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# Capillary gas chromatography for the assessment of cholesterol oxides in the heart*

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**Abstract**: Recent studies indicated the presence of cholesterol oxides in biological tissues such as heart under pathophysiological conditions. Currently, no suitable method is available to separate and quantitate these oxides in biological tissues. This study was undertaken to develop a method suitable for the quantification of cholesterol oxides in heart. Since under normal conditions cholesterol oxidation does not occur, we exposed isolated perfused rat heart to oxygen-derived free radicals, which are known to oxidize cholesterol in heart. Free radical treated hearts were homogenized and lipids extracted by conventional techiques. The extracted lipids were saponified, and non-saponified portions were extracted with ether. Silyl derivatives of the extracted products were subjected to capillary gas chromatography. One microlitre of the sample was injected onto an SE 54 Supelco capillary column and run by stepwise temperature programming from 230 to 260°C at a rate of 20°C $min^{-1}$, and then from 260 to 290°C at 6°C $min^{-1}$. 5-Androsten-3β-ol-17-one was used as internal standard. The separated cholesterol oxides were identified by comparing with authentic standards. This method was suitable to separate 7α-hydroxycholesterol, dihydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol, cholesterol-5α,6α-epoxide, and 5-cholestene in heart. Since some of these oxides are cytotoxic to the heart, identification of these oxides should be important to understand the pathophysiology of myocardial disease and for the successful therapy to prevent them.

**Keywords**: *Capillary gas chromatography; cholesterol; cholesterol oxides; free radicals; heart.*

## Introduction

Cholesterol during storage under ambient conditions undergoes auto-oxidation, giving rise to a myriad of oxidized derivatives [1]. During the heat processing of cholesterol-containing foods, especially under deep frying conditions, a number of cholesterol oxides are formed [2]. Although high cholesterol foods are, in general, susceptible to oxidation, cholesterol oxides can also be formed in biological systems under abnormal pathophysiologic conditions. For example, cholesterol oxidation may occur in biological tissues by the action of free radicals, which may be generated during a variety of disease processes [1]. The role of free radicals in diseases such as ischemic heart disease (viz heart attack) is well known [3, 4]. The presence of oxygen-derived free radicals, including hydroxyl radical ($OH^{\cdot}$), has been demonstrated when an ischemic heart is reperfused [5–7].

A number of cholesterol oxides are considered to be cytotoxic [8]. In addition, many oxides are also mutagenic [9], enzyme inhibitory [10] and atherogenic [11]. Thus, it seems to be important to monitor the presence of cholesterol oxides in addition to cholesterol in foods and the biological system in order to prevent health hazards.

Cholesterol and its oxidized derivatives have been estimated by thin layer chromatography (TLC) [2, 12], gas–liquid chromatography (GLC) [2, 13] and by high-performance liquid chromatography (HPLC) [12]. However, a reliable method is still lacking for the analysis of oxysterols derived in foods, and particularly in biological systems. This study was undertaken to resolve this problem. In this report, a capillary gas chromatographic method is described to accurately estimate the formation of cholesterol oxides in rat heart.

## Experimental

### *Materials*

The authentic standards for the sterols, including 5-androsten-3β-ol-17-one,5-

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cholestene, 5α-cholesten-3-one, 7α-hydroxycholesterol, dihydrocholesterol, 19-hydroxycholesterol, 7β-hydroxycholesterol, 4-cholesten-3-one, cholesterol-5α,6α-epoxide, 20α-hydroxycholesterol, cholestan-3β, 5α, 6β-triol, 25-hydroxycholesterol and 7-ketocholesterol, were purchased from either Steraloids Inc. (Wilton, NH) or from Alltech Associates Inc. (Deerfield, IL). Cholesterol was obtained from Sigma Chemical Co. (St Louis, MO). HPLC grade solvents were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Free radical generating system containing hypoxanthine, xanthine oxidase (XO), $FeCl_3$ and EDTA were obtained from Sigma Chemical Co. (St Louis, MO).

