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Short Communication

Determination of cholesterol in sub-nanomolar quantities in biological fluids by high-performance liquid chromatography

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ABSTRACT

A highly sensitive method for the determination of cholesterol in biological fluids is described. Unsaponifiable lipids from rat serum and thoracic duct lymph chylomicron samples were treated with cholesterol oxidase. The product of the enzymatic reaction, Δ^4-cholestenone, was analysed by normal-phase high-performance liquid chromatography (HPLC) using hexane–isopropanol (95:5, v/v) as a mobile phase and detected with a UV spectrophotometer at 240 nm. When the standard samples containing varying amounts of cholesterol (0.15–3 nmol) were treated with cholesterol oxidase and analysed by HPLC (injected amounts 0.09–1.8 nmol of cholesterol), the peak areas increased proportionally with the amounts of authentic cholesterol with a correlation coefficient of 0.996. The values in these biological fluids determined by the HPLC method were identical to those obtained by enzymatic–colorimetric or gas chromatographic methods. Moreover, the detection limit (0.09 nmol) of the present method (0.15 nmol are required for the sample preparation) is lower than those of conventional methods (approximately 30 nmol). Because of the excellent sensitivity and reproducibility, this method is well suited for the determination of cholesterol in biological fluids where cholesterol concentration is low.

INTRODUCTION

An enzyme reaction catalysed by cholesterol oxidase and producing Δ^4-cholestenone and hydrogen peroxide from cholesterol has provided several specific and reproducible methods to measure the cholesterol concentration in biological fluids and tissues. The amount of the first product, Δ^4-cholestenone, can be determined spectrophotometrically by monitoring the increase in absorbance at 240 nm [1,2]. Alternatively, the amount of the other enzyme product, hydrogen peroxide, can be determined by measuring the absorbance in the visible range after the production of quinoneimine dye by a peroxidase-catalysed enzyme reaction [3,4]. These methods appear sensitive enough (30–150 nmol per assay) to determine the cholesterol concentration in serum of laboratory rats (1500–5000 nmol/ml), and require merely 0.01–0.02 ml of the sample to obtain a reliable value. They also can easily be applied to humans, in whom the cholesterol concen-

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tration is substantially higher than in experimental animals. However, the lack of sensitivity of these methods does not allow the determination of cholesterol concentration in biological fluids such as tissue culture medium, bile and thoracic duct lymph, which contain cholesterol at levels as low as 100–500 nmol/ml and thus require a large volume of sample. An enzymatic–fluorimetric method reported by Gamble *et al.* [5] appears to be sensitive enough (0.13–1.29 nmol per assay) to measure the cholesterol concentration in these samples. However, in our experience, this method gives highly variable values and thus seems to be unsuitable for our purpose. Therefore, we have developed an alternative sensitive assay for cholesterol based on the direct determination by high-performance-liquid chromatography (HPLC) of Δ^4-cholestenone, which is the product of the cholesterol oxidase reaction, with a maximum absorption at 240 nm. We found that this method is highly sensitive, reliable, reproducible and applicable not only for the determination of cholesterol in serum but also for the measurement of lymphatic cholesterol transport by chylomicrons in rats.

EXPERIMENTAL

Collection of thoracic lymph and preparation of the lymph chylomicrons (LCs)

The thoracic duct of male Sprague–Dawley rats weighing 250–300 g was cannulated with polyethylene tubing [6]. Another piece of polyethylene tubing was inserted into the stomach. After surgery, a saline solution containing 5% glucose was continuously infused at the rate of 3 ml/h through the cannula inserted into the stomach for 10–12 h. Before the initiation of the experiment, the lymph fluid was collected for 1 h to measure the basal rate of intestinal lipid transport and then a 5% triacylglycerol emulsion was infused at the rate of 3 ml/h for 1 h. After 1 h, the triacyglycerol emulsion was again replaced by the saline solution containing 5% glucose and was infused at the same rate during the remaining experimental period (5 h). Lymph was collected every hour after initiation of the infusion of triacylglycerol dispersions for 6 h. The 5% triacylglycerol emulsion was prepared by sonicating 5 g of rapeseed oil, 2 g of bovine serum albumin and 1 g of egg yolk phosphatidylcholine in 100 ml of saline solution. The lymph samples thus obtained were centrifuged at $1.8 \cdot 10^4$ *g* for 90 min [7] to isolate LCs. The floating LCs were collected, dispersed in physiological saline solution and washed twice by centrifugation as described above. Purified LCs were adjusted to 3 ml with physiological saline solution.

