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Determination of a cholesterol oxide mixture by a single-run high-performance liquid chromatographic analysis using benzoylation

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ABSTRACT

A rapid, single-run high-performance liquid chromatographic method has been developed to separate and quantify benzoate esters of cholesterol oxides, providing high sensitivity via ultraviolet detection. The mobile phase was 85% isopropanol–water (v/v). Analyses of 7-ketocholesterol, cholestane-triol, epoxycholesterol, 7-hydroxycholesterol and 25-hydroxycholesterol were done using a 30 cm × 3.9 mm I.D. Novapak C_{18} column and a variable-wavelength ultraviolet detector (set at 230 nm). Linearity was excellent since a good correlation was observed. As low as 500 ng of cholesterol benzoate per 20 μl of solution can be detected by this method.

INTRODUCTION

Adverse physiological responses are reportedly associated with several cholesterol oxidation products (COPS) [1–5]. Of particular concern is evidence that cholesterol oxides of dietary origin can be assimilated [6,7] and carried by lipoproteins to arterial tissue where they may initiate the development of atherosclerosis [8–10].

In spite of significant advances in recent years, high-performance liquid chromatographic (HPLC) techniques using UV detectors are not sensitive enough to separate and quantify complex mixtures of cholesterol oxides. It is difficult to analyze most common cholesterol of toxicological interest in a single HPLC run. Using UV detection, a limited number of underivatized cholesterol oxides may be analyzed simultaneously [11–16]. Significant improvements, however, are realized by increasing the UV absorption of cholesterol oxides through derivatization. Attaching a chromophore to the functional groups of cholesterol facilitates the UV detection of very low levels. Although *p*-nitrobenzoylation [17] and picration [18] have been used with UV detection for the analysis of epoxycholesterol epimers, to date no HPLC method

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for the analysis of cholesterol oxide mixtures based on UV-absorbing derivatives has been published in detail.

The present investigation was undertaken to develop an HPLC method based on UV detection of 3,5-dinitrobenzoyl chloride (DNBC) or benzoyl chloride (BC) derivatives of cholesterol oxides for accurate COP analyses.

EXPERIMENTAL

Reagents

The compounds studied, 7-hydroxycholesterol (cholestan-5-ene-3β,7α-diol), α-epoxycholesterol (5,6α-epoxy-5α-cholestane-3-β-ol), 25-hydroxycholesterol (cholest-5-ene-3β,25-diol); 7-ketocholesterol (3β-hydroxycholest-5-ene-7-one) and cholestane-triol (cholestane-3β,5α,6β-triol) were purchased from Sigma (St. Louis, MO, USA), Steraloids (Wilton, NH, USA) or Research Plus (Bayonne, NJ, USA), stored under desiccated nitrogen and used without further preparation. All solvents used were of HPLC grade (Caledon Labs, Georgetown, Canada) degassed by vacuum filtration through 0.45-μm filters (Millipore, Bedford, MA, USA) immediately prior to use. Pyridine (silylation reagent grade, Pierce, Morton Grove, IL, USA) was used as the solvent for the derivatization procedure.

Derivatization procedures

3,5-Dinitrobenzoyl chloride. Derivatization of cholesterol oxides by DNBC (Regis, Morton Grove, IL, USA) was done according to the method of Carey and Persinger [19]. Briefly, 4 ml of tetrahydrofuran, 40 mg of DNBC and 3 drops of pyridine were added to 1 mg of an oxide in a 5-ml of an oxide in a 5-ml Reacti-vial (Pierce), sealed with a Teflon cap and heated to 60°C for 1 h in a Reacti-block (Pierce). The solvent was evaporated under nitrogen with warming (30°C). The residue was dissolved in 3 ml of diethyl ether and washed with 3 volumes of dilute aqueous sodium bicarbonate (0.01 *M*) and once with distilled water.

