherogenic, cytotoxic, mutagenic and possibly carcinogenic [1–3]. Because of these properties, concern has been expressed that substantial amounts may form in cholesterol-containing foods during storage and processing [3,4]. Cholesterol α-oxide along with the β-isomer have received much attention owing to their possible association with carcinogens, atherogenesis and cholesterol metabolism [5]. Gray et al. [6] have found abnormally high concentrations of cholesterol α-oxide in the sera of hypercholesterolaemic and high blood pressure pateints but none in those of normal healthy persons. Addis et al. [7] suggested that the quantification of COPS in human plasma lipoproteins could be used as a tool for studying their potential role in heart disease.

The increasing interest in the biological role of COPS has led to the development of chromatographic methods for their identification and determination [8]. Park and Addis [9] reported the use of capillary gas chromatography (GC) for the determination of COPS in foods. Combined GC and mass spectrometry (MS) has also

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been used for the identification of COPS [10–13]. MS, although being considered the best detection method available, requires expensive instrumentation and is not routinely available to most investigators [4].

The development of high-performance liquid chromatography (HPLC) for the determination of COPS in foods [12] and plasma from rats [4] has also been reported. Tsai and Hudson [14] demonstrated that HPLC could resolve a variety of oxygenated derivatives of cholesterol with polar groups on various carbon atoms of the isoprenoid side-chain. The method proposed by Csiky [15] did not give a good baseline resolution of 25-hydroxycholesterol, as it appeared as a shoulder peak on a large UV absorption peak.

An important issue in the determination of cholesterol oxides concerns the autoxidation of cholesterol [16]. Several studies have appeared in which the extent of cholesterol oxide artifact during analysis was determined by HPLC with radioactivity detection [16] and GC–MS and stable isotope techniques [17].

Stolyhwo et al. [18] reported that laser light-scattering detection (LLSD) is extremely unresponsive to changes in the physical environment that result in the absence of background and baseline drift, even with a gradient elution system. Although LLSD has been reported as a reliable tool in the HPLC of triacylglycerols [19], little is known about its application for the detection of cholesterol and its COPS. As far as the authors are aware, there is no information regarding the use of LLSD in the HPLC of cholesterol and COPS. The objective of this research was to develop a rapid and accurate comparative method for the characterization and determination of cholesterol oxidation products using ultraviolet (UV) and LLS detection.

2. Experimental

2.1. Materials

Standards of cholesterol and its derivatives were purchased from Sigma (St. Louis, MO, USA) and Research Plus (Bayonne, NJ, USA). These standards included cholesta-3,5-dien-7-one (cholestane), 3β-hydrocholest-5-en-7-one (7-ketocholesterol), cholesta-5-ene-3β,7α-diol (7α-hydroxycholesterol), cholesta-5-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-5-ene-3β,20-diol (20-hydroxycholesterol), cholest-5-ene-3β,25-diol (25-hydroxycholesterol), 5,6α-eopxy-5α-cholestan-3β-ol (α-epoxide), 5,6β-epoxy-5α-cholestan-3β-ol (β-epoxide) and 5α-cholestane-3β,5α,6β-triol (cholestanetriol). Chloroform, 2-propanol and hexane (Omnisolv grade) were BDH products (CM Industries, Hawthorne, NY, USA). A mixture was prepared to contain 500 μg/ml of cholestane, cholesterol, 20-hydroxycholesterol, 25-hydroxycholestero, α-epoxide, β-epoxide, 7-ketocholesterol, 7α-hydroxycholesterol and cholestanetriol and also 250 μg/ml of 20-hydroxycholesterol, β-epoxide and 7β-hydroxycholesterol.

2.2. Apparatus

The mixture of cholesterol and oxidation products (COPS) was separated on a μ-Porasil (10 μm) HPLC column (300 × 3.9 mm I.D.) obtained from Waters–Millipore (Bedford, MA, USA). The HPLC system used for the analyses was Beckman Gold (Beckman Instruments, San Ramon, CA, USA) using computerized integration and data handling (Beckman Model 126). The system was equipped with two detectors. The UV absorbance was monitored with a Beckman diode-array UV detector (Model 168) whereas the light scattering was determined with a laser light-scattering detector (Varex, Burtonsville, MD, USA). A Beckman analog interface (Model 406) was used to transfer data from the mass detector to the Gold system. Injection was achieved through a Model 9095 automatic injector (Varian, Walnut Creek, CA, USA) fitted with a 20-μl loop.

