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Determination of the molecular species composition of diacylglycerols in human adipose tissue by solid-phase extraction and gas chromatography on a polar phase

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Abstract

The free diacylglycerols (DAGs) in adipose tissue are involved in the metabolism of stored lipids and hence are related to the supply of fatty acids for other tissues. This paper describes a simple, fast, and reproducible method for the identification and quantification of different molecular species of DAGs in human adipose tissue. The method comprised solid-phase extraction on a diol-bonded phase column combined with capillary GC analysis of silylated DAG derivatives on a polar phase (65% phenylmethylsilicone). Separation of the DAGs was achieved based on chain length, isomeric structure (1,2- and 1,3-DAGs), and degree of unsaturation. The main DAGs were 1,2-OO, 1,2-OP, 1,2-LO and 1,2-LP. The composition was corroborated by analysis of the component fatty acids of the DAGs, 18:1(n-9), 16:0, and 18:2(n-6) being the three major fatty acids obtained.

Keywords: Diacylglycerols; Solid-phase extraction; Human adipose tissue

1. Introduction

Adipose tissue is involved in fat storage and is the most important extrahepatic tissue in regulating carbohydrate and lipid metabolism [1]. The adipose tissue contains predominantly triacylglycerols (TAGs) (97%), followed by phospholipids (2.6%), free cholesterol (0.3%), and esterified cholesterol (0.05%) [2]. Diacylglycerols (DAGs) are formed by lipolysis of TAGs through the mediation of a hormone-sensitive lipase (HSL) [1] or are synthesised as new acylglycerols formed from glucose. In the latter case, DAGs rapidly accumulate up to 20–50% of the newly synthesised acylglycerols [3]. This process is temporal and adrenergically regulated; as a consequence, fewer than 1% of DAGs are found in the lipid stores. In support of this, newly synthesised DAGs are more rapidly hydrolysed by HSL than are TAGs, probably because DAGs are located on the surface layer of lipid droplets and, therefore, more accessible to the lipase [3]. For that reason, DAGs, as well as TAGs, may be precursors of fatty acids, which are released into the blood for transport to other tissues. In addition, it is clear that mobilisation of stored fatty acids depends on the degree of unsaturation and chain length [4,5], although strongly and weakly mobilizable fatty acids are both

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located mainly at the outer positions (*sn*-1 and *sn*-3) on TAG molecules [6].

Free DAGs must be isolated for analysis (DAGs make up less than 1% of total adipose lipids). Injection of samples directly onto the chromatographic column would doubtless involve injection of large amounts of TAGs. Moreover, the phospholipids could interfere with the DAGs. In a previous work, DAGs in bovine adipose tissue were separated from neutral lipids by solid-phase extraction (SPE) with adsorption cartridges containing an aminopropyl-bonded phase [7]. Although separation of the total bulk DAGs was successful, we have observed that aminopropyl bonded-phases produce high isomerisation rates [8]. The undesirable effect of acyl migration has long been discussed as a major problem in the determination of DAGs [9]. Indeed, boric acid impregnation of silica gel plates is commonly employed to block the free hydroxyl groups of DAGs in TLC [10], and additionally immediate derivatisation of DAGs for further chromatographic analysis is always recommended [11].

HPLC has been widely used to resolve isomeric DAGs, and chiral phase HPLC has been very useful in determining the positional distribution of the fatty acids in TAGs by analysis of the 1,2-(2,3)-DAGs generated by Grignard degradation. This technique has helped elucidate TAG metabolism in the intestine and liver [12,13]. In any case, chiral phase HPLC has been used in combination with GLC or reversed-phase HPLC to determine the molecular species of enantiomeric DAGs (1,2- and 2,3-DAGs) but 1,3-DAGs were not analysed in those studies.

Injection of silyl derivatives of DAGs onto a polar-phase column in GLC has produced separations of DAGs according to carbon number, 1,2- and 1,3-isomeric structure, and the number of double bonds [8,11]. This principle of separation has recently been applied to free DAGs from HDL-lipoproteins [14], yielding 26 molecular species of both isomers (1,2- and 1,3-DAGs).

The object of the present study was to develop a simple, fast, and reproducible method for the determination and quantification of molecular species of free DAGs in human adipose tissue by combining diol-SPE and GLC on a polar-phase (65% phenylmethylsilicone).

