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Simultaneous determination of serum lathosterol and cholesterol by semi-micro high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A simple method has been developed for the simultaneous determination of lathosterol and cholesterol by high-performance liquid chromatography with electrochemical detection (HPLC–ECD). Lathosterol was found to be electrochemically oxidized and its current peak height was linearly related to the amount of lathosterol injected, ranging from 0.15 μmol/L to 300 μmol/L (r = 0.995). Similar results were obtained with cholesterol from 15 μmol/L to 600 μmol/L (r = 0.995). The separation was carried out with an ODS column, acetonitrile containing 30 mmol/L lithium perchlorate as a mobile phase, and an applied potential at +2.8 V vs. Ag/AgCl. The detection limit (S/N = 3) of lathosterol as well as cholesterol was 0.03 μmol/L (0.15 pmol). Total lathosterol in control human and rat serum was determined by the present method with a recovery of more than 95.8% and an RSD (n = 5) of less than 7.3%. The present method was applied to an experiment with rats to examine the effect of lathosterol feeding. There were no significant changes in serum lathosterol or cholesterol levels in rats fed with a high-lathosterol diet for six days. Therefore, we found this method to be both simple and useful for the simultaneous determination of lathosterol and cholesterol in serum.

1. Introduction

Lathosterol is a precursor of cholesterol in the later stages of the cholesterol biosynthetic pathway [1]. The serum level of lathosterol or the lathosterol/cholesterol ratio is a well-known marker of endogenous cholesterol synthesis [1–6]. Therefore, it is important to accurately determine the concentrations of lathosterol and cholesterol in serum in order to investigate cholesterol homeostasis *in vivo*.

For the determination of lathosterol, many methods are available although most require derivatization of lathosterol to achieve sufficient sensitivity. Derivatization with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) in the presence of pyridine [7] and BSTFA and trimethylchlorosilane [8] have been used for gas chromatography–mass spectrometry (GC–MS). BSTFA has also been used for gas chromatography–flame ionization detector [9,10]. 4-Bromobenzenesulfonyl chloride has been used for high-performance liquid chromatography with UV detection (HPLC–UV) [11] and picolinic acid has been reported for liquid chromatography–tandem mass spectrometry (LC–MS/MS) [12]. Although the chromatographic separation of lathosterol from cholesterol is difficult due to their similar structures, an LC–MS method for determining serum lathosterol without derivatization has been reported [13].

In addition to these established methods, we have recently developed a new detection method for sterols based on the direct electrochemical oxidation of sterols [14]. This method coupled with HPLC (HPLC–ECD) has been applied to the determination of serum cholesterol [15], serum cholestanol [16], serum phytosterols [17], and oxysterols in oxidatively modified low density lipoprotein [18]. Our current study aims to resolve lathosterol from a vastly greater quantity of cholesterol and then to apply the electrochemical method (HPLC–ECD) to the simultaneous determination of serum lathosterol and cholesterol. In our preliminary experiments, the isocratic separation of lathosterol from cholesterol was difficult using the conditions we had previously established. However, we have carefully examined several HPLC conditions and found that lathosterol can be separated from cholesterol. Furthermore, the new method has been applied to the determination of serum lathosterol in rats fed with a high lathosterol diet.

Abbreviations: IS, internal standard; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry; HPLC–UV, high-performance liquid chromatography with UV detection; HPLC–ECD, HPLC with electrochemical detection; LC–MS/MS, liquid chromatography–tandem mass spectrometry.

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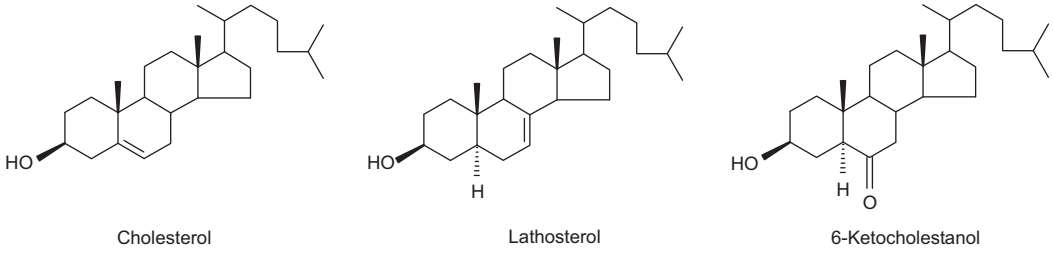

Fig. 1. Structures of cholesterol, lathosterol, and 6-ketocholestanol used as an internal standard (IS).