2.3. HPLC of COPS using UV detection

HPLC of COPS with UV detection were performed according to a modification of the procedure described by Csallany et al. [20]. Elution was carried out with a gradient elution

system, using a mixture of hexane and 2-propanol containing from 99.6% to 79.3% of hexane at a flow-rate of 1 ml/min for 56 min. UV detection was performed simultaneously at two different wavelengths, 206 and 233 nm.

2.4. HPLC of COPS using laser light-scattering detection

The HPLC separation of COPS was performed with a mixture of hexane and 2-propanol, using a linear decreasing gradient from 99.6% to 79.3% of hexane, at a flow-rate of 1 ml/min for 56 min. Detection was performed at a temperature setting of 120°C and a stream of inert gas (N_2) at a flow-rate of 40 ml/min and a sensitivity range of 1.

3. Results and discussion

3.1. Optimization of HPLC analyses

UV maximum absorbance analyses of standard COPS compounds were performed. The results (data not shown) indicated that 7α-and 7β-hydroxycholesterol exhibited one specific maximum absorbance (λ_{max}) at 207 and 208 nm, respectively, whereas 7-ketohydroxycholesterol demonstrated two specific λ_{max} at 207 and 234 nm. Csallany et al. [20] reported that the λ_{max} for 7-ketohydroxycholesterol is 202 nm whereas that of cholesterol, 7α- and 7β-hydroxycholesterol and 25-hydroxycholesterol is 233 nm. Hence, to achieve the optimum detection of cholesterol and COPS, the HPLC–UV analyses were performed using two different wavelengths, 206 and 233 nm.

Preliminary work on the optimization and selection of the most appropriate temperature and N_2 flow-rate for setting the LLS detector indicated that both high sensitivity and a stable baseline were obtained for COPS at 120°C with a flow-rate of inert gas of 30 ml/min. The optimization of the conditions for HPLC–LLSD demonstrated that a gradient elution solvent system from 99.6% to 79.3% of hexane at a flow-rate of 1 ml/min for 56 min provided a chromatogram with well separated and highly resolved peaks. The available literature on LLSD [18,19,21] suggested that, in addition to instrumental factors, optimization of factors such as temperature and gas flow-rate are also important for the sensitivity.

3.2. UV and LLS detection of standard cholesterol and COPS

Fig. 1 shows the chromatograms for the HPLC of cholesterol and COPS using (A and B) UV detection and (C) LLSD. The results demon-

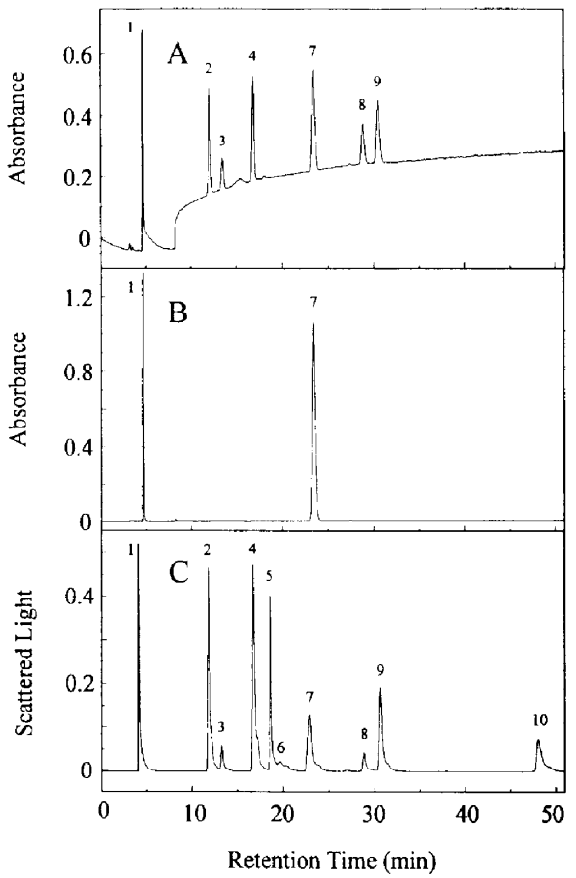

Fig. 1. HPLC of cholesterol and its oxidization products on a (μ-Porasil (10-μm) column (300 × 3.9 mm I.D.) with (A,B) diode-array UV detection at (A) 206 nm and (B) 233 nm and (C) laser light-scattering detection. Peaks: 1 = cholestane; 2 = cholesterol; 3 = 20-hydroxycholesterol; 4 = 25-hydroxycholesterol; 5 = α-epoxide; 6 = β-epoxide; 7 = 7-ketocholesterol; 8 = 7β-hydroxcycholesterol; 9 = 7α-hydroxycholesterol; 10 = cholestanetriol.

