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Analysis of cholesterol and cholesteryl esters in human serum using capillary supercritical fluid chromatography

Dal Ho Kim, Kong-Joo Lee *, Gwi Suk Heo

Organic Analytical Laboratory, Korea Research Institute of Standards and Science, P.O. Box 102, Daejonsi, Daeduk Science Town 305-606, South Korea

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Abstract

Capillary supercritical fluid chromatography (SFC) using carbon dioxide as a mobile phase was applied for the determination of free cholesterol and cholesteryl esters in human serum. Serum samples were extracted with methanol–chloroform (2:1, v/v), and the extracts were analyzed by pressure programmed capillary SFC–flame-ionization detection (FID) without thermal degradation and derivatization. The total cholesterol concentrations obtained from SFC analysis were compared with those from GC or enzymatic analysis. The capillary SFC–FID method having high resolution gave an acceptable average relative standard deviation of 2.6%, and a detection limit of 4–6 pg. The quantitative results were acceptable for the simultaneous analysis of cholesterol and its esters in biological fluids. The concentration profiles of each compound in various samples, normal Korean human serum, Western human serum, and from high-cholesterol patient plasma, have been compared with this method.

1. Introduction

Abnormally high concentrations of serum cholesterol are recognized as the risk factor in atherosclerosis, coronary heart disease *etc.* [1–3]. Accuracy and precision in cholesterol measurement are required for the identification, classification and proper treatment of people with high concentrations of cholesterol in blood. Many analytical methods have been reported describing the determination of cholesterol and/or its esters including gas chromatography (GC) [2,4–6], high-performance liquid chromatography (HPLC) [3,7–10] and an enzymatic method [11]. However, GC is unsuitable for the determination of high-molecular-mass unsaturated cholesterol esters, since they undergo thermal decomposition at the temperatures required for their elution [16]. Chemical derivatization is often used to increase the volatility, and to improve the stability of cholesterol derivatives prior to GC analysis, but this method can not be used to analyze individual cholesteryl esters [2,5,6]. HPLC gives no significant thermal stability problems but it has its limitations in the analysis of cholesterol derivatives in human serum, requiring high column efficiency and sensitive detection [12–15]. The enzymatic methods available for total cholesterol analysis are relatively simple and easily automated, but their data show lack of precision and accuracy, and these methods can

* Corresponding author. Present address: College of pharmacy, Ewha Woman's University, Sŏdaemun-Ku, Seoul 120-750, South Korea.

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not be employed to determine the individual cholesteryl esters [5].

A supercritical mobile phase allows separation at low temperatures and with high chromatographic efficiency. Density- or pressure-programming make it possible to control the solvating power of the mobile phase and provide the same advantages as gradient elution in HPLC. The highly sensitive and universal flame-ionization detector (FID) can be employed in SFC analysis [17]. Capillary SFC is a powerful analytical technique and may be used for the determination of cholesterol and its esters. Although SFC separations of cholesterol and/or its esters have been reported, most have dealt with the separation of simple standard reagents or with instrumental manipulation including supercritical fluid extraction (SFE) or interfacing techniques [18–23].

The main objective of this study was to investigate the applicability of capillary SFC–FID for the individual determination of cholesterol and cholesteryl esters in human serum. Optimization of sample preparation and the chromatographic conditions has been performed and cholesterol and its esters were quantitatively determined in human serum. Capillary SFC is a unique procedure to obtain the fatty acid profiles of cholesteryl esters in clinically important human serum samples.

