paper and note on methodology

Identification and quantification of diacylglycerols in HDL and accessibility to lipase[1]

Claude Vieu, Beatrice Jaspard, Ronald Barbaras, Jeanine Manent, Hugues Chap, Bertrand Perret, and Xavier Collet[2]

Institut National de la Santé et de la Recherche Médicale, Unité 326, Phospholipides membranaires, Signalisation cellulaire et Lipoprotéines, Hopital Purpan, 31059, Toulouse Cedex, France

Abstract We have investigated the presence of diacylglycerols in lipoproteins and especially in HDL. Lipoprotein diacylglycerols are very difficult to isolate and to quantify using classical enzymatic techniques, as they are measured in the presence of triacylglycerols and monoacylglycerols. Using a rapid and very sensitive method of gas–liquid chromatography, developed for neutral lipid analysis on an Ultra 1 Hewlett-Packard fused silica capillary column, diacylglycerols (DG) were identified in HDL and classified into five groups: DG 14–16, DG 16–16, DG 16–18, DG 18–18, and DG 18–20. However, their quantitation was difficult due to only partial resolution of molecular species. HDL lipids were submitted to preparative gas–liquid chromatography and diacylglycerols were then silylated using trimethylsilyl reagents. The trimethylsilyl ethers were analyzed by gas–liquid chromatography on a Restek 50 capillary column and were resolved on the basis of carbon number, degree of unsaturation, and double bond positions. The amount of HDL diacylglycerols was twice that of triacylglycerols. The major molecular species of diacylglycerols consisted of 16:0–18:2n–6, 18:0–18:2n–6, and 16:0–18:1n–9 as the major molecular species (33.4, 22.2, and 16.1 mol % of total diacylglycerols, respectively). Using guinea pig cationic pancreatic lipase in order to test the accessibility of diacylglycerols at the surface of HDL, we measured 59% of diacylglycerol hydrolysis, whereas no triacylglycerol hydrolysis was obtained. In addition, most of diacylglycerols having long chain fatty acids, such as 18–20, were completely hydrolyzed, whereas 18–18 and 16–18 were only partially hydrolyzed (64 and 46%, respectively). This reflects a different partition of diacylglycerol molecular species between the particle's surface and the lipid core in HDL. This is the first analysis of diacylglycerol molecular species and their distribution in native lipoprotein particles.—**Vieu, C., B. Jaspard, R. Barbaras, J. Manent, H. Chap, B. Perret, and X. Collet.** Identification and quantitation of diacylglycerols in HDL and accessibility to lipase. *J. Lipid Res.* 1996. **37:** 1153–1161.

Supplementary key words HDL • diacylglycerols • gas–liquid chromatography

High density lipoproteins (HDL) are involved in the reverse cholesterol transport from peripheral cells to the liver (1). The human plasma HDL are heterogeneous in terms of particle size, density, lipid content, and apolipoprotein composition (2). HDL are subjected to constant remodeling through intricate mechanisms: exchange of surface components with cell membranes or other lipoproteins, specific cellular interactions, and modifications by lipolytic enzymes. The lipid composition of HDL is always given in terms of cholesterol (free and esterified), phospholipids, and triacylglycerols. The latter represent about 4% of the HDL composition (by weight). Triacylglycerols are measured using a kit method, and this procedure is based on the complete enzymatic hydrolysis of triacylglycerols and measurement of the glycerol released. In fact, this method is not specific for triacylglycerols because diacylglycerols and monoglycerols present in HDL will be hydrolyzed and measured in the same way. Thus, diacylglycerols have never been taken into account in the HDL composition and little is known about the diacylglycerol molecular species present in the lipoproteins.

In plasma, diacylglycerols occur as important intermediates in many metabolic processes, such as VLDL or HDL lipolysis under the action of lipoprotein lipase or hepatic triacylglycerol lipase. The diacylglycerols that are transiently produced from triacylglycerols during the action of lipolytic enzymes may account for their presence in the lipoproteins. Hydrolytic enzymes, such as lipoprotein lipase and hepatic lipase, hydrolyze triacylglycerols at acyl ester primary alcohol bonds, producing *sn*-1,2 (2,3)-diacylglycerols. Further hydrolysis of the 1(3)-esters bound by these lipases produces 2-monoacylglycerol which is isomerized to 1(3)-monoacyl-

Abbreviations: DG, diacylglycerol; TG, triacylglycerol; HDL, high density lipoprotein; PC, phosphatidylcholine; TMS, trimethylsilyl; CE, cholesteryl ester.

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[2]To whom correspondence should be addressed.

glycerol before complete hydrolysis. Most, if not all, of these transformations occur at an interface of phospholipids and free cholesterol which surrounds the neutral lipid core of lipoproteins. The characterization of diacylglycerol as a component of HDL will open new perspectives in the physiological processes involving diacylglycerols. However, these extremely polar molecules are very difficult to quantify.

We have used a gas–liquid chromatographic technique to determine in detail the composition and structural characteristics of human plasma HDL diacylglycerols.