2. Experimental

2.1. Materials

Lathosterol and 6-ketocholestanol were obtained from Steralids (Newport, RI, USA). Acetonitrile (HPLC grade) and $LiClO_4$ (reagent grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Human serum-based reference material for cholesterol determination (SRM909b) was from NIST (Gaithersburg, MD, USA). Other reagents were of reagent grade available from commercial sources.

2.2. Animal experiments

Male Wistar rats (eight weeks old, 240–250 g) and male BALB/c mice (four weeks old, 15–17 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Animal experiments were performed under the approval of the institutional ethics committee of the Tokyo University of Pharmacy and Life Sciences. Three rats were given standard chow (CE-2, Clea Japan, Inc., Tokyo, Japan) containing 10% safflower oil and 0.2% lathosterol mixture for one week. They were maintained under a controlled temperature (21–23 °C) and light was provided by artificial illumination for 12 h a day with *ad libitum* access to chow and water. At the end of the experiment, the animals were anesthetized with diethyl ether and 8 mL of blood was taken from the abdominal aorta of each animal to prepare serum.

2.3. HPLC

The HPLC–ECD system consisted of a DG-980-50 vacuum degasser (Jasco International, Tokyo, Japan), an intelligent pump 301M (FLOM Co., Ltd., Tokyo, Japan), a model 7725i Rheodyne injector fitted with a 5 μL injection loop (Rheodyne, Cotati, CA, USA), a YMC-Pack ODS-AL column (250 mm × 2.0 mm i.d., 5 μm, YMC Co. Ltd., Kyoto, Japan), a CTO-10AS column oven (Shimadzu, Kyoto, Japan), an HECS 311B 15-01 potentiostat (Fuso ElectroChemical System, Kanagawa, Japan) and used a mobile phase consisting of acetonitrile containing 30 mmol/L $LiClO_4$. The electrochemical cell (radial flow cell, BAS Inc., Tokyo, Japan) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. The column was maintained at 50 °C in the column oven. The flow rate was set at 250 μL/min. 6-Ketocholestanol was used as an internal standard (IS) for the quantification of lathosterol (Fig. 1).

2.4. Sample preparation

Sample preparation for the determination of human, rat, or mouse serum lathosterol was carried out by saponification and hexane extraction. Briefly, 10 μL of human serum (SRM909b), rat serum (Wistar), or mouse serum (BALB/c) were transferred to a screw-capped vial, and 0.1 mL of freshly prepared 1 mol/L potassium hydroxide in ethanol was added. Alkaline hydrolysis was conducted by vortex for 1 min and sonication (Branson 2200, Yamato Scientific, Tokyo, Japan) for 10 min. This procedure (vortex and sonication) was repeated. To extract lipids, 0.2 mL of distilled water was added to the sample, vortex-mixed with 0.8 mL of hexane for 1 min, and then centrifuged for 5 min at 3000 rpm (Model 3740, Kubota, Tokyo, Japan). The supernatant (hexane layer) was transferred to a new tube and further extracted once with 0.2 mL of water. Separately the water layer was further extracted twice with 0.8 mL of hexane. The hexane extracts were combined, and dried at 90 °C in a water bath. The residue was dissolved in 0.2 mL of the mobile phase containing 100 μmol/L of 6-ketocholestanol and passed through a 0.45 μm membrane filter before injection into the HPLC system.

3. Results and discussion

3.1. HPLC–ECD conditions

HPLC–ECD conditions were optimized in terms of the HPLC columns, the column temperature, the flow rate, and the detection potential. For column selection, 15 ODS columns, two C8 columns, a C4 column, a silica column, two phenyl columns, two cyano columns, a diol column, and a C30 column were examined (Supplementary Table S1). Among the 25 columns examined, the YMC-Pack ODS-AL column was the only one that could separate lathosterol from cholesterol. Therefore, the YMC-Pack ODS-AL column was selected for the determination of lathosterol and cholesterol.

Next, column temperature was examined. From 40 to 50 °C, the elution of lathosterol became faster. Because the peaks of lathosterol and cholesterol were separated at all temperatures examined, 50 °C was selected as the column temperature.

The flow rate of the mobile phase was next examined. From 220 μL/min to 270 μL/min, the elution of lathosterol became faster. However, 250 μL/min was selected as the optimal flow rate on the basis of adequate resolution.