2. Experimental

2.1. Chemicals

SFC-grade carbon dioxide from Scott Specialty Gases (Plumsteadville, PA, USA) was used as a mobile phase. Cholesterol, cholesteryl *n*-valerate, cholesteryl laurate, cholesteryl myristate, cholesteryl palmitoleate, cholesteryl palmitate, cholesteryl linoleate, cholesteryl oleate, cholesteryl arachidonate, cholesteryl stearate, trimyristin, tripalmitin, epicoprostanol (5β-cholestan-3α-ol) and bistrimethylsilylacetamide were purchased from Sigma (St. Louis, MO, USA). Hexane, methanol and chloroform were HPLC grade from Burdick and Jackson (Muskegon, MI, USA). Lyophilized human serum CRM (certified reference material) KRISS-NOR is a Korean reference material from KRISS (Korea Research Institute of Standards and Science, Taejon, South Korea). Additional human control serum, CIBA-NOR was obtained in a lyophilized form from Ciba Corning Diagnostic (Irvine, CA, USA). High cholesterol patient serum was donated from Chungnam National University Hospital (Taejon, South Korea).

2.2. Sample preparation

Extraction of cholesterol and cholesteryl esters

Deionized water was added to lyophilized serum (KRISS-NOR and CIBA-NOR) and vortex-mixed for 1 min. Cholesterol derivatives associated with proteins in serum, which were determined in a preliminary extraction study, were extracted with methanol–chloroform. Methanol–chloroform gave good recovery and reproducibility. Methanol–chloroform (2:1, v/v, 18 ml) was added to 500 μl of the control serum solution or high-cholesterol patient serum, and mixed well for 2 min. Then, 6 ml each of water and chloroform were added. After vigorous mixing for 5 min, the organic phase (chloroform layer) was separated by centrifugation at 1600 *g* for 5 min. The organic layer was evaporated under a stream of nitrogen. The residue was reconstituted in 1 ml of *n*-hexane and further diluted 2–10 times for quantitating the high concentration of cholesteryl esters. For SFC analysis, 5 μl of cholesteryl *n*-valerate (10 mg/ml) was added as an internal standard (I.S.) prior to extraction. For GC analysis, the extract containing epicoprostanol as an internal standard was hydrolyzed and derivatized.

Hydrolysis and derivatization of cholesteryl esters

Cholesteryl esters were hydrolyzed to cholesterol and derivatized to cholesteryl trimethylsilyl ether by the procedure described previously [24]. Total cholesterol was determined by GC and the result was compared with that obtained with SFC. To hydrolyze the cholesteryl esters, the

extract (reconstituted mixture in 1 ml of *n*-hexane) was evaporated in a stream of nitrogen and the residue was dissolved in 500 μl of ethanol. Ethanolic KOH solution (0.6 ml of 8.6 *M* KOH solution mixed with 4 ml absolute ethanol) was added, and the mixture was incubated at 37°C for 3 h. Hydrolyzed cholesterol was extracted by adding of 5 ml of water and 10 ml of *n*-hexane, and the hexane layer was separated by centrifugation at 750 *g* for 5 min after vigorous mixing. The samples were either stored or were evaporated. Total cholesterol after hydrolysis was determined by SFC after adding cholesteryl *n*-valerate as an internal standard. This result was compared with that of GC-FID analysis which was performed after derivatization of hydrolyzed total cholesterol with bistrimethylsilylacetamide using epicoprostanol as an internal standard.

2.3. SFC analysis

The capillary SFC instrument consisted of a Model 260D high-pressure syringe pump (Isco, Lincoln, NE, USA) and a HP 5890 gas chromatograph oven (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame-ionization detector. The pump cylinder was cooled to −1°C when the pump was running. A 0.5-μl internal loop sampling valve (Model CI4W.5, Valco Instruments, Houston, TX, USA) was used for sample injections and the valve body temperature was maintained at −1°C. The SFI-1 SFC inlet splitter (SGE, Argent place, Ringwood, Australia) was connected to the sampling valve to prevent column overload and a fused-silica restrictor (2 m × 25 μm I.D.) was attached to the split exit sidearm. Separations were carried out in a 10 m × 50 μm I.D. column coated with 50% *n*-octyl, 50% methylpolysiloxane (SB-Octyl-50, 0.25 μm film thickness, Dionex, USA). A frit restrictor (Lee Scientific, Salt Lake City, UT, USA) was connected to the end of the column using a butt connector. The mobile phase was carbon dioxide linear pressure-programmed from 137.90 to 275.79 bar at 1.03 bar/min. In all experiments, the oven temperature and FID temperature were maintained at 65°C and 250°C respectively.