EXPERIMENTAL METHODS

Materials

[(N,O)-bis(trimethyl-silyl)]trifluoro-acetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) was obtained from Sigma (St. Quentin Fallavier, France) while pyridine was from Prolabo (Paris, France). Acetonitrile was from Rathburn (Walker Burn, Scotland). Phospholipase C (*C. welchii*, Type XIV); the phosphatidylcholine molecular species: 1-palmitoyl-2-oleoyl- (16:0–18:1), 1-palmitoyl-2-linoleoyl- (16:0–18:2), 1-palmitoyl-2-arachidonoyl- (16:0–20:4), 1-stearoyl-2-oleoyl- (18:0–18:1), 1,2-dioleoyl- (18:1–18:1), 1-stearoyl-2-linoleoyl- (18:0–18:2), 1,2-dilinoleoyl-phosphatidylcholine (18:2–18:2), purified phosphatidylcholines from soybean, bovine brain and liver, and fresh egg yolk; the following diacylglycerols: *sn*-1,3-dicapryloyl- (8:0–8:0), *sn*-1,2-dicaproyl- (10:0–10:0), sn-1,3-dilauroyl- (12:0–12:0), *sn*-1,3-dimyristoyl- (14:0– 14:0), *sn*-1,3-dipentadecanoyl- (15:0–15:0), *sn*-1,2-dipalmitoyl- (16:0–16:0), *sn*-1-palmitoyl-3-stearoyl- (16:0– 18:0), *sn*-1,3-distearoyl- (18:0–18:0), *sn*-1,2-dioleoyl- (18:1–18:1), *sn*-1,3-dilinoleoyl- (18:2–18:2), *sn*-1-stearoyl-2-arachidonoyl- (18:0–20:4), *sn*-1,3-diarachidoyl- (20:0– 20:0) glycerols; cholesterol, stigmasterol, cholesteryl heptadecanoate, triheptadecanoylglycerol; the following monoacylglycerols; myristoyl- (14:0) and stearoyl- (18:0) *sn*-glycerol were obtained from Sigma. Twelve mixed *sn*-1,2 and -1,3 synthetic diacylglycerols were generated from monoacylglycerols (14:0) or (16:0) or (18:0) or (18:1n–9), and fatty acids (14:0) or (16:0) or (18:2n–6) or (20:3n–6) or (20:4n–6) or (20:5n–3) or (22:6n–3), (mol/mol) according to Neises and Steglich (3) using dicyclohexylcarbodiimide and 4-dimethylaminopyridine dissolved in dichloromethane as reagents (1:0.1; mol/mol). The diacylglycerol synthesis reaction was performed at room temperature during 3 h. The lipids were extracted according to Bligh and Dyer (4) in the presence of 0.1 N HCOOH. Purified lipase/phospholipase A_1 from guinea pig pancreas was isolated in our laboratory as described by Fauvel et al. (5).

Measurements of neutral lipid molecular species by gas–liquid chromatography (GLC)

The amount of free cholesterol was assayed enzymatically in each HDL sample as described in Analytical procedures. Total lipids containing about 20 nmol of free cholesterol/ml were extracted by the method of Bligh and Dyer (4), as modified by the addition of formic acid (12 µl per ml of aqueous phase) in 8-ml Teflon-capped vials. Before extraction, four internal standards were added: stigmasterol, 3 µg; *sn*-1,3-dimyristoyl glycerol (DG 14:0–14:0), 4 µg; cholesteryl heptadecanoate (CE-C17:0), 6 µg, and triheptadecanoyl glycerol (triacylglycerol C51), 1 µg. About 8 µg of butylated hydroxytoluene (BHT) was added in each sample as an antioxidant. The chloroform phase was evaporated to dryness and dissolved in 100 µl ethyl acetate (spectroscopy grade, Merck, Darmstadt, Germany). After injection of 2.5 µl, lipids were analyzed by gas–liquid chromatography (GLC) as previously described (6), using an Ultra 1 Hewlett-Packard fused silica capillary column (5 m × 0.31 mm i.d.) coated with crosslinked dimethyl siloxane. Oven temperature was programmed from 205°C to 345°C at a rate of 6°C/min and the carrier gas was hydrogen (0.5 bar). Use of a solventless injector avoids response from phospholipids which are deposited on the moving glass needle. The response factors for lipid classes were determined by a mixture of internal standards.

Preparative gas–liquid chromatography

A micropreparative apparatus was designed to separate major phospholipids from neutral lipids. This apparatus was made by coupling an Ultra 1 capillary column (0.6 m × 0.31 mm i.d.) to an all glass (1/4" o.d.) splitless injector equipped with a cold septum and an outer inlet for carrier gas (N_2; 0.086 bar). The temperatures of the injector and oven were 310°C and 295°C, respectively. The column outlet was bubbling in a small reaction-vial containing 80 µl of pyridine. Total lipid extract (100 µl ethyl acetate) was evaporated to dryness and dissolved in 25 µl of ethyl acetate in a small sharp vial. Aliquots (3 µl) were injected at 30-sec intervals. Ethyl acetate and neutral lipid eluates were trapped in pyridine at 20°C. The recovery of neutral lipids was immediately checked on the same 5-m capillary column as previously described. All cholesterol, stigmasterol, and all diacylglycerols were recovered, whereas cholesteryl esters and triacylglycerols were only partially recovered (50% and 25%, respectively). These values were predicted by the isothermal conditions of oven and injector. After this preparative procedure, the injector was washed five times with 6 µl of ethyl acetate. The temperature of the oven was increased (345°C) and the carrier gas was raised to 0.1 Bar. This wash procedure was essential to

prevent pollution of the next step. Finally, 25 µl of ethyl acetate was injected and trapped in 80 µl of pyridine and the absence of any neutral lipids was then checked on the same capillary column.