strate a much more stable and better baseline with LLSD than that obtained with UV detection. In addition, LLSD provided a higher resolution of COPS than was achieved with UV detection. The results also show that cholesterol, cholestane, 7-ketocholesterol, 7α- and 7β-hydroxycholesterol and 20- and 25-hydroxycholesterol were detected by both UV and LLSD methods, whereas cholestanetriol and α- and β-epoxide were detected only with LLSD. In addition, the use of UV detection at 206 nm allowed an increase in the sensitivity of detection of 7-ketocholesterol.

It has been reported that the use of UV detection in HPLC analyses allowed the determination of cholesterol, 7α- and 7β-hydroxycholesterol, 7-ketocholesterol [9] and 25-hydroxycholesterol [20], whereas the use of refractometric detection also permits the detection of cholestanetriol, α- and β-epoxide and 20-hydroxycholesterol [14]. However, Stolyhwo et al. [18] reported that the LLSD response is more sensitive than that of the refractometer.

3.3. Limits of detection

The minimum detectable concentrations of COPS were calculated on the basis of a signal-to-noise ratio of 3 [20]. The results (Tables 1 and 2) indicate that the limits of detection of cholesterol, cholestane and 7-ketocholesterol (10, 1 and 0.5 μg/ml, respectively) with UV detection were 2.5, 2.5 and 20 times lower than those (25, 2.5 and 10.0 μg/ml, respectively) obtained with LLSD. As the molar absorptivity of 7-ketocholesterol is high [20], its detection by HPLC–UV was the most sensitive. The results (Tables 1 and 2) also show that the limit of detection of 7α-hydroxycholesterol (10.0 μg/ml) with LLSD was 2.5 times lower than that (25 μg/ml) obtained with UV detection. The results also indicate that the limits of detection of 20-hydroxycholesterol (12.5 μg/ml), 25-hydroxycholesterol (10.0 μg/ml) and 7β-hydroxycholesterol (12.5 μg/ml) were the same with both UV and LLS detection. Hence LLSD was the most sensitive for the detection of cholestane, 25-hydroxycholesterol, 7α-hydroxycholesterol, α-epoxide, 7-ketocholesterol and cholestanetriol.

It has been reported that the limit of detection of 25-hydroxycholesterol in serum by HPLC–UV was 10 ng/ml [4], whereas the lowest concentration of this compound in plasma, detected by gas chromatography with flame ionization detection (GC–FID), was 100 ng/ml [7]. The limits of detection of 7α- and 7β-hydroxycholesterol by HPLC–UV were 1.7 and 1.5 μg/ml, respectively, [20] and 50 μg/ml with refractometric detec-

Table 1
Quantitative chromatographic parameters for HPLC of cholesterol and its oxidation products using diode-array UV detection

Compound	Retention time (min)	Detection limit[a] (μg/ml)	Range of linearity[b] (μg/ml)	R^2 [c]	Precision[d]
Cholesterol	11.7	10.0	10.0–500	0.995	1.8
Cholestane	4.2	1.0	1.0–500	0.994	1.5
20-Hydroxycholesterol	13.2	12.5	12.5–250	0.991	6.5
25-Hydroxycholesterol	16.7	10.0	10.0–500	0.991	3.1
7α-Hydroxycholesterol	30.6	25.0	50.0–500	0.999	1.8
7β-Hydroxycholesterol	28.8	12.5	12.5–500	0.992	5.1
7-Ketocholesterol	22.9	0.5	0.5–500	0.996	1.0

[a] Detection limit is the minimum detectable concentration of COPS calculated on the basis of a signal-to-noise ratio of 3.
[b] Range of linearity is determined by the lower and higher limits of quantification of the calibration graph.
[c] Correlation coefficient (R^2) of the calibration graph, calculated on the basis of triplicate injections of each product.
[d] Precision is the percentage deviation of the mean as obtained by three replicate analyses of the sample; the sample contained 50.0 μg/ml of each compound.