Benzoyl chloride. Derivatization of cholesterol oxides by BC was done according to the method of Fitzpatrick and Siggia [20]. Each cholesterol oxide (0.10 mg) was dissolved in 4 ml of pyridine in a 5-ml Reacti-vial (Pierce) to which 0.2 ml of BC (Sigma) was added. The mixture was shaken ten times, heated at 80°C for 20 min and extracted in a separatory funnel containing 50 ml of 0.1 *M* HCl and 50 ml of diethyl ether. The ether phase was washed three times with 50 ml 0.1 *M* HCl to remove pyridine.

Trials using different volumes of pyridine (1–4 ml) and BC (0.01–0.2 ml) or omitting the washing procedure were also performed for comparison.

HPLC procedure

The following COPs were analyzed by HPLC: 7-hydroxycholesterol, α-epoxycholesterol, 25-hydroxycholesterol, 7-ketocholesterol and cholestane-triol. HPLC was performed using a delivery pump (Model 2150, LKB, Bromma, Sweden) equipped with a Rheodyne 20-μl injection loop. Reversed-phase separation was performed with a RCM-100 radial compression unit equipped with a 4-μm Novapak C_{18} column (30 cm × 3.9 mm I.D., Waters Assoc. Milford, MA, USA). Elution of dinitrobenzoates was monitored at 254 nm and benzoates at 230 nm by a variable-

wavelength UV detector (Model 2151, LKB). Chromatograms were processed on an integrator (Model HP 3393A, Hewlett-Packard, Avondale, PA, USA). Spectra were recorded from 200 to 400 nm by means of a photodiode array detector (Model 1040, Hewlett-Packard) connected to a computer (Model 9000, Series 300, Hewlett-Packard). The upslope, apex and downslope were recorded for confirmation of peak purity. Combinations of mobile phase systems were used, *i.e.* isopropanol–water (65:35 to 90:10, v/v) and acetonitrile–water (65:35 to 90:10, v/v). The limit of detection of the COP derivatives was determined by analyzing standard COP solutions of decreasing concentrations (5 μg per 20 μl).

Linear response of COPs as benzoate derivatives

To determine the response linearity for COPs studied, approximately 200 μg of each oxide were dissolved in 5 ml of pyridine, from which appropriate dilutions were made to give concentrations corresponding to 0.8–10 μg of each oxide per 20-μl injection. Plots of area *versus* quantity were made for regression analysis using Stat View 512+ software for Macintosh, version 1986 (Brain Power, Calabasas, CA, USA).

RESULTS AND DISCUSSION

No increase in sensitivity could be obtained using the DNBC method described by Carey and Persinger [19] since it was not possible to derivatize the oxides due to the failure of DNBC to react with certain molecules of high molecular weight. According to these authors, the yield of DNBC derivatives could depend on the solvent employed to extract them. Fitzpatrick and Siggia [20] reported the derivatization of cholesterol using DNBC, but cautioned that rapid hydrolysis occurred spontaneously, returning the derivatives to their original form, probably due to the presence of polar groups. For these reasons, DNBC was not retained for the derivatization of COPs.

For the Fitzpatrick and Siggia [20] procedure, it was necessary to increase the heating time to ensure total derivatization of the cholesterol oxides. The reaction with all COPs was complete after 1 h at 80°C after which no further increase in UV absorption was observed. This is well illustrated with cholestane-triol in Fig. 1.

Derivatization with 0.05 ml of BC in 1 ml of pyridine appeared to provide the best conditions. The molar ratio of BC to cholesterol oxide should be at least 500 to ensure a complete reaction. Beyond this point, no increase in the formation of the cholesterol derivatives was observed. Greater than 0.05 ml of BC per ml of pyridine resulted in supersaturation of the solution and rapid crystal formation.

It was also observed that washing the organic extract caused losses of oxide derivatives of up to 74% (Table I), undoubtedly due to hydrolysis. The washed derivatives were unstable after 2 h, HPLC analysis showing decreased peak area, whereas unwashed benzoyl derivatives remained stable for 2 days when kept in a tightly capped vial at room temperature.