Preparation of trimethyl silyl ether derivatives

Pyridine was rapidly evaporated to dryness in reaction-vial with a vacuum pump, to prevent any trace of water. Then, the sample was dissolved in 80 µl of the silylation reagent; BSTFA (1% TMCS)-acetonitrile dried over KOH pellets (1:1; v/v). The silylation reaction was performed overnight at 20°C. Synthetic *sn*-1,2- or 1,3-diacylglycerols and the natural isomers formed on storage were used as standards. Some phospholipids containing the known fatty acids were hydrolyzed by phospholipase C to diacylglycerols as previously described by Kuksis, Myher, and Geher (7). These generated diacylglycerols were also used to identify more precisely the natural diacylglycerols present in HDL. The mixtures of standards (4 µg of each) were silylated after only 1 h at 20°C in the presence of 100 µl of the silylation reagent.

Measurements of diacylglycerols by gas–liquid chromatography

After injection of 4 µl, the lipid-trimethyl silyl ethers were analyzed by GLC using a Restek 50 capillary column (Restek Corp., Port Matilda, PA) (23 m × 0.25 mm i.d.) coated with phenyl (50%) methyl (50%) siloxane. Oven temperature was programmed from 215°C to 325°C at a rate of 4°C/min and the carrier gas was hydrogen (1 bar). Diacylglycerol TMS ethers molecular species were resolved on the basis of their carbon number, the degree of unsaturation, and the type of isomers. The response factors for lipid classes were determined by a mixture of internal standards.

Analytical procedures

Lipoproteins were isolated as previously described (8). HDL triacylglycerol content was assayed enzymatically in an automatic analyzer (Cobas Bio, Roche, France). Proteins were measured according to Lowry et al. (9). Total and unesterified cholesterols were determined enzymatically (10), using commercial kits (Boehringer, Mannheim, Germany). Phospholipids were estimated according to Böttcher, Van Gent, and Pries (11). Neutral lipids were separated using petroleum ether–diethyl ether–acetic acid 165:35:2. Hydrogel apoA-I/B were also used for the quantitation of apoA-I based on an electroimmunodiffusion technique in an agarose gel.

RESULTS

Measurements of neutral lipid molecular species of HDL by GLC

A typical profile of HDL neutral lipid molecular species was obtained by gas–liquid chromatography according to total carbon number (Cn) where n is the total number of carbon atoms in free cholesterol, diacylglycerols, esterified cholesterol, and triacylglycerols (**Fig. 1**). This method has proved to be very useful in terms of sensitivity, specificity, and speed of analysis. The percentage of cholesteryl arachidonate represents 7.3 ± 0.74% (n = 4) and 7.3 ± 0.50% (n = 5) of total cholesteryl ester molecular species in HDL_2 and HDL_3, respectively (**Table 1**). The free and esterified cholesterol of HDL subfractions appeared to be fully recovered as the value corresponded to the value found in HDL by classical enzymatic measurements. Furthermore, correlations between gas–liquid chromatography and enzymatic measurement gave the following correlation coefficients and slopes; 0.988 (slope 1.209) for total cholesterol (n = 16), 0.973 (slope 1.159) for esterified cholesterol (n = 14), and 0.978 (slope 1.619) for triacylglycerols. Concerning HDL diacylglycerols, there

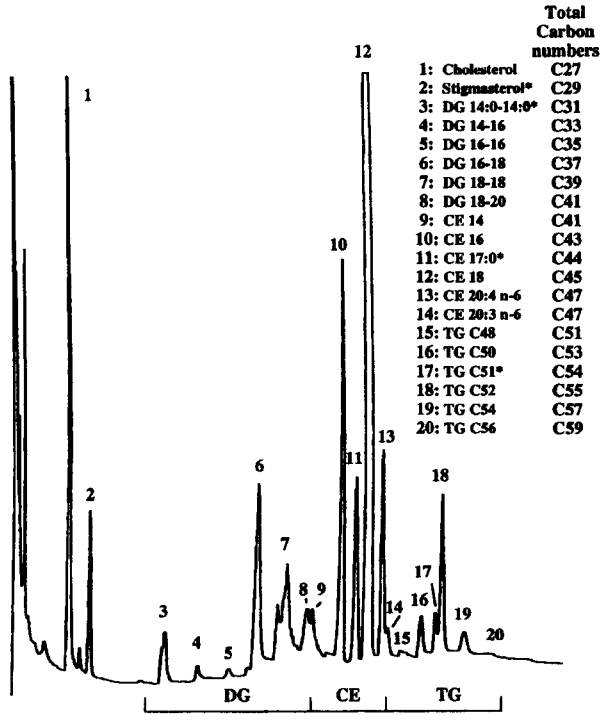

Fig. 1. Analysis of the neutral lipid molecular species of HDL_2 by gas–liquid chromatography. An Ultra 1 Hewlett-Packard fused silica capillary column coated with crosslinked methyl siloxane was used as described in Materials and Methods. Free cholesterol, diacylglycerols, cholesteryl esters, and triacylglycerols were determined based on total carbon numbers; *, internal standards.