2. Experimental

2.1. Materials

Standards of *sn*-1,3-dipalmitoyl glycerol, *sn*-1,2-dipalmitoylglycerol, *sn*-1,3-distearoylglycerol, *sn*-1,2-distearoylglycerol, *sn*-1,3-dioleoylglycerol, and *sn*-1,2-dioleoylglycerol were purchased from Sigma (St. Louis, MO, USA). The SPE columns (3 ml) were from Supelco (Bellefonte, PA, USA).

2.2. Samples

Human adipose tissue was extracted from the anterior abdominal wall as described by Ruiz-Gutiérrez et al. [2]. The tissue portions were washed in saline solution and immediately preserved in liquid nitrogen until processing.

Extraction of the total lipids was carried out following the method of Folch et al. [15]. Tissues were homogenized in ice-cold chloroform–methanol (2:1, v/v) containing 0.01% BHT in an Ultra Turrax Type TP-18-1 blender.

2.3. Solid-phase extraction, isolation, and TLC analysis

An amount of 25 mg of *sn*-1,3-dipentadecanoylglycerol was dissolved in a mixture of 2.5 ml of methylene chloride and 0.25 ml of diethyl ether and then diluted with hexane up to a volume of 50 ml for use as the internal standard. The lipid extract, containing 20–100 mg of total lipids, was evaporated in a nitrogen stream, and 0.1–0.2 ml of the internal standard solution was added immediately.

A diol bonded-phase SPE column was placed in a vacuum elution apparatus and washed with 4 ml hexane under vacuum. After the wash the vacuum was disconnected to prevent the column from drying out. The sample was placed on the column and the solvent drawn through. A volume of 8 ml of hexane–methylene chloride–ethyl ether (89:10:1, v/v/v) was applied to the column and a first fraction was collected. Subsequently, 8 ml of hexane–ethyl acetate (85:15, v/v) was applied to the column and a second fraction (containing the diacylglycerols) was

collected. The SPE column was then successively washed with 4 ml of ethanol and 3 ml of acetone and dried by passing nitrogen through. Columns could be used at least six times.

Both fractions were analysed by TLC on silica gel 60 (Merck, Darmstadt, Germany). The plates were eluted with hexane–diethyl ether–acetic acid (79:20:1, v/v/v) and developed by spraying with H_2SO_4–water (1:1, v/v) and heating.

2.4. GLC analysis of the diacylglycerols

The second fraction from the diol-SPE was evaporated to dryness in a rotary evaporator, and the residue was treated with 0.2 ml of silylating reagent (3 ml of hexamethyldisilazane, 1 ml of trimethylchlorosilane and 9 ml of anhydrous pyridine) and left at room temperature for 3 min. The DAG derivatives (2 μl) were injected into a Chrompack CP9000 gas chromatograph (Middelburg, The Netherlands) fitted with a flame ionisation detection system and a split injection system (split ratio 1:30). A high-temperature fused-silica capillary column (25 m×0.25 mm I.D.) coated with a 0.1 μm-thick film of 65% phenylmethylsilicone (Quadrex, New Haven, CT, USA) was used. The operating conditions were as follows: an oven temperature of 270°C for 4 min, increasing to 295°C at 1°C/min and then held at 295°C for 1 min; injector temperature, 300°C; detector temperature, 325°C; hydrogen as carrier gas at 100 kPa. Data acquisition and processing were carried out by a Chrom-Card Data System (Fisons, Altrincham, UK).

2.5. Fatty acid methyl esters: preparation and analysis

The SPE-fractions containing the diacylglycerols were placed in a volumetric flask and evaporated to dryness. They were then subjected to acidic methanolysis using 5 ml of 6% H_2SO_4 in methanol with refluxing for 40 min. After cooling the fatty acid methyl esters were extracted with 500 ml of hexane. The samples were evaporated and redissolved in 50 ml of hexane. For GLC analysis, 2 μl were injected onto the gas chromatograph, a Hewlett-Packard Series 5890a model with a flame ionisation detector. A Supelcowax 10 fused-silica capillary column (60 m×0.25 mm I.D., film thickness 0.25 μm) was purchased from Supelco. Column temperature was programmed from 180°C (held for 12 min) to 205°C (held for 10 min) at 2°C/min. Injector and detector temperature was 250°C.

2.6. Fatty acid composition of dags

The fatty acid composition (Table 3) was calculated on the basis of the percentage share of each molecular species of DAG (see Table 2).