2.4. GC analysis

For total cholesterol determination, a HP 5890 GC-FID with HP Ultra 2 (crosslinked 5% phenyl methyl silicone, 25 m × 0.32 mm I.D., film thickness 0.17 μm) was used. Helium was used as a carrier gas and the flow-rate was 1.5 ml/min. The sample (1 μl) was introduced in the split mode (1:10). Column, injector and detector temperatures were 270, 270 and 300°C, respectively.

3. Results and discussion

3.1. Optimization of SFC separation

The SFC separations of standard mixtures (100 μg/ml each) of the cholesterol and cholesteryl esters were examined by changing the pressure-programming rate and oven temperature (Fig. 1). Optimum separations were obtained by ramping at 1.03 bar/min from 137.90 bar to 275.79 bar at 65°C oven temperature (Fig. 1A). Under these conditions most of the compounds show good resolution ($R_s > 1.2$) except linoleate, oleate and arachidonate. Resolution between the cholesteryl oleate and arachidonate was acceptable ($R_s = 0.99$), but cholesteryl linoleate and oleate nearly overlapped. As shown in Fig. 1B, elution time and resolution of the compounds decreased by raising the carbon dioxide pressure ramping rate. The effect of oven temperature on the separation is shown in Fig. 1C. At 65°C, cholesteryl linoleate/oleate could not be separated; on the other hand, it was impossible to separate cholesteryl oleate/arachidonate at a temperature raised to 75°C. Resolution and selectivity of the free and esterified cholesterol could be regulated by changing the pressure ramping rate and the oven temperature. In this study, pressure ramping rate and temperature were fixed at 1.03 bar/min and 65°C for qualitative and quantitative analysis of human serum.