Enzymatic oxidation of cholesterol and analysis of the enzyme product by HPLC

Rat serum (0.01 ml) was saponified with ethanolic potassium hydroxide (3% potassium hydroxide in 94% ethanol) at 50°C for 1 h. Unsaponifiable matter was extracted three times with 3 ml of hexane and adjusted to 10 ml with hexane. Aliquots of the extract (0.2–1 ml) were evaporated to dryness under nitrogen and dissolved in 0.02 ml of isopropanol. Following the addition of 5% sodium cholate solution (0.05 ml) and vigorous vortex mixing, a solution (0.4 ml) containing cholesterol oxidase (0.1 unit, Toyobo, Tokyo, Japan), 5 m*M* magnesium chloride and 0.1 m*M* EDTA in 100 m*M* phosphate buffer (pH 7.4) was added to the mixture, which was incubated at 37°C for 1 h. The enzyme reaction was terminated by the addition of 0.47 ml of methanol, and the enzyme product, Δ^4-cholestenone, was extracted three times with 3 ml of hexane. The extract was evaporated to dryness under nitrogen, reconstituted in 0.05 ml of hexane–isopropanol (95:5, v/v), and a 0.03-ml aliquot was analysed by a normal-phase HPLC using a Fine Pak SIL column (250 × 4.6 mm I.D., Japan Spectroscopic, Tokyo, Japan) with a mobile phase of hexane–isopropanol (95:5, v/v) at a flow-rate of 0.8 ml/min and detected at 240 nm. Cholesterol concentrations in rat serum samples were also determined by an enzymatic–colorimetric method [3,4] using a commercially available kit (Cholesterol C-test; Wako, Osaka, Japan). Lipids in 1 ml of LC samples prepared as described above were extracted and purified by the method of Folch *et al.* [8]. The chloroform

phase containing lipids was adjusted to 20 ml with methanol. The unsaponifiable lipid fraction in an aliquot (0.2–1.0 ml) of the lipid extract was prepared and processed as above for the enzymatic oxidation of cholesterol to Δ^4-cholestenone and for the analysis by HPLC. The two other aliquots of the lipid extract were used for the determinations of cholesterol by gas chromatography using 5α-cholestane as an internal standard (10-ml aliquot) [9] and of triacylglycerol spectrophotometrically (2-ml aliquot) [10].

RESULTS AND DISCUSSION

Typical chromatograms obtained by HPLC of the cholesterol oxidase oxidation products of a blank, 0.54 nmol of authentic cholesterol sample (0.9 nmol of cholesterol were processed for the sample preparation) and unsaponifiable lipids from purified LCs monitored by spectrophotometry at 240 nm are shown in Fig. 1A, B and C, respectively. Under the conditions described in the Experimental section, the authentic cholesterol and the LC samples show a sharp single peak having a retention time of 6.5 min. Although this peak was also detected in a blank sample, the peak area was much lower than in the authentic cholesterol and LC samples. When the oxidation products of the standard samples containing varying amounts of cholesterol (0.15–3 nmol; injected amounts 0.09–1.8 nmol) were analysed, the peak areas increased proportionally with the amounts of authentic cholesterol (r = 0.996). Moreover, the authentic cholesterol sample which was treated in the absence of cholesterol oxidase did not show any detectable peak at this retention time. Thus, we concluded that this peak represents Δ^4-cholestenone formed by the enzymatic reaction of cholesterol oxidase. As no peak was detected after 10 min of the HPLC assay in blank, authentic cholesterol and LC samples, 12 min appears to be long enough to wait before injecting the next sample. The existence of a small but detectable amount of Δ^4-cholestenone in blank samples (Fig. 1A) limited the sensitivity of the present method. When 0.15 nmol (injected dose 0.09 nmol) of cholesterol were assayed, the peak area having a retention time of 6.5 min was approximately three times higher than that of the blank sample. Thus this amount is the minimum able to obtain a reliable cholesterol value. We routinely injected samples for HPLC analyses containing 0.18–1.2 nmol of cholesterol (0.3–2 nmol were required for processing).

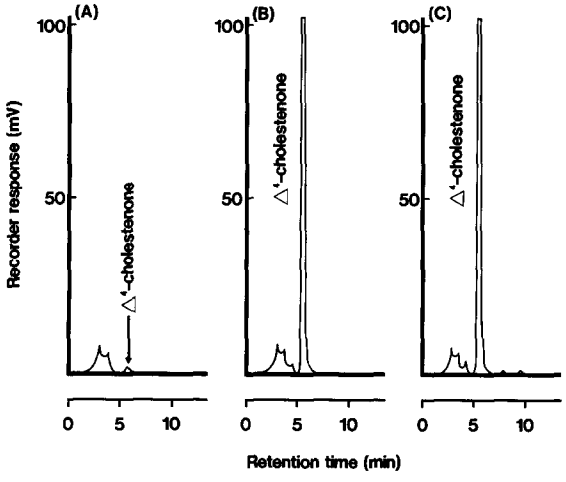

Fig. 1. Chromatograms of a sample of (A) blank, (B) authentic cholesterol (0.9 nmol of cholesterol were processed for the analysis as described in the Experimental section; injected dose 0.54 nmol), and (C) unsaponifiable matter from rat LCs.