Nomenclature of fatty acids (Table 3): 14:0= tetradecanoic acid, myristic acid; 16:0= hexadecanoic acid, palmitic acid; 18:0=octadecanoic acid, stearic acid; 16:1=*cis*-9-hexadecenoic acid, palmitoleic acid; 18:1=*cis*-9-octadecenoic acid, oleic acid; 18:2 *cis,cis*-9,12-octadecenoic acid, linoleic acid.

3. Results and discussion

The main lipid components of human adipose tissue are triacylglycerols (TAGs), followed by phospholipids, free cholesterol, and esterified cholesterol [2]. Using a diol-SPE minicolumn, DAGs were completely resolved from the TAGs and phospholipids. The first mobile phase (hexane–methylene chloride–ethyl ether) eluted TAGs and esterified cholesterol, the second mobile phase (hexane–ethyl acetate) eluted the DAGs and free cholesterol, whereas the phospholipids and MAGs were retained on the stationary phase (Table 1). To our knowledge, diol bonded-phases are the most effective chemically bonded phase for preventing isomerisation of the DAGs [8]. As a further means of avoiding isomerisation, the fraction containing the DAGs was immediately derivatised with trimethylchlorosilane and hexamethyldisilazane in the presence of pyridine.

The conditions of the GLC analysis were designed to separate the DAGs on the basis of chain length, 1,2- and 1,3-isomeric structure, and degree of unsaturation. Peaks were identified by comparing retention times with those of the standards or using the equivalent carbon number (ECN) of each DAG (Table 2) in combination with the fatty acid and

Table 1
Solvents used in the elution procedure and lipid fractionation by diol-SPE

Fraction	Eluent	Lipid eluted
First	1% diethyl ether, 10% methylene chloride in hexane	TAGs, esterified cholesterol
Second	15% ethyl acetate in hexane	DAGs, free cholesterol
Column wash	Ethanol	Phospholipids, MAGs

Abbreviations: TAGs = triacylglycerols; DAGs = diacylglycerols; MAGs = monoacylglycerols.

TAG compositions of human adipose tissue [2]. The ECN was estimated as follows [16]:

$$ECN = CN + 0.15n_1 + 0.22n_2 + 0.29n_3$$

$$(ECN)_{1,3} = (ECN)_{1,2} + 0.32$$

where CN is the number of carbons in the acyl group, n_1 is the number of double bonds in the monounsaturated acyl group, n_2 is the number of double bonds in the linoleoyl group, and n_3 the number of double bonds in the linolenoyl group. The second equation describes the relationship between homologues of the 1,2- and 1,3-series. Retention times differ according to whether double bonds are present and, if so, whether they are present in one or both of the acyl groups, because in the former polarity is greater. The solutes eluted in order of increasing ECN, and this parameter was related to overall molecule polarity.

Table 2
Reproducibility data on diacylglycerol concentrations in human adipose tissue by SPE–GLC

DAG[a]	ECN	Mean[b] (%)	S.D.$_r$	R.S.D.$_r$ (%)
1,2-PP	32.00	3.65	0.31	8.55
1,2-PPo	32.15	2.20	0.02	1.09
1,2-MO	32.15	2.38	0.08	3.16
1,3-PP	32.32	3.54	0.03	0.97
1,3-PPo + 1,3-MO	32.47	1.21	0.10	8.60
1,2-PS	34.00	1.06	0.03	2.64
1,2-OP	34.15	22.60	1.13	4.98
1,2-OPo	34.30	2.92	0.04	1.42
1,2-LP	34.44	7.49	0.29	3.90
1,3-OP	34.47	7.14	0.10	1.47
1,3-OPo	34.62	1.32	0.12	9.09
1,3-LP	34.76	1.15	0.08	6.75
1,2-OS	36.15	2.56	0.20	7.84
1,2-OO	36.30	23.65	2.04	8.62
1,2-LO	36.59	11.81	0.30	2.58
1,3-OO	36.62	5.83	0.02	0.30
1,2-LL	36.88	1.18	0.04	3.75
1,3-LO	36.91	1.28	0.01	7.17
Total DAGs (mg/g)		1.8	0.13	7.54

[a] See Fig. 1 for DAG nomenclature.
[b] $n = 6$.