To determine the detection potential, hydrodynamic voltammograms of lathosterol and cholesterol were measured (Fig. 2). The potential was changed from 2.6 to 3.0 since the oxidation current of 6-ketocholestanol (IS) appeared at a more positive potential than the 2.6 V vs. Ag/AgCl observed in previous studies [16]. The oxidation current peak was observed around at 2.9 V vs. Ag/AgCl in both lathosterol and cholesterol. However, an applied potential of 2.8 V vs. Ag/AgCl was selected because of the best signal-to-noise ratios of lathosterol.

3.2. Determination of lathosterol by HPLC–ECD

Fig. 3 shows a chromatogram of standard lathosterol and cholesterol under optimized HPLC conditions. The theoretical plate numbers were 9800 for lathosterol and 8500 for cholesterol. Lathosterol and cholesterol were separated completely with a resolution (*Rs*) of 1.6. To assess the performance of the HPLC–ECD

Table 1
Linear range of calibration curve, detection limit, and relative standard deviation of lathosterol and cholesterol.

Compound	Linear range, μmol/L (pmol)	Calibration curve			Detection limit, μmol/L (pmol)	RSD, %	
		y, sample/I.S. = $a + bx$, μmol/L				Intra-day ($n = 5$)	Inter-day ($n = 3$)
		a	b	r			
Lathosterol	0.15–300 (0.75–1500)	0.0462	0.0076	0.995	0.03 (0.15)	0.58	1.29
Cholesterol	0.15–600 (0.75–3000)	0.0900	0.0068	0.995	0.03 (0.15)	0.73	0.65

To draw the calibration curve, various concentrations of lathosterol or cholesterol and 100 μmol/L of 6-ketocholestanol (IS) were injected into HPLC–ECD. The value of y was calculated on the basis of the ratio of the current peak heights of lathosterol or cholesterol and IS. r indicates the correlation coefficient. RSD indicates the relative standard deviation. To obtain RSD, 7.5 μmol/L of lathosterol and 15 μmol/L of cholesterol were repeatedly injected into HPLC–ECD in a day (intra-day) or three different days (inter-day).

for determining lathosterol and cholesterol, calibration curves of lathosterol (eight-point) and cholesterol (nine-point) using 6-ketocholestanol as the IS, were made. The linear ranges, correlation coefficients, detection limits, and RSDs (inter-day and intra-day precision) are listed in Table 1. The linear ranges and correlation coefficients of the calibration curves showed good linearity. Lathosterol (7.5 μmol/L) and cholesterol (15 μmol/L) were determined with an intra-day RSD ($n = 5$) and an inter-day RSD ($n = 3$) of less than 1.5%, respectively. These results indicate that the method is precise. The detection limit ($S/N = 3$) was 0.03 μmol/L (0.15 pmol) for both of lathosterol and cholesterol showing that the method is highly sensitive and the detection limit is comparable to GC–MS (0.40 μmol/L) [8].

3.3. Determination of serum total lathosterol by HPLC–ECD

The applicability of the method to human serum as a biological sample was examined. In the preliminary stages of this study, we tested whether simultaneous determination of cholesterol, lathosterol, and cholestanol was possible. However, under the conditions we optimized for the determination of lathosterol and cholesterol, the retention time of cholestanol was 70 min, and its peak was not observed in human serum. Fig. 4 shows a representative chromatogram of lathosterol and cholesterol in human serum. The concentration of lathosterol was determined to be 8.9 μmol/L in human serum (SRM909b). This result is in good agreement with a reported serum lathosterol level (4.4–13.7 μmol/L) in human serum [1,19]. A recovery test was conducted between 6 μmol/L, a lathosterol level in normal human serum indicated by several reports [1,19], and 750 μmol/L. The upper limit is higher than the highest reported plasma lathosterol level (650 μmol/L) of patients with lathosterolosis, a very rare inborn error of cholesterol synthesis caused by lathosterol 5-desaturase deficiency [20–24]. The results of the recovery test are listed in Table 2. The lathosterol recoveries from the spiked samples were more than 95.8% with RSD values less than 6.9% in human serum. In addition, the accuracy of total cholesterol in SRM909b, Level I was confirmed to be 98.6% with RSD values ($n = 5$) of less than 3.7%, enabling simultaneous determination of lathosterol and cholesterol. These results suggest that the present HPLC–ECD is suitable for the simultaneous determination of lathosterol and cholesterol in human serum.