To test the validity of the present method, a correlation between the cholesterol values obtained with a conventional enzymatic–colorimetric method [3,4] using a commercially available assay kit (Cholesterol C-test) and those obtained with the proposed HPLC method was examined. As shown in Fig. 2, an excellent correlation (r = 0.996) was obtained between the values obtained by these two methods. Moreover, the values obtained with the proposed method were almost identical to those determined by a commercial assay kit ($y = 1.016x - 3.785$).

We then applied the present method for the measurement of cholesterol transport by LCs in thoracic lymph where the cholesterol concentration is one-tenth that of serum. As Fig. 3 shows, a 1-h infusion of triacylglycerol dispersion greatly increased triacylglycerol concentration in LCs during the following 2–3 h of the experimental

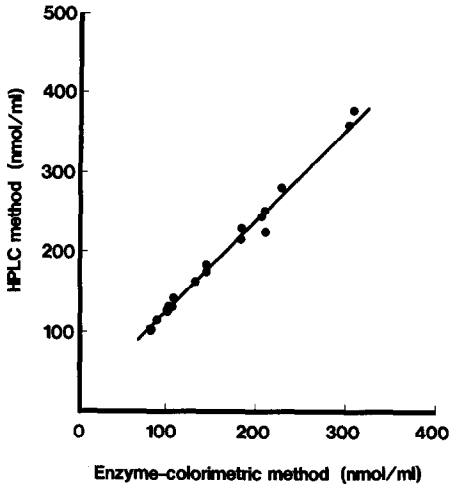

Fig. 2. Correlation between the serum cholesterol values determined by enzymatic–HPLC and enzymatic–colorimetric methods. The correlation coefficient is 0.996 ($y = 1.016x - 3.785$).

TABLE I

CONCENTRATIONS OF LYMPH CHYLOMICRON CHOLESTEROL DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) AND GAS CHROMATOGRAPHIC (GC) METHODS

Values represent means of duplicate determinations.

Sample No.	Cholesterol (nmol/ml of lymph)		Difference [(A − B)/B × 100]
	HPLC method (A)	GC method (B)	
1	426.3	471.9	− 9.66
2	309.5	307.0	0.81
3	244.9	239.5	2.25
4	205.6	190.2	8.10
5	194.4	200.0	− 2.80
6	182.5	201.7	− 9.52
7	112.0	112.3	0.27
8	95.5	95.9	− 0.42

period. A similar feature was confirmed in the transport of cholesterol by LCs. The molar ratio of cholesterol to triacylglycerol in LCs was initially low (6.85 ± 3.38) but increased sharply during and after the cessation of the infusion of triacylglycerol emulsion. The values obtained during 1–2 h (48.7 ± 9.8) and 2–3 h (40.2 ± 16.3) of the experimental period were similar to those reported for chylomicrons isolated from rat lymph [11,12] and serum [13] during active fat absorption.

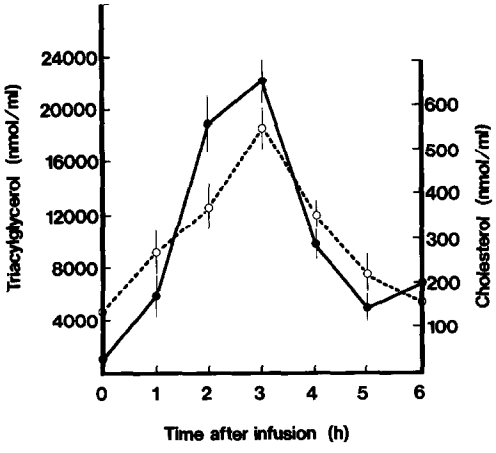

Fig. 3. Lymphatic transport of triacylglycerol (●) and cholesterol (○) in chylomicrons after initiation by the infusion of triacylglycerol emulsion. Each value represents the mean ± S.D. (n = five rats).

Cholesterol concentrations in some of the LC samples were also determined by gas chromatography and the values obtained were compared with those obtained by the present HPLC method (Table I). An excellent coincidence is found between the values determined by gas chromatography and the present method. Differences in values due to the analytical methods employed herein were less than 10% in the various LC samples differing in cholesterol concentration.

In conclusion, the present study shows that the enzymatic oxidation of cholesterol by cholesterol oxidase and the subsequent determination of the enzyme product, Δ^4-cholestenone, by HPLC provides an alternative sensitive method to determine the cholesterol concentration in biological fluids. The lowest detection limit of 0.09 nmol (0.15 nmol of cholesterol is required for the sample preparation) is much lower than those in enzymatic–spectrophotometric [1–4] and gas chromatographic methods (approximately 30 nmol) [8]. This highly sensitive method employed routinely in our laboratory allows the determination of cholesterol concentration in chylomicrons iso-

lated from 0.01 ml of thoracic duct lymph and 0.001 ml of serum of rats, and may be applicable to other biological fluids such as tissue culture medium and rat bile, in which the cholesterol concentration is very low.

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