The best resolution of benzoylated COPs was obtained using a mixture of isopropanol–water (85:15) (Fig. 2, Table II). At this ratio, the relative retention time (k') of all COPs was ≤ 7.32. Other solvent combinations failed to elute all cholesterol oxides or gave poor resolution of some. However, even with this mobile phase, the isomeric oxides were not resolved (*e.g.* 7α- and 7β-hydroxy; α- and β-epoxide); the

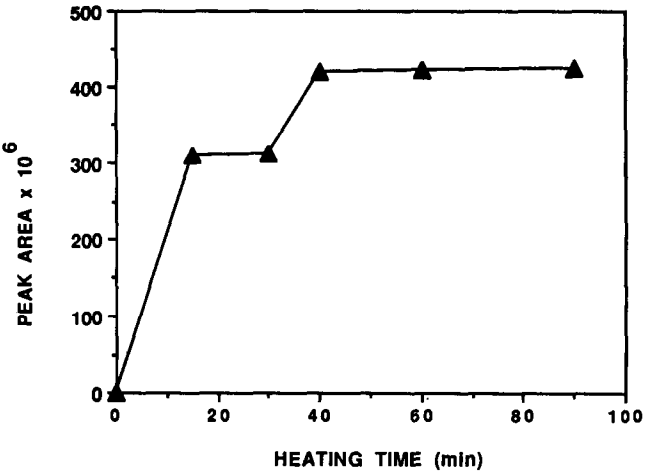

Fig. 1. Response of cholestane-triol absorption peak at 230 nm to heating time during derivatization with benzoyl chloride.

isomers were eluted at the same retention time. Also, triol retention time reported by Tsai *et al.* [15] is greater than 55 min, too long for the simultaneous determination of this compound in a COP mixture. With the present method, however, the retention time for triol is only 12.5 min. This may be attributable to decreased hydrophobicity as a result of benzoylation of the hydroxyl groups of cholestane-triol.

Confirmation of peak purity of each oxide was obtained by concordance of spectra taken at upslope, apex and downslope of the peak. Small differences may be attributable to the background effect of solvent (Fig. 3).

Spectral analysis of COPs also confirmed that all derivatized compounds showed high absorbance at 230 nm. This is well illustrated by the 25-hydroxy compound which is normally detected at 205 nm [13] or at 210 nm [12]. When detected as the benzoate derivative, the maximum sensitivity for 25-hydroxycholesterol was observed at 230 nm (Fig. 4). Absorbance at 230 nm for 7-hydroxycholesterol, 23-hy-

TABLE I

INFLUENCE OF WASHING PROCEDURE ON DERIVATIZED COPs RECOVERIES

Analyses were performed in triplicate.

COP	Unwashed (mg)	Washed (mg)	Losses[a] (%)
7-Ketocholesterol	3.2	0.82	74.4
Cholestane-triol	3.5	2.04	41.6
Epoxycholesterol	6.9	3.66	46.9
7-Hydroxycholesterol	2.9	0.99	65.5
25-Hydroxycholesterol	3.4	1.45	57.3

[a] Losses due to washing with respect to the unwashed procedure.

