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Determination of α-tocopherol, free cholesterol, esterified cholesterols and triacylglycerols in human lipoproteins by high-performance liquid chromatography

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ABSTRACT

The determination of α-tocopherol, free cholesterol, esterified cholesterols and triacylglycerols in human plasma and in fractions containing individual lipoproteins was achieved by reversed-phase high-performance liquid chromatography (HPLC). The lipoprotein fractions, such as chylomicron, VLDL, LDL, HDL_2 and HDL_3,were collected by ultracentrifugation of human plasma. The chromatographic separation was accomplished with a column packed with Hitachi Gel 3057, which is a spherical octadecylsilica of particle size 3 μm. The mobile phase was acetonitrile–2-propanol (75:25, v/v),and the eluate was monitored with ultraviolet absorption and fluorescence detectors connected in series. Qualitative analysis of the main chromatographic peaks collected during the HPLC of a plasma sample was done with the use of field-desorption mass spectrometry. The determination analysis of α-tocopherol, free cholesterol and esterified cholesterols was effected with a single chromatographic run with *n*-hexane extracts of plasma or lipoprotein fraction. The separation and determination of these fat-soluble components required as little as 5 μl of plasma or lipoprotein fraction.

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INTRODUCTION

The lipids of the living body are a heterogeneous group of compounds related, either actually or potentially, to the fatty acids. They have the common property of being relatively insoluble in water and soluble in non-polar solvents. Fat serves as an insulating material in the subcutaneous tissues and around certain organs. Combinations of lipids and protein (lipoprotein) are important cellular constituents, occurring both in the cell membrane and in the mitochondria within the cytoplasma, and serving also as the means of transporting lipids in the blood[1].

Pure fat is less dense than water; it follows that as the proportion of lipid to protein in lipoproteins creases, the density decreases. Use is made if this property in separating the various lipoproteins in plasma by ultracentrifugation[2,3]. By this separation method, lipoproteins are classified as chylomicron, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). Lipoproteins are known to be composed of free cholesterol (FC), esterified cholesterols (cholesteryl esters, CE), triacylglycerols (triglycerides, TG), phospholipids, apoproteins, tocopherols and others[4,5]. Nevertheless, the distribution and the behaviour of these components in lipoproteins and the steric structure of lipoproteins are not yet clear[6]. Further, these components are correlated with the states of various diseases such as atherosclerosis and hyperlipaemia[7].

Numerous analytical procedures are used for the measurement of these lipids because of their clinical significance. The most commonly used method is spectrophotometry, which is based on the colour reactions of these components with acid reagents[8,9]. However, this method can be used for the determination of only one component or the total homologues in plasma. Chromatographic methods are capable of determining fat-soluble components; gas–liquid[10], thin-layer[11], and high-performance liquid chromatography (HPLC)[12–16] have been applied. HPLC is useful for the determination of various types and numbers of components of plasma[14,15] and TG[14] in plasma. Various HPLC methods have been reported for determining tocopherols[6,12–16], FC[6,14,15], CE[14,15] and TG[14] in plasma. Unfortunately, the separation of these components in fractions containing individual proteins has not yet been achieved.

In this paper, we report an HPLC method for the determination of these fat-soluble components in human plasma and in individual lipoprotein fractions.

EXPERIMENTAL

Chemicals

n-Hexane, acetonitrile, 2-propanol and ethanol of liquid chromatography grade were purchased from Wako (Osaka, Japan). Cholesterol and cholesteryl benzoate, caprylate, linoleate, oleate, palmitate and stearate were purchased from Nacalai Tesque (Kyoto, Japan), cholesteryl arachidonate and cholesteryl heptadecanoate from Sigma (St. Louis, MO, U.S.A.), cholesteryl myristate from Tokyo Kasei (Tokyo, Japan), triolein, trilinolein, 1,2-dioleoylstearin, 1,2-dipalmitoylolein, 1,3-dipalmitoylolein and 1,2-dipalmitoylstearin from P. L. Biochemicals (Milwaukee, WI, U.S.A.) and pig liver triglycerides from Serdary Research Labs. (London, Canada).

Preparation of samples

Plasma samples from individual normal healthy adult male volunteers were obtained from the National Defence Medical College (Saitama, Japan). All centrifugations of samples were carried out in a Hitachi Model 55P-7 ultracentrifuge at 4°C.