*Methods*

*Animal preparation.* Sprague–Dawley male rats weighing 250–300 g were divided into two groups. One group was fed normal rat chow (RMH 3200 meal from Agway Prolab, Syracuse, NY) containing 0.01% cholesterol for 60 days, whereas the other group was given the same rat chow, but supplemented with 0.2% cholesterol, for 60 days.

Rats were anaesthetized with intraperitoneal pentobarbital (200 mg $kg^{-1}$). After sodium heparin (500 units $kg^{-1}$) was administered, hearts were removed. Isolated hearts were prepared and perfused with a non-recirculating Krebs–Henseleit buffer (KHB), pH 7.4 according to the Langendorff technique (Fig. 1), as described previously [14]. Hearts were perfused for 15 min with the buffer for equilibration. Each group of rats was further divided into four subgroups: (1) hearts were perfused with buffer only for 60 min; (2) hearts were perfused with a mixture of hypoxanthine (0.1 mM) and XO (8 mU) for 60 min; (3) hearts were perfused with $H_2O_2$ (1 mM) for 60 min; and (4) hearts were perfused with hypoxanthine (0.1 mM), XO (8 mU), $FeCl_3$ (0.1 mM) and EDTA (0.1 mM) for 60 min [15]. At the end of each experiment, heart was freeze-clamped at liquid nitrogen temperature and kept at −70°C until assayed for cholesterol and cholesterol oxides.

*Assay for cholesterol oxides — extraction and saponification*

The rat hearts (approximately 1 g, tissue, water content = 80%) were homogenized in chloroform, methanol and water (1:2:0.8, v/v/v) using a Polytron homogenizer (Brinkman Instruments, Westbury, NY), and lipids were extracted by the method of Bligh and Dyer [16]. Chloroform and water were added to the homogenates to obtain a ratio of chloroform, methanol and water of 2:2:1.8, v/v/v, respectively. After rehomogenization and low-speed centrifugation, the chloroform layer was removed and saved. An equal volume of chloroform corresponding to the amount removed

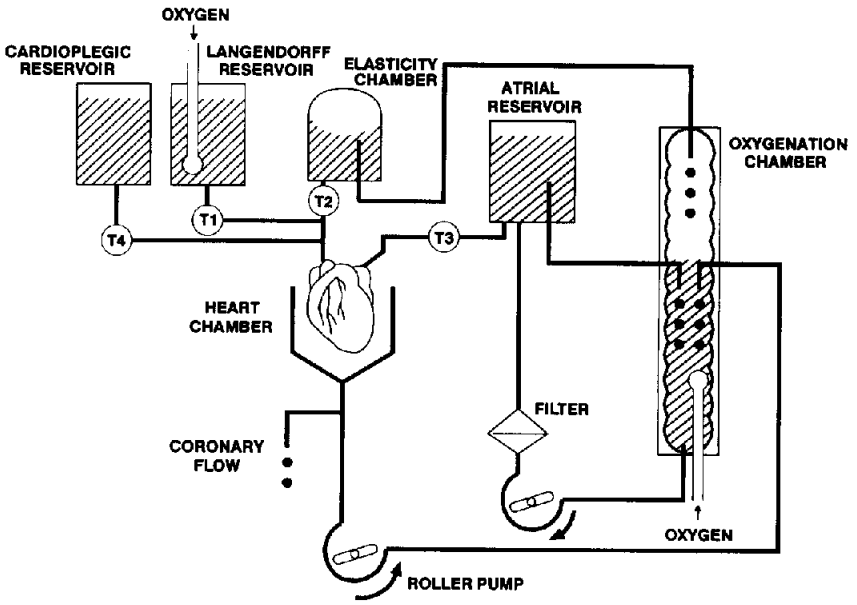


**Figure 1**
Rat heart perfusion apparatus.

was added to the remaining methanol and water followed by rehomogenization and centrifugation. The chloroform layer was removed and added to the first chloroform extract. The chloroform was evaporated at 40°C under $N_2$, and the lipid pellets were resuspended in 1 ml of 2N KOH in methanol per wet gram original heart tissue. The mixture was refluxed under $N_2$ overnight at room temperature [2, 12], the reflux was evaporated at 40°C under $N_2$, 1 ml of water was added, and the non-saponified portions were extracted twice with 2 ml of diethyl ether.