TABLE 1. Neutral lipid molecular species of HDL_2 and HDL_3

Molecular Species	HDL_2 (n = 4)	HDL_3 (n = 5)
	%	
Triacylglycerol		
C48	15.19 ± 8.19	11.01 ± 5.70
C50	25.83 ± 3.43	22.13 ± 3.07
C52	47.66 ± 3.77	44.55 ± 4.9
C54	13.65 ± 2.29	18.24 ± 5.05
C56	traces	traces
Esterified cholesterol		
C14	4.08 ± 2.82	5.07 ± 1.59
C16	16.57 ± 1.85	16.30 ± 1.62
C18	71.44 ± 5.48	69.80 ± 7.3
C20:4n-6	7.30 ± 0.74	7.30 ± 0.50
C20:3n-6	1.41 ± 0.60	1.36 ± 0.42

HDL were isolated by ultracentrifugation and the lipid composition was analyzed by gas–liquid chromatography as described in Materials and Methods. Triacylglycerol molecular species were determined based on total acyl carbon number (Cn). Cholesterol esterified molecular species are identified as a Cn CE where n is the number of carbons in the fatty acid chain.

was a partial resolution of the molecular species but they were eluted as broad peaks. This made the quantitation of these molecules difficult, by direct integration.

Identification and quantitation of HDL diacylglycerols

In order to obtain a better resolution in the diacylglycerol separation using an intermediately polar capillary column, we modified diacylglycerols into trimethylsilyl ether derivatives with a quantitative yield. However, we observed a partial inhibition of polar lipid silylation in the presence of phospholipids, even after drying the lipid extract under vacuum. We thus designed a micropreparative apparatus to separate major phospholipids from neutral lipids as the phospholipids remained on the glass injector. This apparatus was obtained by coupling an ULTRA 1 capillary column to the splitless injector previously described in Materials and Methods. Cholesterol, stigmasterol, and all diacylglycerols were completely recovered, whereas cholesteryl esters and triacylglycerols were only partially recovered (50% and 25%, respectively) (not shown). Sterols and diacylglycerols were derivatized into trimethylsilyl ethers and then analyzed using a Restek 50 capillary column. It is noteworthy that injection of pure phospholipids in the ULTRA 1 capillary column showed a profile with no detectable peaks. We have determined that 96% of lipid phosphorus remained on the glass needle of the solventless injector (not shown). The molecular species of the HDL diacylglycerols were identified by reference to the retention times of two mixtures of different molecular species of diacylglycerols (**Fig. 2,** upper and lower panels). *sn*-1,2-Diacylglycerols were generated from different phosphatidylcholines treated with phospholipase C and mixed *sn*-1,2- and *sn*-1,3-synthetic diacylglycerols were generated from monoacylglycerols and fatty acids. Some of the diacylglycerol standards present both *sn*-1,2- and *sn*-1,3-isomers which are very well separated after silylation with *sn*-1,2-diacylglycerols eluting before the *sn*-1,3-isomers. A typical profile of HDL TMS-derivatized diacylglycerols is presented in **Fig. 3** and approximately 27 peaks were identified on the chromatogram including TMS-derivatized cholesterol and stigmasterol (**Table 2**). A 100% recovery was achieved upon silylation, as non-silylated cholesterol and stigmasterol, which have different retention times, were not present on the chromatogram. We found five families, 14–16, 16–16, 16–18, 18–18, and 18–20, which were identified on the chromatogram and represented 1.24 ± 0.70, 1.29 ± 0.7, 50.89 ± 1.94, 42.84 ± 1.50, and 3.74 ± 1.18% of total diacyl-

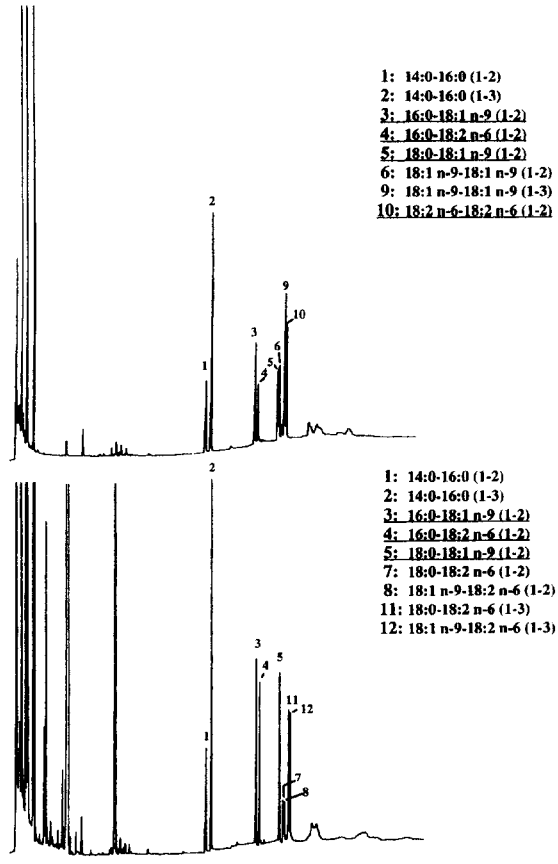

Fig. 2. Gas–liquid chromatograms of *sn*-(1-2) and -(1-3) diacylglycerol standards (TMS ether derivatives). *sn*-(1,2) Diacylglycerols were obtained from synthetic phosphatidylcholines after treatment by phospholipase C (underlined; compounds: 3, 4, 5 and 10). Other diacylglycerols (*sn* 1-2 and 1-3) were synthesized by esterification of monoacylglycerol with unsaturated fatty acids (compounds: 1, 2, 6–9, 11, and 12). Diacylglycerols were converted to trimethylsilyl ether derivatives and analyzed by GLC (Restek 50 capillary column) as described under Materials and Methods. The upper panel corresponds to a mixture of TMS diacylglycerols (1, 2, 3, 4, 5, 6, 9, and 10) and the lower panel to another mixture of TMS diacylglycerols (1, 2, 3, 4, 5, 7, 8, 11, and 12). Compounds 1–5 are present in upper and lower chromatograms.