Lipoprotein fractions of human plasma were prepared by the standard method of flotation ultracentrifugation[2,6] using potassium bromide to adjust the density (d) of the plasma. The plasma was centrifuged sequentially at densities of 1.006, 1.063, 1.125 and 1.21 to separate plasma lipoproteins. The first fraction ($d < 1.006$), obtained by centrifugation for 30 min at 27 000 rpm (60 000 g) contains chylomicrons, the second fraction ($1.006 > d > 1.063$), obtained by centrifugation for 18 h at 39 000 rpm (120 000 g) contains VLDL, the third ($1.063 < d < 1.125$) contains LDL, the fourth ($1.125 < d < 1.21$) contains HDL_2 and the fifth ($d > 1.21$) contains HDL_3 and plasma proteins.

Preparation of sample extracts

A 100-μl plasma or lipoprotein sample was pipetted into a 115 mm × 23 mm I.D. glass test tube and 1 ml of water and 1 ml of ethanol were added and mixed on a vortex mixer for 2 min. Then 5 ml of n-hexane (containing 15 μg of cholesteryl benzoate or heptadecanoate as an internal standard) was added, and the tube was shaken mechanically for 10 min. A 4-ml aliquot of the n-hexane layer was pipetted into a 110 mm × 16 mm I.D. glass test-tube and evaporated to dryness in a water-bath at 45°C under reduced pressure using a rotary evaporator. The residue was then dissolved in 100 μl of ethanol and the extract was used for sampling for injection (usually 20 μl) onto the chromatographic column.

Apparatus

A Toyo Soda Model 803-B liquid chromatograph with a Schoeffel Model 770 UV detector and a Schoeffel Model 970 fluorescence detector connected in series was used. A 150 mm × 4 mm I.D. stainless-steel column packed with Hitachi Gel 3057 (octadecylsilica) of particle size 3 μm was used for the reversed-phase HPLC of lipids.

For routine plasma and lipoprotein analyses, an isocratic system was used with a mobile phase of acetonitrile–2-propanol (75:25, v/v). The eluent flow-rate was 1 ml/min with UV monitoring at 205 nm (0.04 a.u.f.s.) and with fluorescence monitoring at $\lambda_{ex.}$ 215 and $\lambda_{em.}$ 320 nm. The column temperature was maintained at 50°C. Field-desorption mass spectrometry (FD-MS) of cholesteryl esters and triglycerides collected during the HPLC of individual samples was carried out with a Hitachi Model M80-A mass spectrometer.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained from (A) the HPLC separation of a standard solution containing α-tocopherol, FC and individual CE and (B) the HPLC separation of a standard solution containing pig liver TG. A chromatographic run was completed in *ca.* 20 min. As shown by the solid line, CE with saturated fatty acids, cholesteryl caprylate ($C_{8:0}$), myristate ($C_{14:0}$), palmitate ($C_{16:0}$), heptadecanoate ($C_{17:0}$) and stearate ($C_{18:0}$) were eluted in that order owing to the differences in partitioning of the fatty acid chain length on the column. On the other hand, CE with