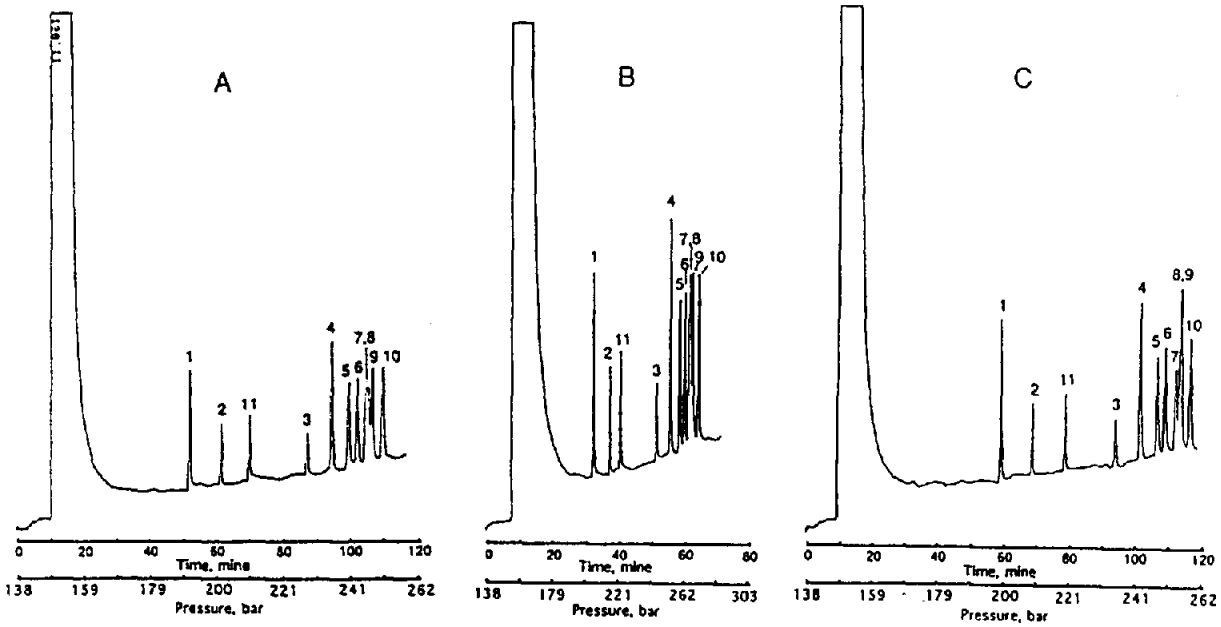

Fig. 1. Effect of pressure and temperature on capillary SFC separation of standard mixtures of cholesterol and cholesteryl esters (100 μg/ml each). Carbon dioxide mobile phase programmed from 138 bar to 276 bar with a ramping rate of 1 bar/min at 65°C (A), 2 bar/min at 65°C (B), 1 bar/min at 75°C (C). Peaks: 1 = cholesterol, 2 = cholesteryl *n*-valerate (I.S.), 3 = cholesteryl laurate (12:0), 4 = cholesteryl myristate (14:0), 5 = cholesteryl palmitoleate (16:1), 6 = cholesteryl palmitate (16:0), 7 = cholesteryl linoleate (18:2), 8 = cholesteryl oleate (18:1), 9 = cholesteryl arachidonate (20:4), 10 = cholesteryl stearate (18:0), and 11 = trimyristin.

3.2. Separation of cholesterol and its esters in human serum

Fig. 2 illustrates a series of typical SFC chromatograms of extracts of (A) KRISS-made lyophilized serum (KRISS-NOR, Korean normal serum), (B) commercially available control serum (CIBA-NOR, Western normal serum), (C) high-cholesterol patient serum I, (D) high-cholesterol patient serum II, (E) KRISS-NOR after hydrolysis of cholesteryl ester, and (F) representative standard mixture of triglycerides and fatty acids. As demonstrated in Figs. 1A and 2, the run time of the SFC separation was nearly 110 min. The resolution between cholesteryl linoleate and oleate was poor under the present SFC conditions. The order of elution in SFC was determined based on the polarity of the compounds. The elution time decreased for shorter carbon chains and with a lower degree of saturation of the fatty acyl chain of the cholesteryl ester. Less polar compounds were retained more on a non-polar stationary phase. Cholesterol derivatives in serum were identified by comparing their retention times with those of the calibration standards. These identifications were reconfirmed by adding the individual standards to serum extracts and surveying the changes of area, height, width and shape of the individual peaks after analysis under the same conditions. The main components in human serum were cholesterol, cholesteryl linoleate, oleate, arachidonate and palmitate. Interferences by representative lipids, trimyristin and tripalmitin and several fatty acids, were examined in Fig. 2F. The fatty acids were eluted with solvent, and the triacylglycerides had retention times between 60 and 80 min. In Fig. 2A–D, even though the individual peaks were not identified in this study, the unknown peaks in Fig. 2A (with elution