Fig. 1 illustrates the composition of the molecular species of DAGs in human adipose tissue as elucidated by GLC. At least 18 DAGs were identified, distributed in three groups according to their CNs. As expected, the content of 1,2-DAGs was significantly higher than that of the isomers, because the β-position in the TAGs is more resistant to the action of lipolytic enzymes and 1,2-DAGs are preferred intermediates in both the phosphatidic acid and 2-monoacylglycerol pathways for TAG synthesis [1]. Human adipose tissue was particularly rich in 1,2-OP, 1,2-OO, 1,2-LO and 1,2-LP (Table 2), which account for up to 70% of the total DAGs, and the three constituent fatty acids account for up to 80% of the total fatty acids [2]. These results cannot be compared to those of any other workers, because we have not found any previous work on the DAG composition of human adipose tissue.

The experimental fatty acid composition of the DAGs was clearly correlated with the calculated values (Table 3). The positional isomers (n-7 and n-9) of palmitic acid (16:1) and oleic acid (18:1) were separated in the experimental data. However, the calculated values were based on the molecular species composition of the DAGs, which did not differentiate between the two isomeric forms. The study of the fatty acid composition of the DAGs showed that oleic acid was the main fatty acid, contributing about 60%. The next highest contributors were palmitic acid at 25% and linoleic acid at 9%. The high amounts of oleic acid in the lipids stored in the human adipose tissue in this study were

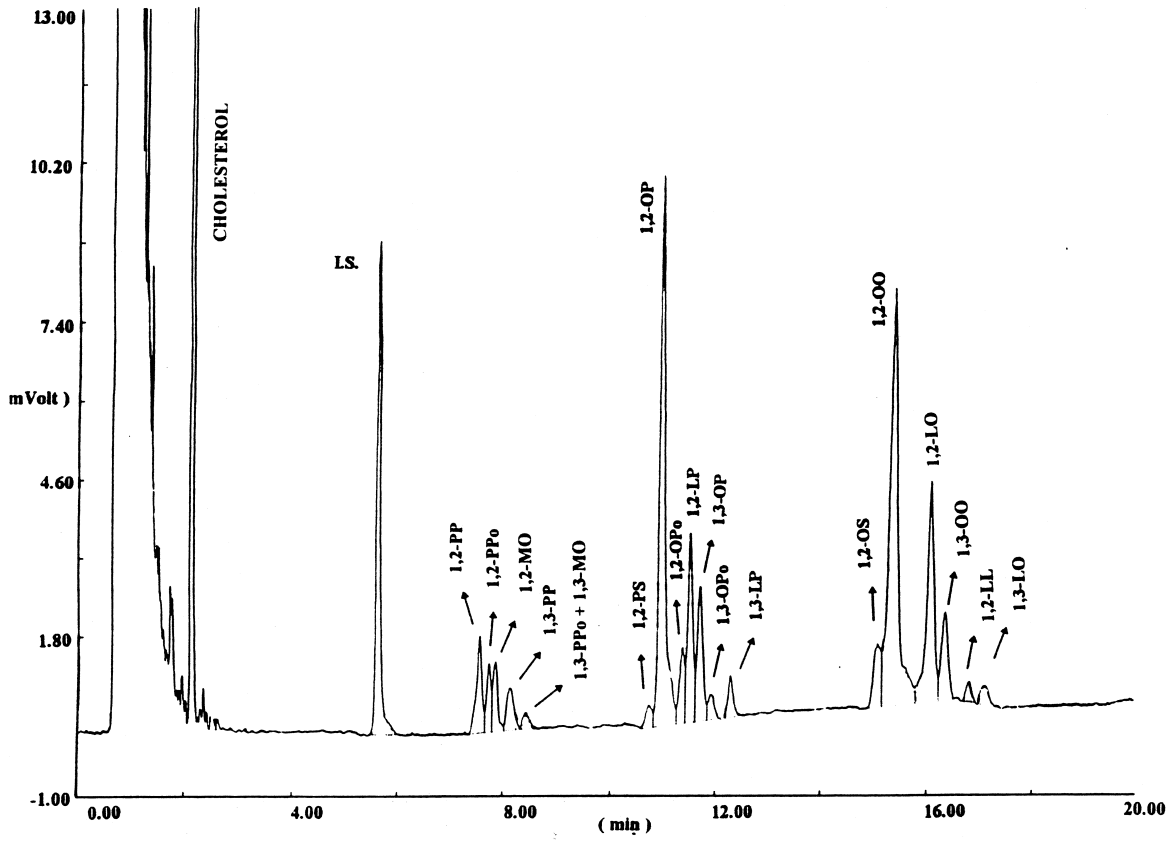

Fig. 1. Gas chromatogram of the polar fraction isolated from human adipose tissue. Nomenclature: fatty acids M = myristic acid, 14:0; P = palmitic acid, 16:0; S = stearic acid, 18:0; Po = palmitoleic acid, 16:1; O = oleic acid, 18:1; L = linoleic acid, 18:2. Diacylglycerols: 1,2-OO = X-1,2-dioleoylglycerol; 1,3-OP = X-1,3-oleoylpalmitoilglycerol; 1,2-OPo = X-1,2-oleoylpalmitoleoylglycerol, etc.