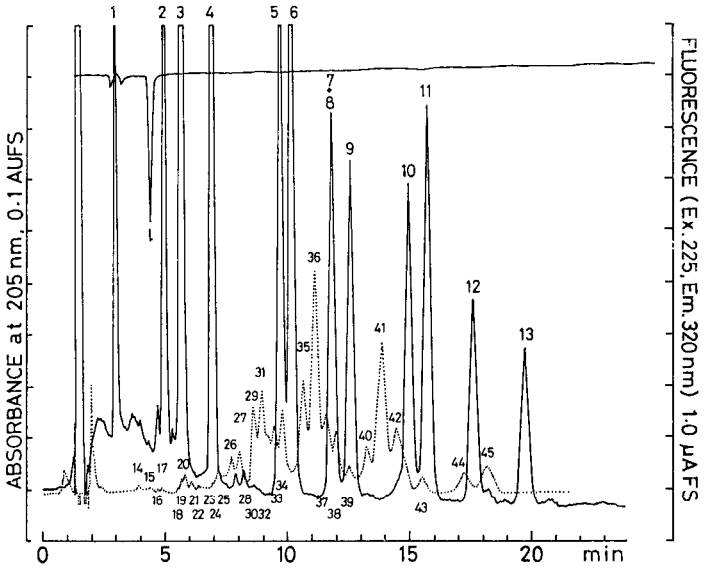

Fig. 1. Chromatograms of standard compounds. Conditions: column, stainless steel (15 × 0.4 cm I.D.) packed with Hitachi Gel 3057; eluent, acetonitrile–isopropanol (75:25, v/v); flow-rate, 1 ml/min; temperature, 50°C; pressure, 10.3 MPa; sample, (a) standard solution containing α-tocopherol (145 ng), cholesterol (2.23 μg) and cholesteryl esters (1.5–6.85 μg) (solid line) and (b) standard pig liver triglyceride (10 μg) (dotted line). For identification of individual peaks, see Table I.

unsaturated fatty acids, cholesteryl linolenate ($C_{18:3}$), arachidonate ($C_{20:4}$), linoleate ($C_{18:2}$) and oleate ($C_{18:1}$) were eluted in that order owing to the differences in the number of double bonds in the fatty acid chain. The peak for cholesteryl palmitoleate was eluted between those for cholesteryl myristate and linoleate.

TABLE I

CAPACITY FACTORS (k') OF MAIN FAT-SOLUBLE COMPOUNDS ON THE HITACHI GEL 3057 COLUMN

No.	*Compound*	k'	*No.*	*Compound*	k'
1	α-Tocopherol	1.25	23	Trilinolein (LLL)	4.35
2	Cholesterol	3.01	39	Triolein (OOO)	8.59
3	Cholesteryl benzoate	3.22	41	1,2- or 1,3-Dipalmitoylolein (PPO or POP)	9.59
4	Cholesteryl caprylate ($C_{8:0}$)	4.16			
5	Cholesteryl linolenate ($C_{18:3}$)	6.18	43	Tripalmitin (PPP)	10.61
6	Cholesteryl arachidonate ($C_{20:4}$)	6.51	44	1,2-Dioleoylstearin (OOS)	11.44
7	Cholesteryl linoleate ($C_{18:2}$)	7.72			
8	Cholesteryl palmitoleate ($C_{16:1}$)	7.72			
9	Cholesteryl myristate ($C_{14:0}$)	8.34			
10	Cholesteryl oleate ($C_{18:1}$)	10.06			
11	Cholesteryl palmitate ($C_{16:0}$)	10.68			
12	Cholesteryl heptadecanoate ($C_{17:0}$)	12.08			
13	Cholesteryl stearate ($C_{18:0}$)	13.65			

Similarly, as shown by the dotted line, standard pig liver TG were also separated under the same conditions as standard cholesteryl esters. From the chromatogram, it is clear that the sample contains about 30 kinds of individual TG. As the TG consist of three fatty acids and glycerol, the chromatographic behaviour of TG is more complicated than that of CE. However, as a rule, the retention of TG depends on the structure of the three fatty acids as with CE.

The retention characteristics of these standard compounds are given in Table I as capacity factors (k'). The HPLC separation of some CE and a few TG have been reported by other investigators[14,15] using columns packed with 10-μm reversed-phase octadecylsilica packings, but the resolutions of individual CE and/or TG were not satisfactory. With regard to the mobile phase, we chose acetonitrile–2-propanol (75:25, v/v) to measure fat-soluble components in blood at low UV wavelengths. The fluorescence detector was also employed for the sensitive measurement of α-tocopherol. Fig. 2 shows a chromatogram of a plasma extract corresponding to 8 μl of original human plasma. The completeness of extraction of α-tocopherol, FC and CE from plasma was investigated by the use of an internal standard. The recoveries of each component were also excellent when known amounts of standard compounds were added to plasma together with the internal standards before the extraction. The use of antioxidants was not necessary for the determination of α-tocopherol and other constituents.