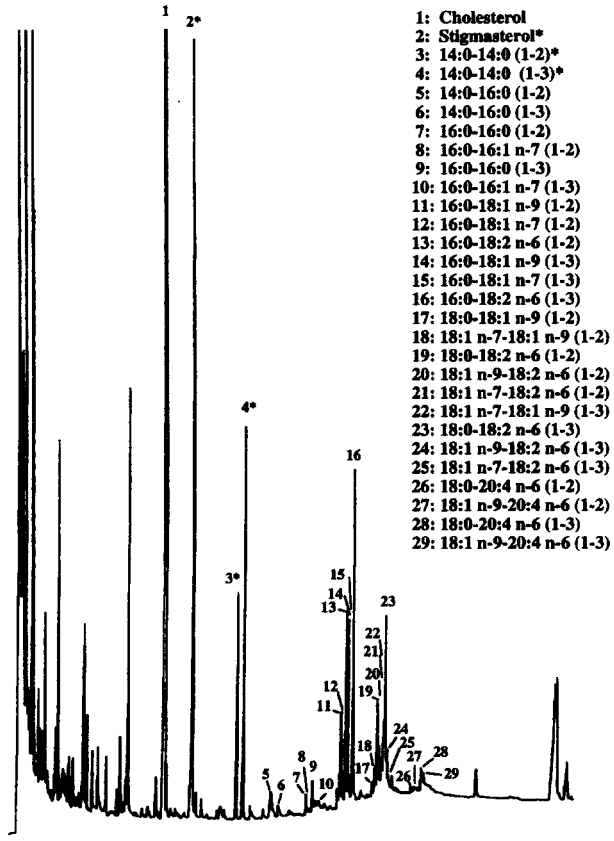

Fig. 3. A typical profile of HDL *sn*-(1-2), (1-3) diacylglycerol (trimethylsilyl ether derivatives) molecular species. HDL lipids were extracted and submitted to micropreparative chromatography to separate major phospholipids from neutral lipids, as the phospholipids remained onto the glass injector. This apparatus was obtained by coupling an ULTRA 1 capillary column to the splitless injector previously described in the Materials and Methods. The lipids were immediately silylated into trimethylsilyl ethers (TMS ethers). Then, TMS ethers were analyzed by gas–liquid chromatography (RESTEK 50 capillary column coated with 50% phenyl, 50% methyl polysiloxane) as described in Materials and Methods; *, internal standards.

glycerols, respectively (Table 2). The identification of the HDL TMS-derivatized *sn*-1,2- and *sn*-1,3-diacylglycerol isomers are indicated in Table 2, with their relative retention time and relative proportions (mole %) of the total diacylglycerols. The peaks were resolved and exhibited a symmetrical shape. For a given number of carbons in the acyl groups, TMS-derivatized diacylglycerol molecular species are eluted in the order of increasing number of double bonds, and decreasing with double bonds position from n–9, n–7, and n–6. For instance, the 16:0–18:1 n–9 diacylglycerols was eluted ahead of the 16:0–18:1n–7 and of the 16:0–18:2n–6. The 18:0–18:1n–9 was eluted ahead of the 18:0–18:2n–6 and of the 18:1n–9–18:2n–6. The diacylglycerols were found to be *sn*-(1,2) isomers and *sn*-(1,3) isomers; *sn*-(1,2) were eluted ahead of the *sn*-(1,3). The three major diacylglycerol species appear to be the 16:0–18:2n–6, 18:0–18:2n–6, and 16:0–18:1n–9 (33.4, 22.2, and 16.1 moles % of total diacylglycerols, respectively). The distribution of diacylglycerols derived from HDL phosphatidylcholines, as obtained after treatment with phospholipase C, indicates that 16:0–18:2n–6 and 18:0–18:2n–6 are also the major molecular species, but represent different percentages. Furthermore, 18:0–20:4n–6 and 18:1n–9–20:4n–6 DG represented 8.7% of total diacylglycerols in phosphatidylcholines isolated from HDL, whereas these molecular species represented only 3.8% in native HDL diacylglycerols.

We have compared the relative proportion of diacylglycerols relative to triacylglycerols in the HDL_2 composition. As shown in **Table 3,** diacylglycerol molecules exceed by 2.2 the amount of triacylglycerols. In HDL_3, the molar ratio of diacylglycerols upon triacylglycerol represented 6.7. Furthermore, the molar ratio of diacylglycerol upon free cholesterol is significantly different between HDL_2 and HDL_3 and represent 1.24 and 1.91, respectively. The calculated molecular composition of HDL_2 and HDL_3 particles are presented in **Table 4.** When the neutral lipids of HDL_2 were separated by thin-layer chromatography, and the diacylglycerols and triacylglycerols were transmethylated, the molar free fatty acid compositions show the presence of more saturated fatty acids in diacylglycerol than in triacylglycerols, 85.22 ± 3.76% and 58.25 ± 4.33% (n = 3), respectively. Furthermore, there was an equimolar distribution of diacylglycerols and triacylglycerols in HDL (not shown). This suggests a loss of diacylglycerol during the thin-layer chromatography procedure and specifically of unsaturated diacylglycerol.