*Derivatization*

The combined diethyl ether extracts were evaporated at 40°C under $N_2$, and a 1 ml ampule of Sylon BTZ (Supelco Inc., Bellefont, PA) was added to each extract and heated at 40°C overnight. The trimethylsilyl ethers were concentrated under $N_2$ at 40°C and dissolved in hexane for injection onto the GLC [12]. The authentic cholesterol oxide standards were similarly derivatized.

*Capillary gas chromatography*

One microlitre of the sample containing 100 ng of internal standard (5-androsten-3β-ol-17-one) was injected at a split ratio of 100:1 onto an SE-54 Supelco fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) in a Hewlett–Packard (Avondale, PA) Model HP5890A gas chromatograph equipped with a model HP3393A integrator, Model HP7673A automatic sampler, and a hydrogen flame ionization detector. Helium was used as the carrier gas at a flow rate of 1.7 ml $min^{-1}$ (20 psi). A stepwise temperature program was run from 230 to 260°C at a rate of 20°C $min^{-1}$, and then from 260 to 290°C at 6°C $min^{-1}$. 5-Androsten-3β-ol-17-one was used as an internal standard, and the separated cholesterol oxides were identified by comparison with authentic standards.

## Results

The separation of the oxidation products of cholesterol by capillary GC is shown in Fig. 2. Relative retention times and response factors are depicted in Table 1. The response factors of the various cholesterol oxides to the internal standard 5-androsten-3β-ol-17-one ranged

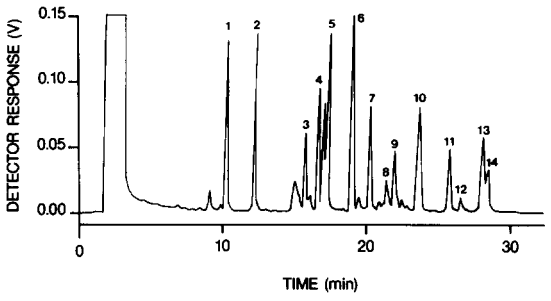


**Figure 2**
Resolution of authentic standards of cholesterol oxides using capillary GC. One microlitre of sample containing 100 ng of the internal standard 5-androsten-3β-ol-17-one and 10–75 ng of each of the silyl derivatives was injected at a split ratio of 100:1 and chromatographed as described in Methods and numbered: (1) 5-androsten-3β-ol-17-one; (2) 5-cholestene; (3) 5α-cholesten-3-one; (4) 7α-hydroxycholesterol; (5) cholesterol; (6) dihydrocholesterol; (7) 19-hydroxycholesterol; (8) 7β-hydroxycholesterol; (9) 4-cholesten-3-one; (10) cholesterol-5α, 6α-epoxide; (11) 20α-hydroxycholesterol; (12) cholestan-3β, 5α, 6β-triol; (13) 25-hydroxycholesterol; (14) 7-ketocholesterol.