With regard to plasma determination, the values obtained for α-tocopherol, FC and individual cholesteryl esters are given in Table II. These values are in agreement with those reported in the literature for α-tocopherol[13,16] and FC and CE[14,15]. In order to ascertain the identity of the chromatographic peaks of CE, each peak fraction was collected during a run with plasma, and then analysed by FD-MS. The mass

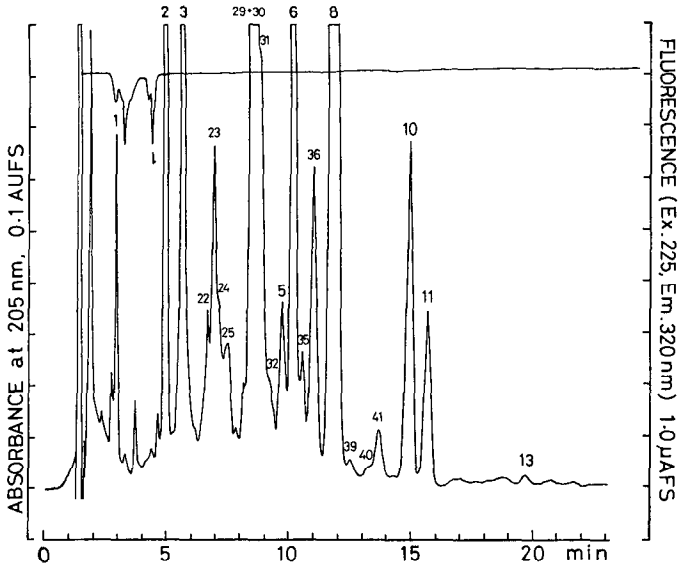

Fig. 2. Chromatogram of fat-soluble components of human plasma extracts. Conditions as in Fig. 1.

TABLE II

CONCENTRATIONS OF α-TOCOPHEROL, FREE CHOLESTEROL, INDIVIDUAL CHOLESTERYL ESTERS AND TOTAL CHOLESTEROL IN HUMAN PLASMA AND IN INDIVIDUAL LIPOPROTEIN FRACTIONS DETERMINED BY HPLC

Compound	*Concentration (mg/dl plasma) (mean ± S.D., n=5)*					
	Plasma	*Lipoprotein*				
		Chylomicron	*VLDL*	*LDL*	*HDL_2*	*HDL_3*
α-Tocopherol	0.93 ± 1.6	0.39 ± 0.33	0.20 ± 0.14	0.35 ± 0.21	0.14 ± 0.1	0.02 ± 0.02
Free cholesterol[a]	40.9 ± 4.7	5.50 ± 2.4	4.7 ± 2.6	19.7 ± 4.0	3.2 ± 0.6	1.7 ± 0.4
Cholesteryl linolenate[b]	4.2 ± 1.4	0.5 ± 0.2	0	3.2 ± 1.4	1.0 ± 0.1	0.5 ± 0.2
	2.5[a]	0.3[a]	0[a]	1.9[a]	0.6[a]	0.3[a]
Cholesteryl arachidonate[b]	34.2 ± 9.0	3.4 ± 1.6	2.8 ± 2.6	22.0 ± 9.9	7.4 ± 1.4	4.7 ± 1.1
	19.6[a]	2.0[a]	1.6[a]	12.6[a]	4.3[a]	2.7[a]
Cholesteryl linoleate[b]	191.2 ± 36.6	19.4 ± 6.8	11.8 ± 8.6	123.2 ± 34.0	40.0 ± 8.7	26.2 ± 2.6
Cholesteryl palmitoleate[b]	113.9[a]	11.6[a]	7.0[a]	73.4[a]	23.8[a]	15.6[a]
Cholesteryl oleate[b]	27.7 ± 5.6	2.6 ± 1.4	1.8 ± 1.4	21.5 ± 7.7	5.5 ± 0.6	3.2 ± 1.0
	16.4[a]	1.5[a]	1.1[a]	12.8[a]	3.3[a]	1.9[a]
Cholesteryl palmitate[b]	11.9 ± 2.3	1.6 ± 0.6	0.7 ± 0.6	10.1 ± 3.9	2.6 ± 0.3	2.2 ± 0.9
	7.4[a]	1.0[a]	0.4[a]	6.2[a]	1.6[a]	1.4[a]
Cholesteryl stearate[b]	1.7 ± 0.9	0	0	0	0	0
	1.0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
Cholesteryl ester[b]	270.9 ± 55.8	27.5 ± 10.6	17.1 ± 13.2	180.0 ± 56.9	56.5 ± 11.1	36.8 ± 5.8
Cholesteryl ester[a]	160.8	16.4	10.1	106.9	33.6	21.9
Total cholesterol[a]	201.7	21.9	14.8	126.6	36.8	23.6

[a] Value as free cholesterol.
[b] Value as individual cholesteryl estr.