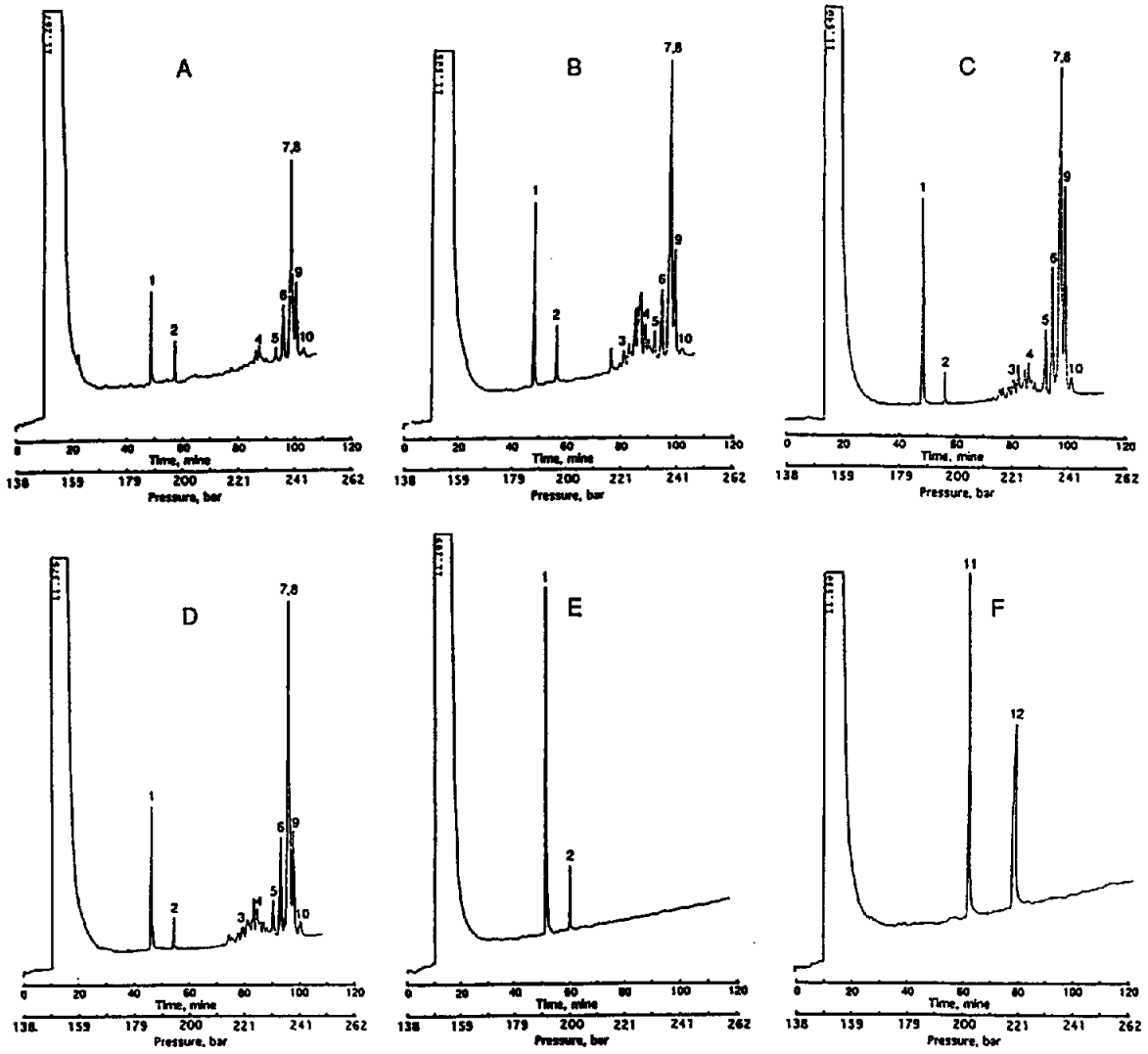

Fig. 2. Chromatograms of capillary SFC separations of extract of KRISS (A), extract of CIBA (B), extract of patient serum 1 (C), extract of patient serum 2 (D), hydrolyzed solution of the extract of KRISS (E), and standard mixture of triglycerides and fatty acids (F). Conditions were the same as in Fig. 1 (A). Peaks: 1 = cholesterol, 2 = cholesteryl *n*-valerate (I.S.), 3 = cholesteryl laurate (12:0), 4 = cholesteryl myristate (14:0), 5 = cholesteryl palmitoleate (16:1), 6 = cholesteryl palmitate (16:0), 7 = cholesteryl linoleate (18:2), 8 = cholesteryl oleate (18:1), 9 = cholesteryl arachidonate (20:4), 10 = cholesteryl stearate (18:0), 11 = trimyristin, and 12 = tripalmitin.

times in the range of 70 ~ 85 min) may be attributed to triacylglycerides (or another unknown compounds).