**Table 1**
Relative retention times and response factors of the elutions of silyl derivatives of cholesterol and cholesterol oxide standards by capillary gas chromatography

| Cholesterol oxide standard | Relative retention time | Response factor* |
|---|---|---|
| 5-Androsten-3β-ol-17-one | 1.000 | 1.00 ± 0.019 |
| 5-Cholestene | 1.186 | 1.28 ± 0.025 |
| 5-Cholesten-3-one | 1.539 | 1.32 ± 0.021 |
| 7α-Hydroxycholesterol | 1.618 | 1.41 ± 0.029 |
| Cholesterol | 1.686 | 1.33 ± 0.017 |
| Dihydrocholesterol | 1.863 | 1.36 ± 0.023 |
| 19-Hydroxycholesterol | 1.980 | 1.40 ± 0.027 |
| 7β-Hydroxycholesterol | 2.098 | 1.39 ± 0.026 |
| 4-Cholesten-3-one | 2.157 | 1.32 ± 0.017 |
| Cholesterol-5α,6α-epoxide | 2.304 | 1.39 ± 0.022 |
| 20α-Hydroxycholesterol | 2.529 | 1.38 ± 0.023 |
| Cholestan-3β,5α,6β-triol | 2.598 | 1.47 ± 0.035 |
| 25-Hydroxycholesterol | 2.755 | 1.41 ± 0.027 |
| 7-Ketocholesterol | 2.784 | 1.38 ± 0.022 |

*Mean ± SD with $n = 6$.

from 1.28 for 5-cholestene to 1.47 for cholestan-3β, 5α, 6β-triol.

Control hearts without any treatment did not exhibit the appearance of any oxidized cholesterol derivatives (Fig. 3). When treated with $O_2$ generating system, a small amount of 7α-hydroxycholesterol appears. With $H_2O_2$ treatment, a large amount of 7α-hydroxycholesterol is seen plus trace amounts of 5-cholestene, dihydrocholesterol, 7β-hydroxycholesterol, cholesterol-5α,6α-epoxide and 20α-hydroxycholesterol. In control hearts, the $OH^-$ generating system has similar results to $H_2O_2$ except for an increase in 5-cholestene.

Without treatment, the rats fed the high-cholesterol diet also did not produce any oxidized cholesterol (Fig. 4). Treatment with the $O_2^-$ generating system produced a higher level of 7α-hydroxycholesterol than rats fed the normal diet. A trace amount of 5-cholestene also occurred. $H_2O_2$ treatment and the $OH^-$ generating system produced the same six cholesterol oxides as with the normal diet; however, the $OH^-$ generating system produced greater levels of these six cholesterol oxides in the rats fed the high cholesterol diet.

## Discussion

Although some oxidation products of

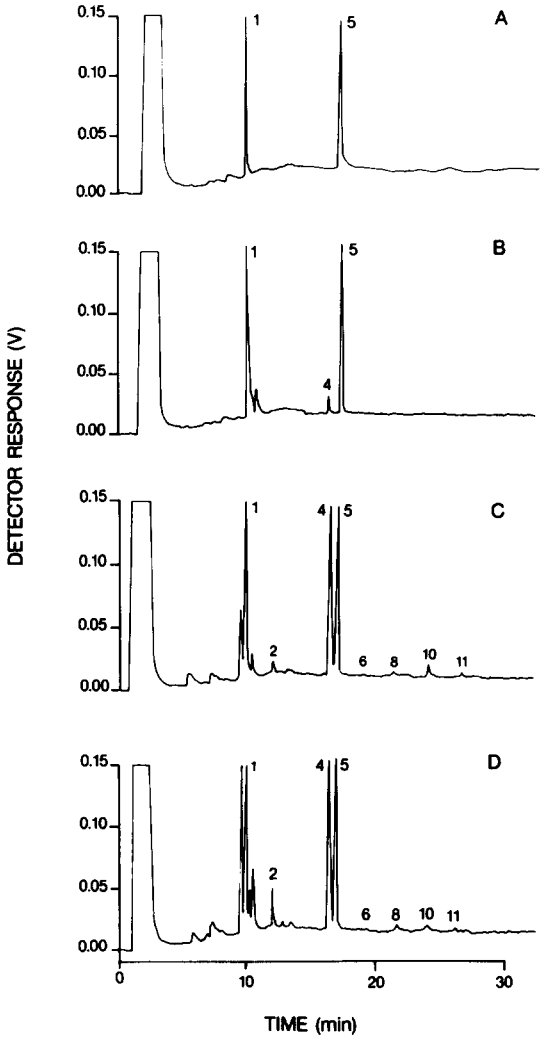


**Figure 3**
Resolution of cholesterol and cholesterol oxidation products obtained from myocardial samples of rats fed standard chow. Oxides chromatographed and numbered as in Fig. 2. (A) No treatment. (B) Hearts perfused with $O_2^-$ generating system. (C) Hearts perfused with $H_2O_2$. (D) Hearts perfused with $OH^-$ generating system.