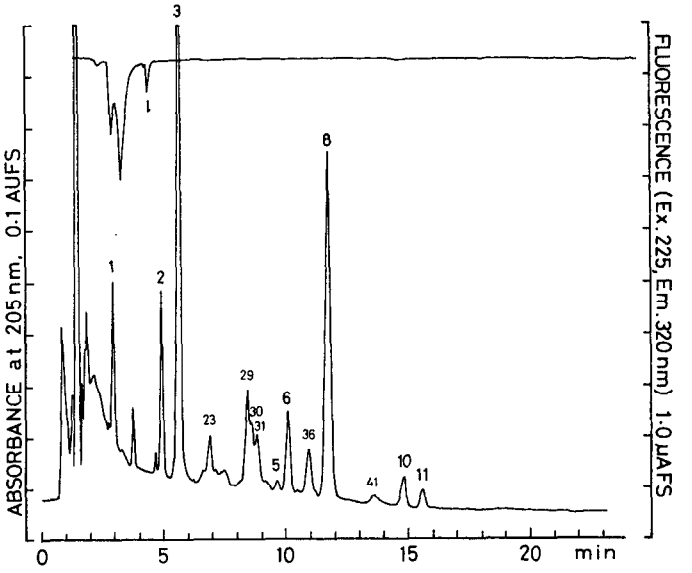

Fig. 3. Chromatogram of fat-soluble components of extracts from chylomicron. Conditions as in Fig. 1.

spectra of each peak of CE showed the molecular ion of each CE and fragment ions of cholesterol.

As Fig. 2 shows, not only tocopherols, FC and CE, but also many other peaks are separated and detected. These peaks from 22 to 41 were assigned as individual

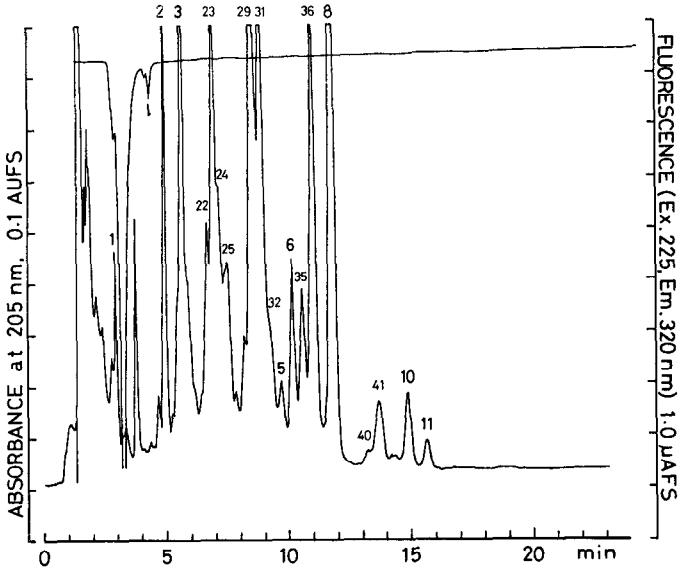

Fig. 4. Chromatogram of fat-soluble components of extract from VLDL. Conditions as in Fig. 1.

glycerols by FD-MS. Of these glycerol peaks, 23 and 41 were assigned as trilinolein and triolein, respectively. From the chromatogram, the profile of human plasma TG is apparently different from that of pig liver TG, as shown in Fig. 1. Unsaturated fatty acids have larger UV absorption coefficients than saturated fatty acids. Fatty acids of glycerols are combined with one, two or three fatty acids. Therefore, it is necessary to identify the TG peak and to prepare the individual TG standard compounds for the determination of these components in plasma.

Each lipoprotein fraction was chromatographed under the same conditions as for plasma extracts. A series chromatograms of fractions of chylomicron, VLDL, LDL, HDL_2 and HDL_3 from the same subject are illustrated in Figs. 3–7.