Diacylglycerol isomer formation

Diacylglycerols (40 nmol) were obtained from soybean phosphatidylcholine by phospholipase C. When these diacylglycerols were immediately silylated and analyzed using the Restek 50 capillary column, only 1,2 isomers were identified. 16:0–18:2n–6-, 18:1n–9–18:2n–6-, and 18:2n–6–18:2n–6-(*sn*) glycerols were the major molecular species as previously described by Myher and Kuksis (12) (**Fig. 4,** lower panel). We then checked whether the formation of 1,3 diacylglycerols could occur in the micropreparative step. Thus, we have injected, under the same conditions, 20 nmol of diacylglycerols prepared as described above, mixed with cholesterol (50 nmol), stigmasterol (7 nmol), *sn*-dimyristoyl glycerol (12 nmol), cholesteryl ester (CE-C18:2n–6; 80 nmol) and triheptadecanonoyl glycerol (triacylglycerol-C51; 7 nmol). The samples were trapped in 80 µl of pyridine, evaporated, and silylated overnight. The diacylglycerols were analyzed using the Restek 50 capillary column (Fig. 4, upper panel). Under these conditions, we observed the presence of *sn*-(1,2) and *sn*-(1,3)

TABLE 2. Identification of HDL (*sn*)-1,2 and -1,3-diacylglycerols separated by gas–liquid chromatography

Molecular Species	Molecular Weight	Isomers	Relative Retention Time	Mol % (n = 8)	Peak No.
			min		
Cholesterol	386.6		12.00 ± 0.19		1
Stigmasterol			14.19 ± 0.18		2[a]
Diacylglycerol					
14:0–14:0	512.8	1-2	17.87 ± 0.13		3[a]
14:0–14:0		1-3	18.41 ± 0.13		4[a]
14:0–16:0	540.8	1-2	21.14 ± 0.40	0.89 ± 0.55	5
14:0–16:0		1-3	21.94 ± 0.55	0.35 ± 0.19	6
16:0–16:0	568.9	1-2	23.67 ± 0.18	0.48 ± 0.25	7
16:0–16:1n–7	566.9	1-2	24.20 ± 0.50	traces	8
16:0–16:0	568.9	1-3	24.26 ± 0.19	0.80 ± 0.45	9
16:0–16:1n–7	566.9	1-3	24.67 ± 0.42	traces	10
16:0–18:1n–9	595	1-2	26.19 ± 0.10	5.62 ± 0.17	11
16:0–18:1n–7		1-2	26.38 ± 0.13	0.56 ± 0.18	12
16:0–18:2n–6	593	1-2	26.57 ± 0.11	12.77 ± 0.61	13
16:0–18:1n–9	595	1-3	26.74 ± 0.10	10.52 ± 0.91	14
16:0–18:1n–7		1-3	26.91 ± 0.13	0.83 ± 0.19	15
16:0–18:2n–6	593	1-3	27.11 ± 0.03	20.59 ± 0.97	16
18:0–18:1n–9	623	1-2	28.80 ± 0.11	1.26 ± 0.62	17
18:1n–7–18:1n–9	621	1-2	28.90 ± 0.10	3.46 ± 0.45	18
18:0–18:2n–6		1-2	29.09 ± 0.10	8.39 ± 0.66	19
18:1n–9–18:2n–6	619	1-2	29.23 ± 0.10	3.59 ± 0.37	20
18:1n–7–18:2n–6		1-2	29.44 ± 0.10	2.05 ± 0.91	21
18:1n–7–18:1n–9	621	1-3	29.52 ± 0.10	5.79 ± 0.92	22
18:0–18:2n–6		1-3	29.64 ± 0.10	13.78 ± 0.53	23
18:1n–9–18:2n–6	619	1-3	29.77 ± 0.11	3.37 ± 0.23	24
18:1n–7–18:2n–6		1-3	30.00 ± 0.14	1.14 ± 0.34	25
18:0–20:4n–6	645	1-2	31.76 ± 0.15	0.69 ± 0.26	26
18:1n–9–20:4n–6	643	1-2	32.00 ± 0.21	0.62 ± 0.26	27
18:0–20:4n–6	645	1-3	32.51 ± 0.17	1.35 ± 0.51	28
18:1n–9–20:4n–	643	1-3	32.70 ± 0.16	1.10 ± 0.34	29

HDL 1,2- and 1,3-diacylglycerols were extracted, derivatized, and identified by gas–liquid chromatography as described under Experimental Procedures. Each molecular species was numbered based upon its retention time.

[a]Standards.

isomers, which represented 36% and 64%, respectively (not shown). This result contrasts with the analysis of the diacylglycerols that are obtained from soybean phosphatidylcholines by phospholipase C and are silylated immediately. The isomers were generated by the relative high temperature of the apparatus. These results are very similar to that found in HDL diacylglycerols (*sn*-(1,2) and *sn*-(1,3) isomers, which represented 41% and 58%, respectively) (Table 2). Thus, during the micropreparative step, isomers of HDL diacylglycerols are formed. This was confirmed, as after partial silylation of HDL total lipids with no preparative step, diacylglycerols were identified as nonsilylated and trimethylsilyl ethers (1,2 isomers only) (not shown).

TABLE 3. Chemical composition of HDL_2 and HDL_3

Fraction	Molar Ratio			PL	TC	FC	DG	TG
	FC/PL	DG/TG	DG/FC					
						μmol/mg apoA-I		
HDL_2	0.37 ± 0.02[a]	2.25 ± 0.08	1.24 ± 0.09[a]	0.88 ± 0.07	1.56 ± 0.27[a]	0.33 ± 0.04	0.41 ± 0.06	0.17 ± 0.04
HDL_3	0.25 ± 0.03	6.70 ± 2.33	1.91 ± 0.18	0.85 ± 0.12	0.92 ± 0.11	0.21 ± 0.02	0.41 ± 0.14	0.07 ± 0.01

Results are expressed as means ± SE. HDL were isolated as described in Materials and Methods. Lipids were analyzed by gas–liquid chromatography and apoA-I was measured as described in Materials and Methods. PL, phospholipid; TC, total cholesterol; FC, free cholesterol; DG, diacylglycerol; TG, triacylglycerol.