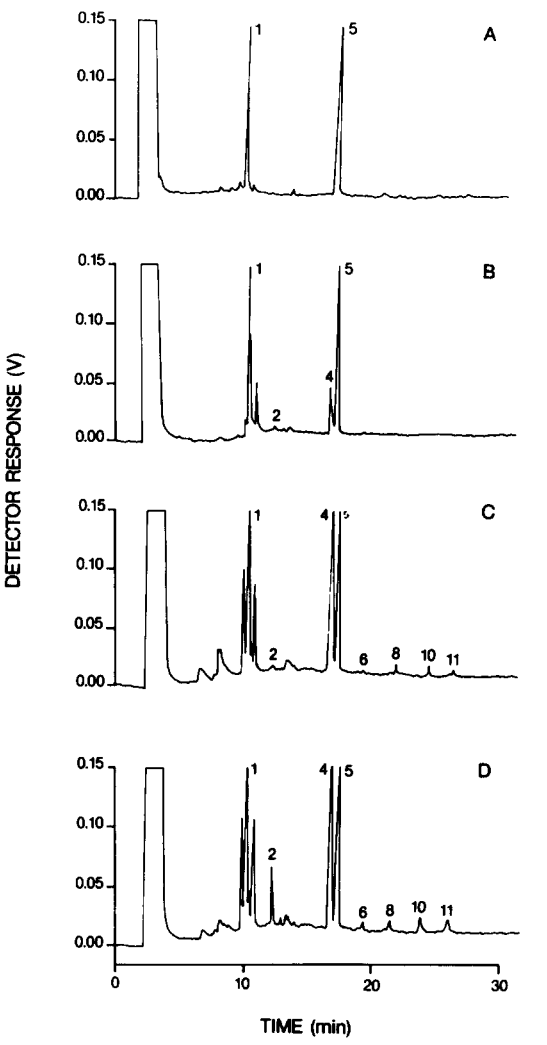


**Figure 4**
Resolution of cholesterol and cholesterol oxidation products obtained from myocardial samples of rats fed high cholesterol diet. Oxides chromatographed and numbered as in Fig. 2. (A) No treatment. (B) Hearts perfused with $O_2^-$ generating system. (C) Hearts perfused with $H_2O_2$. (D) Hearts perfused with $OH^-$ generating system.

cholesterol have been identified in normal tissues such as liver and brain, oxysterols are not present in normal heart. In liver, 7α-hydroxycholesterol has been identified, which is the major substrate in bile acid biosynthesis [1]. In human brain, significant amounts of 24-hydroxycholesterol and 26-hydroxycholesterol have been identified, but their functional role remains unknown [17]. 26-Hydroxycholesterol has also been identified in human aorta, but the amount is directly proportional to the degree of atherosclerosis [18, 19]. In severe atherosclerotic tissue, a number of cholesterol oxides have been found, including 7α-hydroxycholesterol, cholestan-3β,5α,6β-triol,3,5-cholestadien-7-one and 4,6-cholestadien-3-one, 7-ketocholesterol, 25-hydroxycholesterol, and 7β-hydroxycholesterol [1].