Fig. 3 shows a chromatogram of the chylomicron fraction, corresponding to 25 μl of original plasma. Chylomicron is derived from the intestinal absorption of TG and has a molecular weight of several hundred million, but its concentration in plasma is low compared with those of other lipoproteins. Chylomicron is also characterized by relatively large amounts of TG and by small amounts of FC, phospholipids and apoprotein. As expected, the peaks of α-tocopherol and TG in the chromatogram of chylomicron are larger than those of plasma or other lipoproteins. In particularly, chylomicron has 35% of the total α-tocopherol in plasma.

Figs. 4 and 5 show chromatograms of VLDL and LDL, respectively. VLDL and LDL have higher contents of FC and CE than chylomicron, but less TG. These two chromatograms resemble that of the original plasma as shown in Fig. 2. However, the contents of α-tocopherol, FC and CE of LDL are apparently higher than those of VLDL. Therefore, it is confirmed that almost all of the lipids in plasma are involved in the LDL. It is known that VLDL is formed to a lesser extent from dietary lipids but is mainly derived from the liver for the export of TG. The bulk of plasma VLDL is of

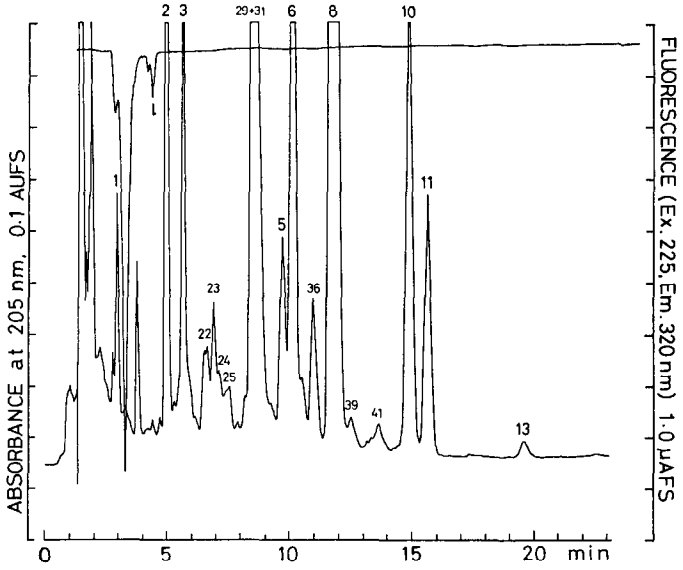

Fig. 5. Chromatogram of fat-soluble components of extract from LDL. Conditions as in Fig. 1.

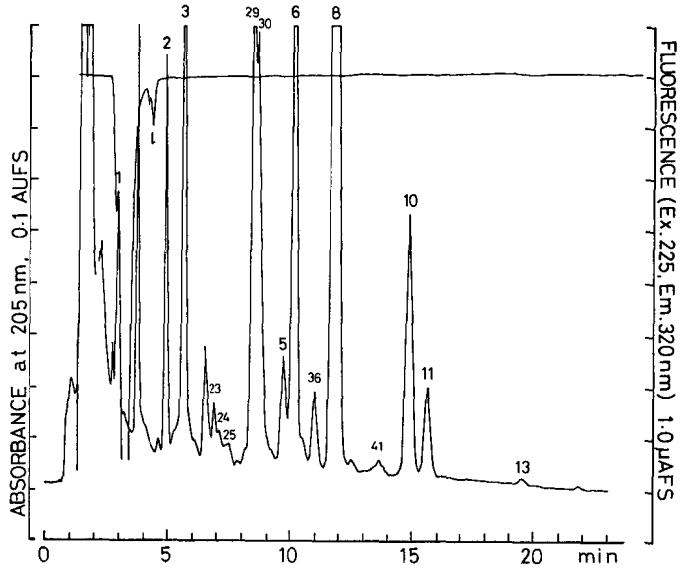

Fig. 6. Chromatogram of fat-soluble components of extract from HDL_2. Conditions as in Fig. 1.