Comparison of SFC chromatograms of extracts of the Korean normal serum KRISS-NOR with Western normal serum CIBA-NOR and high-cholesterol Korean patient serum was made using the same separation conditions as shown in Fig. 2A–D. The results indicated that sera containing high cholesterol were enriched in saturated fatty acid esters, cholesteryl myristate and laurate. In addition, the peak area of the

unknown peaks, which are presumed to be triacylglycerides, was increased. A SFC chromatogram of total cholesterol after hydrolysis of cholesteryl esters in KRISS-NOR extract (Fig. 2E) was compared with that of KRISS-NOR extract without hydrolysis (Fig. 2A). The total amount of cholesterol in KRISS-NOR serum was compared among the capillary SFC method (Fig. 2A), the SFC method after hydrolysis (Fig. 2E), and the GC method after hydrolysis and additional derivatization as described in Experimental (data not shown). Sound agreement of these values indicates that capillary SFC is acceptable for the analysis of individual cholesterol and its esters.

3.3. Calibration

Calibration curves of the peak-area ratio of the standards and the internal standard plotted against the concentration of the standards were obtained (Fig. 3). Each calibration curve shows an acceptable correlation coefficient range (r) of 0.997–1.000 in the normal human serum concentration range of 25–200 μg/ml. The total concentrations of linoleate and oleate were determined by calibration of the total peak area since they gave the same response to the FID detector (peak-area ratio of linoleate to internal standard was 40 and that of oleate to internal standard was 38). To obtain the sum of these two esters, a standard calibration solution was made by mixing the stock standard solutions of the two esters at the same concentration and dilution to the desired concentration. Cholesteryl n-valerate was used as internal standard. The calibration curve shows a correlation coefficient of $r = 0.999$.

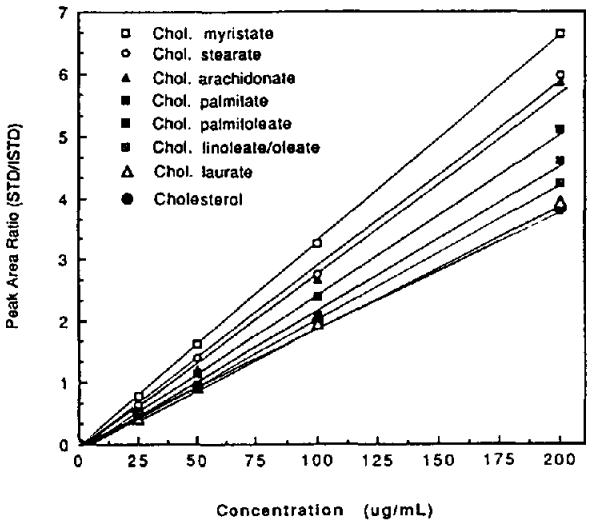

Fig. 3. Calibration curves for cholesterol and cholesteryl esters.

3.4. Quantitative analysis

Quantitative analysis was achieved using calibration of the peak-area ratio of the compounds to the internal standard. The determinations of the free and esterified cholesterols in KRISS-NOR, CIBA-NOR and high-cholesterol patient serum are shown in Table 1. We assessed the inter-day precision of the method by repeated analysis ($n = 3$). The relative standard deviations (R.S.D.), ranging from 0.3 to 4.7 (Table 1), are acceptable. The capillary SFC system shows a reasonable detection limit of 4–6 pg at a signal-to-noise ratio of 5. As shown in Table 1, KRISS-NOR, CIBA-NOR and patient serum show a similar fatty acid composition profile for the cholesteryl esters, except for cholesteryl laurate. In the clinical analysis, the cholesteryl ester composition in high-cholesterol Korean patient serum was similar to that in Korean normal serum, even though the total cholesterol concentrations were dramatically different. The saturated fatty acid fraction (sum of 14:0, 16:0 and 18:0) of Western human serum CIBA-NOR was higher than that in Korean normal human serum KRISS-NOR. KRISS-NOR, CIBA-NOR and high-cholesterol patient serum show a percentage of the total ester concentration of 86% (KRISS), 84% (CIBA), 83% (patient I) and 85% (patient II), respectively. The fractions of total and individual cholesteryl esters in human serum were comparable to those obtained with an HPLC assay [7]. Total cholesterol concentrations calculated by adding up the amounts of the individual compounds were 1379 ± 15 μg/ml for KRISS and 1532 ± 14 μg/ml for CIBA, which were in agreement (less than 8% difference) with the amounts measured by an en-