Table 3
Fatty acid composition of molecular species of free DAGs in human adipose tissue

Fatty acid[a]	Composition (%)	
	Calculated[b]	Experimental[c]
14:0	1.05	2.24±0.60
16:0	25.21	24.19±2.85
16:1(n-9)		0.6±0.46
16:1(n-7)	2.50	2.42±1.20
18:0	1.28	1.78±0.86
18:1(n-9)	60.55	56.53±2.42
18:1(n-7)		2.5±0.44
18:2(n-6)	9.39	9.74±2.39

[a] See nomenclature in Section 2.6.
[b] See Section 2.
[c] Results are the mean±S.D., $n=6$.

mainly due to the Mediterranean diet, in which olive oil is a major component.

Using the methodology employed in this study, it was possible to examine the composition of X-1,3-DAGs. These DAGs contributed approximately 20% of the total free DAGs; 1,3-PP, 1,3-PPo, 1,3-MO, 1,3-OP, 1,3-OPo, 1,3-LP, 1,3-OO and 1,3-LO have been identified. We think that the molecular species of 1,3-DAGs hold out considerable interest, because they only could be hydrolysed by HSL, generating fatty acids that could be incorporated into other tissues. The X-1,2-DAGs are, additionally, substrates for the enzyme diacylglycerol acyl transferase (DGAT), which catalyses TAG synthesis from 1,2-(2,3)-DAGs [17].

In conclusion, we think that knowledge of the fatty acid composition and isomeric structure of the

free DAGs in adipose tissue could contribute to nutritional and biomedical studies relating to fat mobilisation, particularly when diet and certain pathologies may modify the composition of the lipids stored in adipose tissue [18]. This paper describes a fast, simple, and reproducible method for determining the molecular species of free DAGs in adipose tissue.

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References

[1] R. Lehner, A. Kuksis, Prog. Lipid Res. 35 (1996) 169.
[2] V. Ruiz-Gutierrez, E. Montero, J. Villar, J. Chromatogr. 581 (1992) 171.
[3] N.K. Edens, R.L. Leibel, J. Hirsch, J. Lipid Res. 31 (1990) 1351.
[4] T. Raclot, R. Groscolas, J. Lipid Res. 34 (1993) 1515.
[5] V.C. Gavino, G.R. Gavino, Lipids 27 (1992) 950.
[6] T. Raclot, C. Leray, A.C. Bach, R. Groscolas, Biochem. J. 311 (1995) 911.
[7] M.A. Kaluzny, L.A. Duncan, M.V. Merrit, D.E. Epps, J. Lipid Res. 26 (1985) 135.
[8] M.C. Pérez-Camino, W. Moreda, A. Cert, J. Chromatogr. A 721 (1996) 305.
[9] D.R. Kodali, A. Tercyak, D.A. Fahey, D.M. Small, Chem. Phy. Lipids 52 (1990) 163.
[10] A.E. Thomas, J.E. Scharoun, H. Ralston, J. Am. Oil Chem. Soc. 42 (1965) 789.
[11] J.J. Myher, A. Kuksis, J. Chromatogr. B 671 (1995) 3.
[12] L.Y. Yang, A. Kuksis, J. Lipid Res. 32 (1991) 1173.
[13] L.Y. Yang, A. Kuksis, J.J. Myher, G. Steiner, J. Lipid Res. 36 (1995) 125.
[14] C. Vieu, B. Jaspard, R. Barbaras, J. Manent, H. Chap, B. Perret, X. Collet, J. Lipid Res. 37 (1996) 1153.
[15] J. Folch, M. Less, G.H. Sloane Stanley, J. Biol. Chem. 226 (1957) 497.
[16] N. Frega, F. Bocci, G. Lercker, Riv. Ital. Sostanze Grasse 70 (1993) 153.
[17] R. Lehner, A. Kuksis, J. Biol. Chem. 268 (1993) 8781.
[18] L.C. Hudgins, J. Hirsch, E.A. Emken, Am. J. Clin. Nutr. 53 (1991) 474.