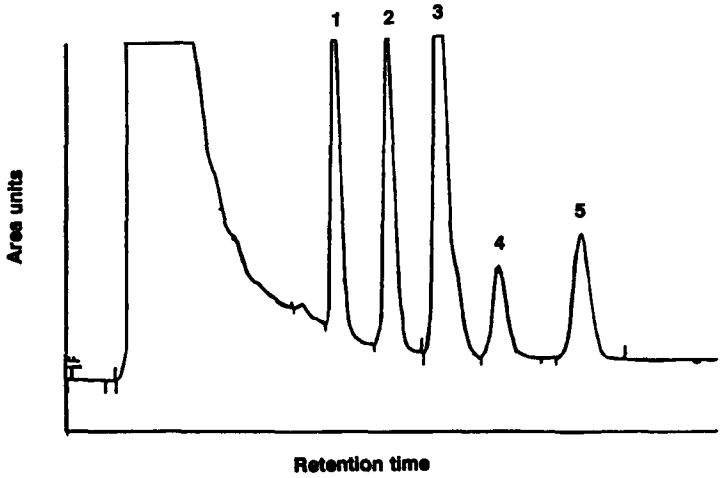

Fig. 2. Typical chromatogram of a mixture of derivatized COPs at 230 nm. Individual oxide concentration, 3 μg per 20 μl approximately; mobile phase, isopropanol–water 85:15 (v/v) at 1 ml/min and recorder chart speed 0.5 cm/min. Peaks: 1 = 7-ketocholesterol; 2 = cholestane-triol; 3 = epoxycholesterol; 4 = 7-hydroxycholesterol; 5 = 25-hydroxycholesterol. For retention times see Table II.

droxycholesterol, epoxycholesterol or cholestane-triol was increased by a factor of at least 10^6 by derivatization. Fitzpatrick and Siggia [20] reported a similar multiple for the amount of underivatized sterol to give an absorbance equivalent to that of the derivatives.

A minor improvement was observed for 7-ketocholesterol, of which the benzoyl derivative gave an absorbance increased by a factor of 3. This compound, however, gave an excellent response at 233 nm without derivatization.

In the present study, the minimum detectable amount of derivatized cholesterol oxides varied among the different compounds, averaging 500 ng, the detection limit being established at concentrations where integration was no longer possible. Also, omission of the washing procedure resulted in the presence of a large solvent peak which could interfere with the integration of small COP peaks.

This improvement in UV sensitivity offers a significant advantage over previous HPLC methods which allow analysis of only a limited number of oxides (such as

TABLE II

RELATIVE RETENTION TIMES OF COPs

Peak No.	COP	Retention time (min)	Relative retention time (k')
1	7-Ketocholesterol	10.40	3.36
2	Cholestane-triol	12.50	4.25
3	Epoxycholesterol	14.30	5.00
4	7-Hydroxycholesterol	16.70	6.01
5	25-Hydroxycholesterol	19.82	7.32

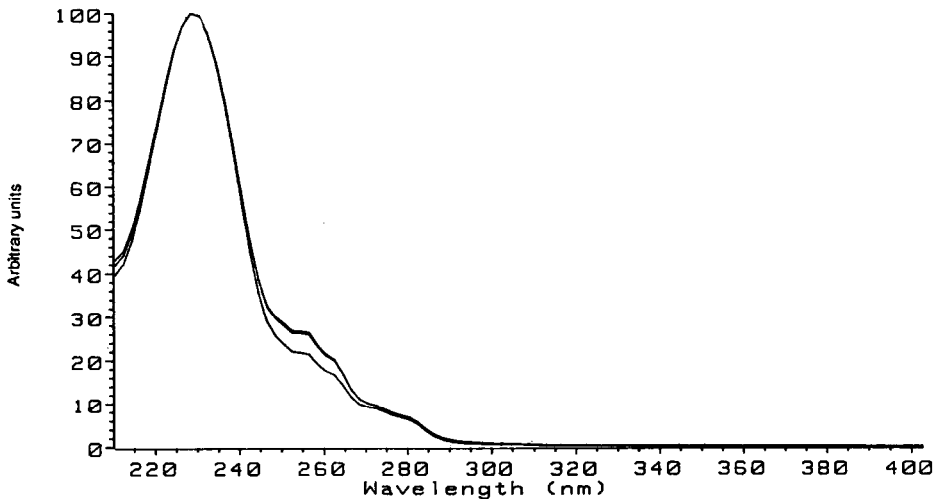

Fig. 3. Comparison between spectra of upslope, apex and downslope of cholestane-triol.