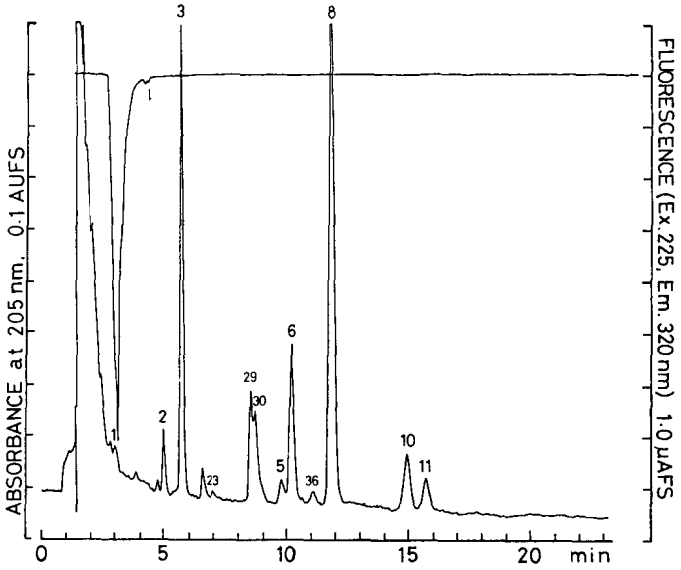

Fig. 7. Chromatogram of fat-soluble components of extract from HDL_3. Conditions as in Fig. 1.

hepatic origin, being the vehicle for transportation of TG from the liver to the extrahepatic tissues. This suggests a high content of TG in VLDL and it agrees with the results presented here. On the other hand, LDL represents the final stage in the catabolism of VLDL and possibly chylomicron.

Figs. 6 and 7 show chromatograms of HDL_2 and HDL_3, corresponding to 32 and 17 μl of original plasma, respectively. The characteristic profiles of these two chromatograms show a relatively high content of CE and low contents of FC and TG. In particular, HDL_3 has more small peaks of TG than other lipoproteins. This result is in agreement with the result for the chemical composition of HDL reported in the literature[17].

The results of the determination of α-tocopherol, FC and CE in plasma or individual lipoprotein fractions are listed in Table II. In the plasma lipoprotein, the FC value averaged 15.8% for chylomicron, 13.5% for VLDL, 56.6% for LDL, 9.2% for HDL_2 and 4.9% for HDL_3. Similarly, the α-tocopherol values averaged 35.5% for chylomicron, 18.2% for VLDL, 31.8% for LDL, 12.7% for HDL_2 and 1.8% for HDL_3. The total value of α-tocopherol, FC, individual CE and total cholesterol of five lipoprotein fractions are almost equal to the values for plasma. These results demonstrate that the present HPLC method is applicable to the determination of fat-soluble components in plasma and lipoprotein fractions.

The data obtained by the HPLC method were compared with those obtained by other HPLC or spectrophotometric methods. It was found that the plasma levels of α-tocopherol and FC are similar to those obtained by the other methods[14,16], but the plasma levels of individual CE are different[14,15]. These differences were considered to be due to the use of plasma samples from different human races.

Lipoproteins from human plasma have been isolated in narrow size ranges, and their chemical compositions have been determined[6,17]. Calulations based on these results were consistent with a model having FC, apoproteins and phospholipids present in a thin film around an inner core of CE and TG. The results from the analyses of lipoprotein fractions by HPLC method appear to be compatible with this model.

CONCLUSION

The physiological importance of the physical exchange of α-tocopherol and free cholesterol between lipoproteins and tissues is not yet clear although cholesterol[18] and α-tocopherol[19] are rapidly taken up by tissues from plasma lipoproteins. Glomset[17] reported a scheme for cholesterol transport involving the physical exchange of free cholesterol between the membrane of tissues and plasma lipoprotein (particularly HDL) which is mediated by the action of lecithin–cholesterol acyltransferase (LCAT). By the use of the present HPLC method, physical exchange of fat-soluble components may be analysed clearly.

The use of HPLC made it possible to increase the separability of analytical procedures for many compounds contained in body fluids and tissues[20–22]. The HPLC method reported here was developed to determine many components not only of plasma but also of individual lipoprotein fractions. The HPLC method provides results that compare favourably with those obtained with widely used methods and yields important information about many fat-soluble components from one easily prepared extract. An additional advantage is that with even as little as 5 μl of plasma,

serum or lipoprotein fraction, it is possible to measure the content of the main fat-soluble components. Further, the HPLC method is applicable to the diagnosis of the state of many diseases which relate to the content of marker components in the body, and to the evaluation of the effects of the treatment of diseases with drugs[23].

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