Table 2
Quantitative chromatographic parameters for HPLC of cholesterol and its oxidation products using laser light-scattering detection

Compound	Retention time (min)	Detection limit[a] (μg/ml)	Range of linearity[b] (μg/ml)	R^2 [c]	Precision[d]
Cholesterol	11.7	25.0	25.0–500	0.999	18.2
Cholestane	4.2	2.5	2.5–100	0.991	3.0
20-Hydroxycholesterol	13.2	12.5	12.5–500	0.999	12.1
25-Hydroxycholesterol	16.7	10.0	10.0–500	1.000	8.2
7α-Hydroxycholesterol	30.6	10.0	10.0–1000	0.998	6.6
7β-Hydroxycholesterol	28.8	12.5	12.5–500	0.997	2.9
α-Epoxide	18.4	10.0	10.0–500	0.999	5.0
β-Epoxide	19.4	50.0	50.0–500	0.999	11.7
7-Ketocholesterol	22.9	10.0	10.0–1000	0.997	8.3
Cholestanetriol	47.7	10.0	10.0–1000	0.993	8.6

[a–d] See Table 1.

tion [5]. The lowest concentrations of 7α- and 7β-hydroxycholesterol detected by GC–FID were 1 μg/ml [8] and 0.4 μg/ml [7]. The limit of detection of 7-ketocholesterol was reported to be 0.38 μg/ml using HPLC–UV [20], whereas the lowest concentration detected by GC–FID was 0.9 μg/ml [2]. It has also been reported that α- and β-epoxides and cholestanetriol could be determined only with GC–FID and their lowest concentration reported with 1 μg/ml [8,22]. Pie et al. [23] reported that the lowest concentration of 20-hydroxycholesterol detected by GC–FID was 0.57 μg/ml.

Although α- and β-epoxides and cholestanetriol have been separated by HPLC with refractometric detection [14], the determination of these compounds was not reported. Fig. 1 demonstrates that the HPLC–LLSD method developed here allowed the quantification of α- and β-epoxides and cholestanetriol that had previously only been determined using GC–FID [8,22,24]. However, GC–FID may be considered as a destructive method as it requires the derivatization of COPS and heating of the sample, whereas HPLC can be regarded as a non-destructive and rapid method [25]. Hence the development of HPLC–LLSD could provide a reliable method for the determination, under mild conditions, of COPS, particularly α-and β-epoxides and cholestanetriol. However, special attempts to suppress autoxidation of cholesterol should be included in the analyses of foodstuffs and biological samples [16,17].

3.4. Linearity and precision of UV and LLSD responses

In order to test the applicability of HPLC–UV and –LLSD for the determination of cholesterol and major COPS, each compound was investigated for its linear response. Calibration graphs were constructed for cholesterol and each of the COPS studied in the range 0–1000 μg/ml.

The plots of compound concentration versus peak area obtained in HPLC–UV analyses are shown in Fig. 2. The results (Table 1) show that good linearity was obtained for the calibration graphs for HPLC–UV of 7-ketocholesterol in the range 0.5–500 μg/ml whereas the ranges of linearity were 1–500 μg/ml for cholestane, 10–500 μg/ml for cholesterol and 25-hydroxycholesterol, 12.5–500 μg/ml for 7β-hydroxycholesterol, 12.5–250 μg/ml for 20-hydroxycholesterol and 50–500 μg/ml for 7α-hydroxycholesterol. The correlation coefficients (Table 2) for COPS were between 0.991 and 0.999.

The plots of compound concentration versus peak area obtained in HPLC–LLSD analyses demonstrate a non-linear response. However, a linear correlation with LLSD was obtained by

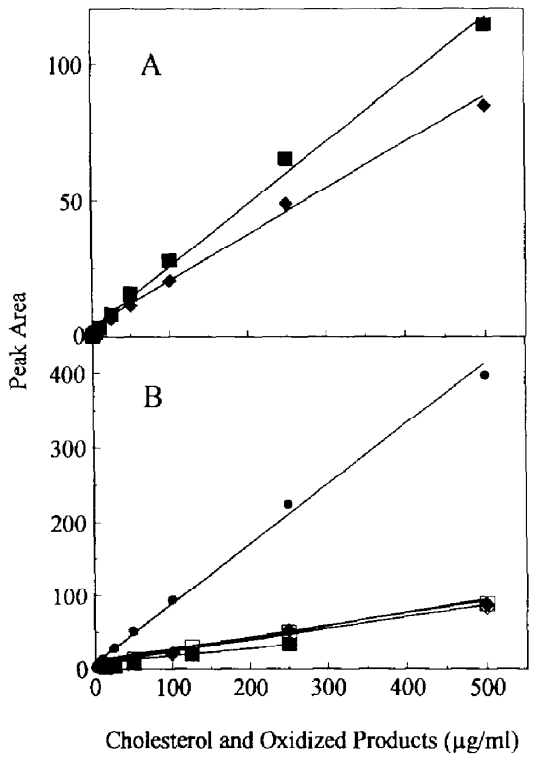

Fig. 2. Calibration graph for HPLC of cholesterol and its oxidization products using diode-array UV detection. (A) ◆ = cholesterol; ● = cholestane. (B) ■ = 20-hydroxycholesterol; ◆ = 25-hydroxycholesterol; □ = 7β-hydroxycholesterol; ◇ = 7α-hydroxycholesterol; ● = ketocholesterol.