7-ketocholesterol and 7-hydroxycholesterol or epoxycholesterols) as underivatized compounds [14–16]. Oxides such as 25-hydroxycholesterol are detectable only at shorter UV wavelengths [12,13] while others such as 7-ketocholesterol require longer wavelengths. In the present study, 25-hydroxycholesterol could be simultaneously analyzed with the others since all were detectable at one single wavelength. Also, a good baseline was observed in comparison to those obtained at the short UV wavelength in the Csiky study [12].

Linearity of response was good for benzoyl derivatives throughout the range of concentrations studied (0.8–10 μg pr 20 μl). Regression analysis of plots of weight

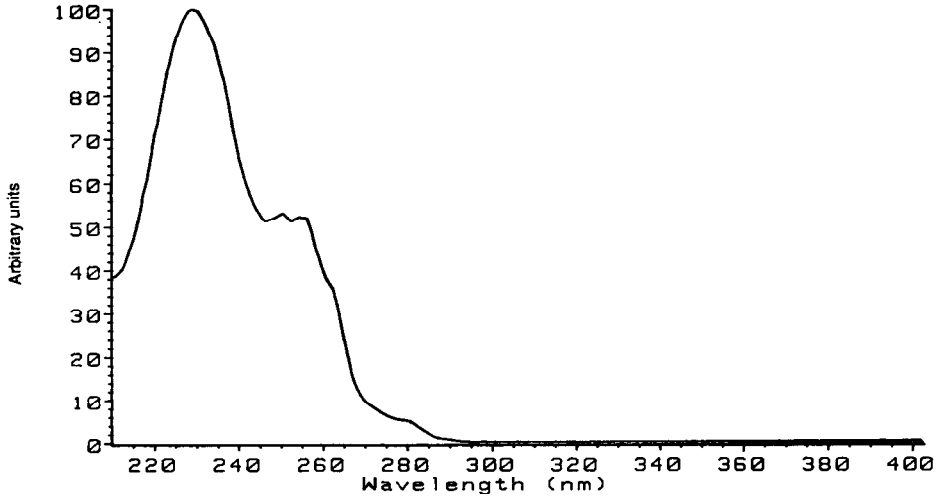

Fig. 4. UV spectrum of 25-hydroxycholesterol.

TABLE III

LINEARITY OF RESPONSE OF COPs AS BENZOATE DERIVATES

COPs	r^2	Slope[a]	y-Intercept[b]	Standard error
7-Ketocholesterol	0.997	1.002	0.155	0.22
Cholestane-triol	0.987	2.095	−1.012	0.76
Epoxycholesterol	0.980	0.432	−0.084	0.05
7-Hydroxycholesterol	0.944	0.644	−0.025	0.41
25-Hydroxycholesterol	0.999	0.008	0.000	0.00

[a] Based on six values, except for 25-hydroxycholesterol (five values).
[b] Area values were divided by a factor of 10^9.

versus area for each benzoyl derivative gave high r^2 values ($r^2 \geq 0.944$), suggesting that quantitation of the major oxidation products by the HPLC technique reported herein is reliable and reproducible (Table III).

For further application of this method to food samples, COPs may be separated from cholesterol and concentrated by filtration through a silica gel column prior to HPLC [14]. This clean-up procedure not only allows the detection of low levels of COPs usually found in foods, but also permits the suppression of autoxidation of cholesterol during extraction and quantification of COPs [21].

CONCLUSION

Results indicate that derivatization of COPs by BC using isocratic reversed-phase HPLC with UV detection is an efficient method for the separation and quantitation of cholesterol oxides when analyzed as their benzoate derivatives. This HPLC method is particularly useful for separating mixtures of autoxidation products of cholesterol having a wide range of UV absorbance maxima in the underivatized form. Moreover, the method is rapid, reproducible and suitable for routine quantitation of oxidized cholesterol products in foods. Application of this technique to meat products will be reported in another paper.

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