zymatic method or the GC method after hydrolysis and derivatization (1260 ± 35 μg/ml for KRISS and 1470 ± 16 μg/ml for CIBA). These results suggest that capillary SFC has advantages over HPLC, GC and enzymatic methods in the quantitative analysis of cholesterol and its esters. Further studies should be performed by applying SFC–MS for the identification of the unknown peaks in human serum.

4. Conclusion

Our studies have demonstrated the utility of capillary SFC for the analysis of free cholesterol and cholesteryl esters in human serum, without thermal degradation of the high-molecular-mass unsaturated cholesteryl esters and derivatization, and with high resolution and high sensitivity. The detection limit of SFC–FID system was 4–6 pg. With a simple extraction procedure, it was possible to measure free, total and individual esterified cholesterol from 50–500 μl of serum. With the capillary SFC column most of the compounds can be separated more clearly and efficiently than with HPLC. In addition, the quantitation data were comparable to those reported in previous studies. In a clinical study it was found that the fatty acid composition of the cholesteryl esters in Korean normal human serum is similar to that in a high-cholesterol Korean patient, and that the saturated fatty acid fraction (sum of 14:0, 16:0 and 18:0) in Western human serum is higher than that in Korean normal human serum. Capillary SFC can be a powerful tool in studies on cholesterol and cholesteryl ester metabolism in biological fluids.

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Table 1
Cholesterol and cholesteryl ester concentrations and their inter-day precisions determined in KRISS-NOR, CIBA-NOR human reference serum and patient serum by SFC-FID method

Compound	KRISS-NOR			CIBA-NOR			PATIENT I			PATIENT II		
	Concentration (μg/ml)	R.S.D (%)	Percentage[a]	Concentration (μg/ml)	R.S.D (%)	Percentage[a]	Concentration (μg/ml)	R.S.D (%)	Percentage[a]	Concentration (μg/ml)	R.S.D (%)	Percentage[a]
Cholesterol	191 ± +2.1	1.1	14	244 ± 4.9	2.0	16	888 ± 27.0	3.0	17	576 ± 15.6	2.7	15
Cholesteryl laurate (12:0)			0	56 ± 2.1	3.8	4	160 ± 3.7	2.3	3	52 ± 1.7	3.3	1
Cholesteryl myristate (140)	27 ± 0.8	3.0	2	163 ± 1.5	0.9	11	129 ± 3.2	2.5	3	87 ± 2.3	2.7	2
Cholesteryl palmitoleate (16:1)	42 ± 1.3	3.1	3	70 ± 1.4	2.0	5	365 ± 11.7	3.2	7	130 ± 3.0	2.3	4
Cholesteryl palmitate (16.0)	130 ± 4.9	3.8	9	125 ± 2.8	2.2	8	536 ± 8.6	1.6	11	382 ± 11.8	3.1	10
Cholesteryl linoleate (18:2) -oleate (18:1)	783 ± 5.5	0.7	57	688 ± 2.1	0.3	45	2139 ± 59.0	2.8	42	2093 ± 39.8	1.9	56
Cholesteryl arachidonate (20:4)	181 ± 7.6	3.9	13	168 ± 3.5	2.1	11	783 ± 15.3	2.0	15	374 ± 9.0	2.4	10
Cholesteryl stearate (18:0)	25 + 0.6	2.3	2	18 ± 0.7	4.0	1	96 ± 4.5	4.7	2	67 ± 2.3	3.5	2
Average of R.S.D		2.6			2.2			2.8			2.7	
Total cholesterol concentration	1379 ± 15	1.1	100	1532 ± 14	0.9	100	5096 ± 88.0	1.7	100	3761 ± 60.0	1.6	100

[a] Percentage of each compound to total cholesterol.

6. References

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