Atherosclerosis is generally associated with an ischemic insult. The atherosclerotic plug obstructs the delivery of oxygenated blood to the affected tissue, causing ischemia. Although atherosclerosis is known to cause cholesterol oxidation, the direct effects of ischemia on the formation of oxysterol is not known. Many ischemic insults are not related to atherosclerosis. Angioplasty and cardiac as well as vascular surgeries are always associated with ischemia. Reperfusion of these ischemic tissues causes tissue injury, known as reperfusion injury [20]. Oxygen-derived free radicals are known to play a significant role in the pathogenesis of reperfusion injury [3–7, 21].

During reperfusion of ischemic tissue, $O_2^-$ may be formed from the incomplete reduction of molecular $O_2$ [Reaction (I)]. $O_2^-$ from Reaction (1) undergoes Haber–Weiss reaction in the presence of $H_2O_2$, forming highly toxic $OH^\cdot$ [Reaction (II)]. Although this reaction is thermodynamically feasible, and the overall stoichiometry is widely accepted under *in vitro* conditions, this reaction is kinetically very slow or even negligible. The modified reaction has been suggested to occur in two steps by iron-catalysed Fenton-type reactions [22] as described in Reactions (III–V)

$$O_2 + e^- \rightarrow O_2^- \quad \text{(I)}$$
$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot \quad \text{(II)}$$
$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad \text{(III)}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\cdot \quad \text{(IV)}$$
$$O_2 + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot \quad \text{(V)}$$

In this study, we attempted to simulate the conditions of myocardial ischemia and reperfusion with respect to free radical generation. The isolated hearts were perfused in the presence of $O_2^-$, $H_2O_2$ and $OH^\cdot$, which are likely to be present in an ischemic and reperfused heart.

Our results demonstrated the formation of several cholesterol oxidation products. We were unable to identify any oxysterol either in normal heart or in atherosclerotic heart from the rats fed high-cholesterol diets. Perfusion of the heart with $O_2^-$ resulted in the formation of a minimal amount of 7α-hydroxycholesterol and 5-cholestene. However, both $H_2O_2$ and $OH^\cdot$ caused significant oxidation of cholesterol to its oxides, including 7α-hydroxycholesterol, 5-cholestene, dihydrocholesterol, 7β-hydroxycholesterol, cholesterol-5α, 6α-epoxide and 20α-hydroxycholesterol. The degree of cholesterol oxidation seems to be slightly higher for $OH^\cdot$ compared to $H_2O_2$. In all cases, the atherosclerotic hearts are more susceptible for cholesterol oxidation.

The formation of cholesterol oxides by free radicals was previously reported in *in vitro* studies. Oxygen free radicals generated *in vitro* caused the oxidation of cholesterol [22]. The authors also demonstrated the formation of several cholesterol oxides following γ-irradiation of liposomes consisting of cholesterol and phospholipids. Co-oxidation of salicylate and cholesterol during the oxidation of metmyoglobin by $H_2O_2$ has also been reported [23]. However, this is probably the first report concerning the cholesterol oxidation during perfusion of isolated heart with oxygen free radicals.

The results of the study are highly significant with respect to the cytotoxicity of cholesterol oxides. Cholesterol-α-oxide has been reported to induce tumour formation in rats and mice [24]. Cholesterol-5α,6α-epoxide is a direct-acting mutagen [9]. 25-hydroxycholesterol, 7-keto-cholesterol and cholesterol-5α,6α-epoxide were found to markedly inhibit the growth of human aortic smooth muscle cells [25]. Various mechanisms of cytotoxicity have been proposed, including inhibition of 3-hydroxy-3-methyl-glutaryl CoA reductase [10], enhanced cellular cholesterol esterification [26], and decreased DNA synthesis [27, 28]. Cholesterol oxides are also believed to accelerate the atherosclerosis and may play a role in heart attack [11].

Since ischemic heart disease remains a serious threat to humans and free radicals play

a significant role in the disease process, the identification of several cytotoxic and mutagenic cholesterol oxides may add another problem to an already large list of promoters of ischemic heart disease. The results of this study no doubt suggest a role of cholesterol oxidation in ischemic heart disease.

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