[a]$P < 0.05$, statistical comparison between HDL_2 and HDL_3.

TABLE 4. Molecular composition of HDL_2 and HDL_3 particles

Fraction	FC	DG	EC	TG	PL	ApoA-I
	number of molecules					
HDL_2	44.00 ± 3.19[a]	54.46 ± 5.05	163.20 ± 9.43[a]	22.45 ± 4.45[a]	117.90 ± 2.77[a]	4.78 ± 0.32[a]
HDL_3	17.04 ± 1.41	32.84 ± 5.66	56.70 ± 11.25	5.48 ± 0.85	67.59 ± 7.95	2.85 ± 0.08

Results are expressed as means ± SE of 4 determinations. On the basis of a molecular weight of 400,000 Da for HDL_2 and 200,000 Da for HDL_3, the predicted molecular composition of HDL particles was calculated from the molecular weight values of 28,000 Da for apoA-I, 386 for free cholesterol (FC), 645 for esterified cholesterol (EC), 880 for triacylglycerol (TG), 775 for phospholipids (PL), and 600 for diacylglycerol (DG).

[a] $P < 0.01$, statistical comparison between HDL_2 and HDL_3.

Accessibility of HDL diacylglycerols to lipase

In order to test the accessibility of diacylglycerols at the surface of HDL_2, we used a purified cationic pancreatic lipase (1 or 10 IU/ml). This enzyme presents a low surface pressure and the hydrolysis of triacylglycerols varied considerably (0–70%) between experiments. Thus, we selected the experiments where triacylglycerol hydrolysis was less than 10%. Under these conditions, after 120 min of incubation in absence of detergent, 59% of HDL_2 diacylglycerols were found hydrolyzed (not shown). Interestingly, in all cases, the diacylglycerol 18–20 were completely degraded while the diacylglycerols 18–18 and 16–18 were only partially degraded: 64.0% and 46.6%, respectively. In contrast, the incubation in the presence of deoxycholate for 120 min induced a complete hydrolysis of diacylglycerols and also of triacylglycerols. This argues for a complete accessibility of diacylglycerols 18–20 at the surface of HDL whereas 18–18 and 16–18 would be partly located in the particle lipid core.

DISCUSSION

The present study provides the first detailed description of the molecular species of diacylglycerol in human HDL. A variety of techniques used for the determination of glycerides (thin-layer chromatography, gas–liquid chromatography, and HPLC) (13–15). Previous reports have described the analysis of total neutral lipids in plasma obtained after dephosphorylation by the action of a phospholipase C (7). This treatment generates a large amount of 1,2 diacylglycerols and also of ceramides. The phospholipase C isolated from *C. welchii* also exerts a sphingomyelinase activity and thus produces ceramides. The major ceramide generated by phospholipase C corresponds to the N-hexadecanoyl D sphingosine, which, like other ceramides, is not naturally present in HDL (C. Vieu and X. Collet, personal communication).

No direct measurements of diacylglycerols have been made in any vertebrate lipoprotein. In contrast, the major neutral glycerolipid of insect lipophorin is diacylglycerol, with much lower levels of monoacylglycerols and triacylglycerols present in all species (for a review see ref. 16). The diacylglycerol content of lipophorin varies among insect species. Interestingly the diacylglycerols in lipophorin particles reside mostly in the lipid core (17). Lipophorins would act as a reusable lipid shuttle transferring diacylglycerols (18). A lipid transfer protein has been identified to have a specificity for

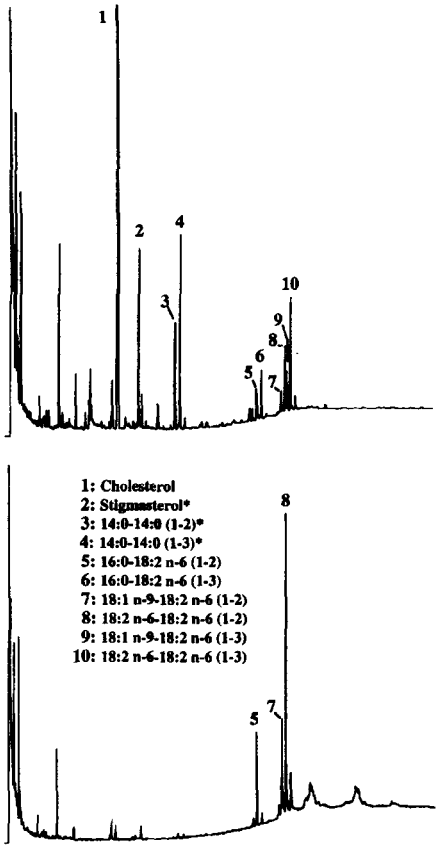

Fig. 4. Gas–liquid chromatogram of *sn*-(1-2) and -(1-3) diacylglycerols (TMS ether derivatives). *sn*-(1,2) Diacylglycerols were obtained from soybean phosphatidylcholines after treatment by phospholipase C (compounds: 5, 7, and 8) (lower profile). Diacylglycerols were converted to trimethylsilyl ether derivatives and analyzed by GLC (Restek 50 capillary column as described in Materials and Methods. Gas–liquid chromatogram of the same *sn*-(1-2) diacylglycerols mixed with standards, cholesterol (50 nmol), stigmasterol (7 nmol), DG 14:0–14:0 (12 nmol), CE 18:2 n–6 (80 nmol), TG C51 (7 nmol) (upper panel). After the micropreparative gas–liquid chromatography, the lipids were silylated and analyzed as described in Fig. 3; *, internal standards.