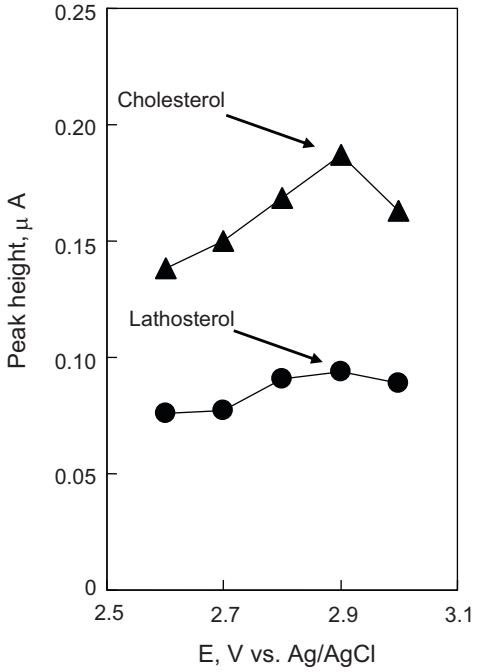

Fig. 2. Hydrodynamic voltammogram of lathosterol. Lathosterol (●) or cholesterol (▲) dissolved in the mobile phase (7.5 μmol/L for lathosterol and 15 μmol/L for cholesterol) was injected into HPLC–ECD. HPLC conditions; mobile phase, acetonitrile containing 30 mmol/L $LiClO_4$; flow rate, 250 μL/min; column temperature, 50 °C; column, YMC-Pack ODS-AL column (250 mm × 2.0 mm i.d., 5 μm).

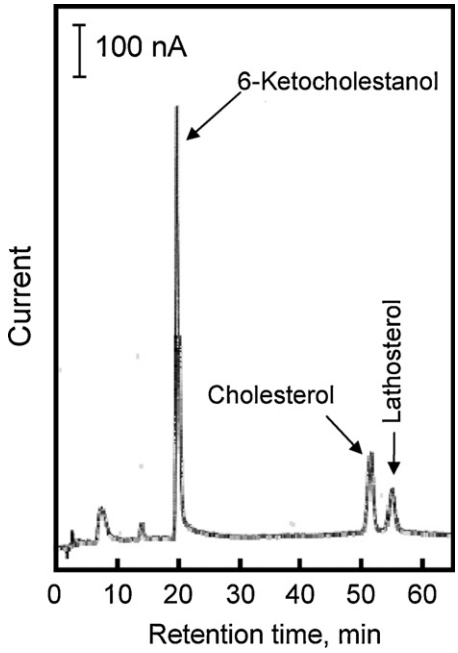

Fig. 3. Chromatogram of standard lathosterol and cholesterol. Lathosterol (7.5 μmol/L), cholesterol (15 μmol/L), and 6-ketocholestanol (100 μmol/L) were dissolved in the mobile phase and injected into HPLC–ECD. The HPLC conditions; mobile phase, acetonitrile containing 30 mmol/L $LiClO_4$; flow rate, 250 μL/min; column, YMC-Pack ODS-AL column (250 mm × 2.0 mm i.d., 5 μm); column temperature, 50 °C; applied potential, +2.8 V vs. Ag/AgCl; injection volume, 5 μL.

Table 2
Recovery of lathosterol from human serum (SRM909b), rat serum and mouse serum spiked with a standard.

Concentration added, μM	Human		Rat		Mouse	
	Recovery, %	RSD, % (n = 5)	Recovery, %	RSD, % (n = 5)	Recovery, %	RSD, % (n = 5)
6	97.2	6.9	96.4	7.3	98.5	5.4
300	103.4	3.4	98.7	3.7	97.0	2.9
750	95.8	2.4	105.5	2.6	96.0	3.1

For the recover test, each concentration of lathosterol was spiked into human serum, rat serum and mouse serum, and analyzed as described in Section 2. Rat serum was obtained from male Wistar rats (8–10 weeks old) and mouse serum was obtained from male BALB/C mice (4–6 weeks old) fed standard chow (CE-2).