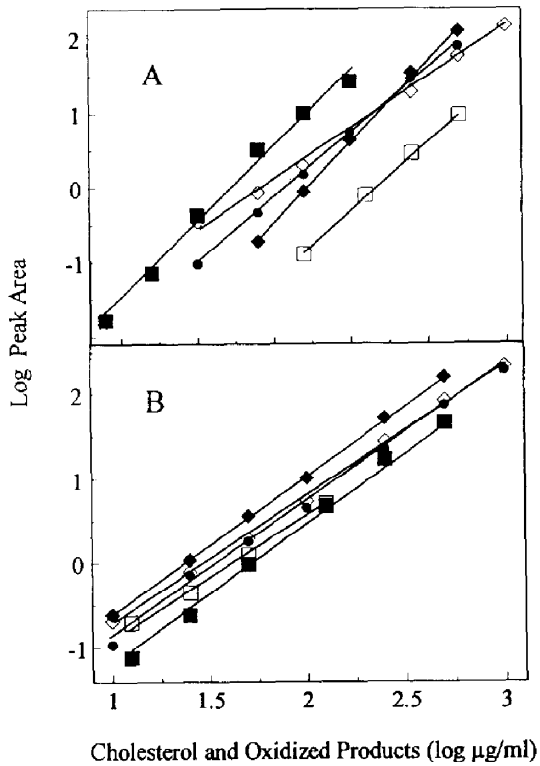

Fig. 3. Calibration graph for HPLC of cholesterol and its oxidization products using laser light-scattering detection. (A) ◆ = cholesterol; ■ = cholestane; ● = α-epoxide; □ = β-epoxide; ◇ = cholestanetriol. (B) ■ = 20-hydroxycholesterol; ◆ = 25-hydroxycholesterol; □ = 7β-hydroxycholesterol; ◇ = 7α-hydroxycholesterol; ● = 7-ketocholesterol.

plotting log peak area versus log (product concentration) (Fig. 3). The results (Table 2) show that good linearity in logarithmic coordinates was obtained for cholestane in the range of 2.50–100 μg/ml whereas the ranges of linearity were 10–1000 μg/ml for 7α-hydroxycholesterol, 7-ketocholesterol and cholestanetriol, 10–500 μg/ml for 25-hydroxycholesterol and α-epoxide, 12.5–500 μg/ml for 20- and 7β-hydroxycholesterol, 25–500 μg/ml for cholesterol and 50–500 μg/ml for β-epoxide. The correlation coefficients (Table 2) were between 0.991 and 1.000. Stolyhwo et al. [18] reported that a plot of peak area versus sample size for dioctyl phthalate was linear in logarithmic coordinates over a ratio of sample size of 500:1. Similar correlation coefficients have been obtained in analyses of cholesterol and COPS using a GC–FID system equipped with a capillary column [26].

The results (Tables 1 and 2) also indicate that the accuracy (1.0–6.5%) of UV detection of COPS was better than that obtained using LLSD (2.9–18.2%). A comparative study of cholesterol analyses [27] indicated that the relative standard deviations (R.S.D.s) for GC and HPLC–UV analyses were 9 and 8%, respectively. Pie et al. [23] reported that the R.S.D.s of GC analyses of COPS varied from 0.4 to 15%. The results obtained here (Tables 1 and 2) demonstrate that

the determination of cholesterol and COPS by HPLC with UV and LLS detection is very reliable.

4. Conclusions

The results suggest that the use of LLSD in the HPLC of cholesterol and COPS could provide a reliable tool for the determination of these compounds. In addition, the HPLC-LLSD analyses allowed the detection of α- and β-epoxides and cholestane–triol under mild conditions, which previously could only be achieved by GC–FID. The present study demonstrated an excellent analytical resolution among cholesterol and nine commonly encountered oxidization of these toxicologically important compounds. The method should be useful in research dealing with cholesterol and COPS in foodstuffs and biological samples.

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