diacylglycerols (19). Furthermore, in vitro incubations of insect lipid transfer protein catalyze the transfer of diacylglycerols from lipophorin to human LDL (20). Lipid transfer protein may also transfer phospholipids and triacylglycerols from a human apoA-I-stabilized phospholipid/triolein microemulsion to human LDL (21). In human plasma, lipid transfer proteins such as the cholesteryl ester transfer protein and the phospholipid transfer protein are involved in the transfer of cholesteryl ester, triacylglycerols, as of phospholipids between lipoproteins (22, 23). However, there is no evidence that these proteins may facilitate the transfer of diacylglycerols between the human lipoproteins.

In membrane bilayers, ^{13}C-NMR spectroscopy showed that the diacylglycerol molecules have an orientation similar to that of the phosphatidylcholine molecules in bilayers and that the backbone of diacylglycerols is approximately parallel to the acyl chains (24). Chemical shift data indicated a preference of diacylglycerol for the inner leaflet of the bilayer, at lower contents of diacylglycerols (8%), and a progressive change to a distribution similar to that of phosphatidylcholine at higher levels of diacylglycerols. As regards lipophorins, diacylglycerols are mostly located in the lipid core of the particles (17). In our study we have used a pancreatic lipase with a high affinity for diacylglycerols in an attempt to assess their accessibility at the surface of human HDL. We observed that about 60% of diacylglycerols were accessible to the enzyme. In the presence of detergent, the diacylglycerols were completely hydrolyzed. As the ceramides emerge with the diacylglycerols very close to the diacylglycerol 14–16, and as it is highly unlikely that this enzyme also reacts against the ceramides (i.e., ceramidase), this argues for the absence of ceramides in HDL.

Nonenzymatic racemization between *sn*-1,2- and *sn*-2,3-diacylglycerol is very rapid. Furthermore, 1,2-*sn*-diacylglycerols may be isomerized to the 1,3-form as the latter is thermodynamically more stable. This is the case concerning the mixture of isomers found in our diacylglycerol standards. The treatment of phospholipids by phospholipase C followed by immediate derivatization produced, as expected, only *sn*-1,2-diacylglycerols. Hence, synthetic diacylglycerol standards may be isomerized during storage, but the silylation prevents this interconversion (not shown). Furthermore, the thermal lability of glycerides limits the use of gas–liquid chromatography. However, the derivatization to the more stable silyl ether forms circumvents this problem (13). It has been shown that nonenzymatic racemization can occur when diacylglycerols are present as a surface component in a phosphatidylcholine bilayer. This is not the case at the surface of lipoproteins. Indeed, we have observed the formation of 1,3-diacylglycerols from 1,2-diacylglycerols during the preparative gas–liquid chromatography, but native HDL were found devoid of 1,3-diacylglycerols. Formation of the 1,3-isomer from the 1,2-isomer can be monitored by NMR or thin-layer chromatography methods. However, using thin-layer chromatography, the contact of diacylglycerol with silica can alter the distribution of the isomers (25).

In this work, we report the development of a sensitive method for the quantitation and identification of diacylglycerols present in HDL. The free naturally occurring diacylglycerols having long saturated and unsaturated fatty acids are not able to cross membranes to activate protein kinase C, when added to intact cells such as platelets. The presence of diacylglycerols in HDL is of great interest as HDL interact with cells and thus may bring diacylglycerols into the cell membrane. The diacylglycerols inserted in the outer leaflet of the plasma membrane are expected to undergo rapid transbilayer movement (26, 27). The transbilayer movement of a sulfhydryl analogue of diacylglycerol in phospholipid vesicles takes place within 15 sec, compared to 8 days for a phospholipid sulfhydryl analogue (28). In human red cell membranes, diacylglycerol flip-flop was shown by chemical means to occur within 1 min (24, 26). Diacylglycerol is an essential known physiological regulator of cellular protein kinase C, an enzyme that modulates a wide array of cellular responses (29), and temporal fluctuations in the concentration of diacylglycerol may be another control of these enzymes. Interestingly, protein kinase C has been involved in cellular cholesterol efflux mediated by HDL (30–32). The mass measurements of HDL diacylglycerols may be of first interest to argue for a new role of HDL in signal transduction. However, the role of HDL diacylglycerols is unknown and must be studied further before their function as a second messenger can be appraised.

The origin of HDL diacylglycerols is not well elucidated as yet. Preliminary results showed that the diacylglycerols are present in fresh plasma, and there is no increase during time (until 3 h) after incubation at 37°C (C. Vieu and X. Collet, unpublished observation). Thus, it is unlikely that the diacylglycerols are formed under the action of an eventual phospholipase C, which was never detected in plasma. The diacylglycerols that are produced from triacylglycerols during the action of lipolytic enzymes (hepatic triacylglycerol lipase and lipoprotein lipase) may contribute to their presence in the lipoproteins. Interestingly, the hydrolysis of diacylglycerols by hepatic lipase occurred at a rate slower than that of triacylglycerols, leading to the accumulation of diacylglycerols (33). Further studies will be necessary to elucidate the origin of these molecules in lipoproteins.

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