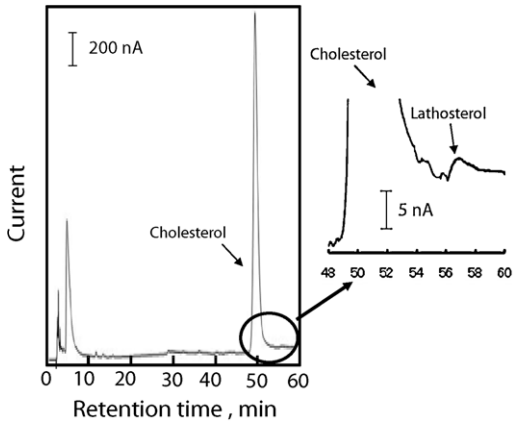

Fig. 4. Chromatogram of lathosterol and cholesterol in human serum (SRM909b). The preparation of the serum was described in Section 2. HPLC conditions used are the same as Fig. 3.

The utility of the method for the analysis of rat serum was also examined. Fig. 5 shows a representative chromatogram of lathosterol and cholesterol in rat serum. The concentration of lathosterol was determined to be 15.1 μmol/L in rat serum. In a similar way to human serum, a recovery test was conducted in rat serum and the results are also listed in Table 2. The lathosterol recoveries from the spiked samples were more than 96.4% with the RSD values less than 7.3%. Therefore, it is suggested that the current method is appropriate for the determination of rat serum lathosterol. In addition, the utility of this method for mouse serum was examined by a recovery test (Table 2), suggesting the applicability of the method to mouse serum.

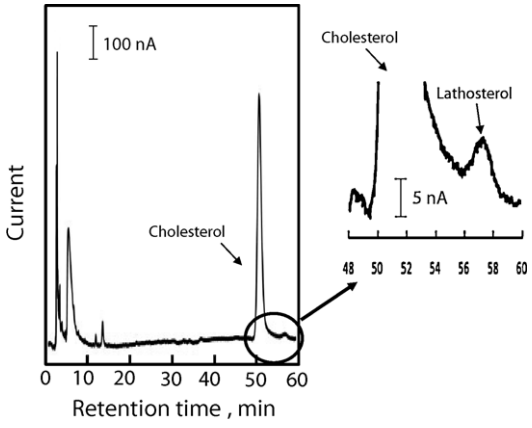

Fig. 5. Chromatogram of lathosterol and cholesterol in rat serum. The preparation of the serum was described in Section 2. HPLC conditions used are the same as Fig. 3.

Table 3
Concentrations of serum lathosterol and cholesterol, and the lathosterol/cholesterol ratio in rats fed a high-lathosterol diet.

	Day 0	Day 6
Lathosterol, μmol/L	15.0 ± 4.80	14.9 ± 5.16
Cholesterol, mmol/L	1.19 ± 0.111	1.25 ± 0.122
Lathosterol/cholesterol ratio, μmol/mmol	14.1 ± 10.6	12.2 ± 5.36

Data are expressed as means ± S.D. (n = 3).

3.4. Effect of lathosterol feeding on serum lathosterol in rats

It is well known that the serum level of lathosterol or the lathosterol/cholesterol ratio is used for monitoring the changes in endogenous cholesterol synthesis [1–6]. However, the effect of lathosterol feeding on the serum lathosterol level has not yet been examined in any animals. Therefore, we have determined the serum lathosterol concentration in rats fed a high-lathosterol diet. Table 3 shows the results of this dietary study. No significant changes were observed in serum lathosterol or cholesterol levels in rats fed a high-lathosterol diet for six days. These results suggest that lathosterol is less accumulative than cholestanol [16] and phytosterols [17]. In addition, these results may support the notion that serum lathosterol can be used as a marker of endogenous cholesterol synthesis as it seems to be relatively uninfluenced by food-derived lathosterol.

4. Conclusions

In the present study, we have developed a simple method for the simultaneous determination of lathosterol and cholesterol by HPLC–ECD. At present, this method is the only HPLC method enabling isocratic separation of underivatized lathosterol from cholesterol. The detection limits for lathosterol and cholesterol were 0.03 μmol/L and were found to be sensitive enough to determine serum lathosterol levels. The applicability of the present method to biological samples was shown by the determination of total lathosterol in human as well as rat serum by an internal standard method. Moreover, this method was used to determine serum lathosterol in rats fed a high-lathosterol diet, showing that lathosterol feeding did not affect the serum lathosterol levels. From these observations, the present method provides an alternative tool to investigate lathosterol physiology by determining serum lathosterol levels.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.02.017.

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