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Note

Separation and determination of diacylglycerols as their naphthylurethanes by high-performance liquid chromatography

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Natural glycerolipids are made up of complex mixtures of molecular species, which are believed to influence the biological properties of the cell membranes containing them [1, 2]. The immediate precursors in the biosynthesis of glycerolipids like phosphatidylcholine, phosphatidylethanolamine and triacylglycerol are the intramicrosomal diacylglycerols [3—5]. The species pattern of the phospholipids changes with physiological activity [6] and may be subject to metabolic regulation [7]. Much effort has therefore been expended in elaborating appropriate methods for the analysis of the species pattern and for detecting changes in it.

The present communication describes a quantitative resolution of the *sn*-1,2-diacylglycerol moieties by high-performance liquid chromatography (HPLC) in the picomole range after derivatization with α-naphthylisocyanate. Besides the separation of a mixture of commercial diacylglycerol species the separation of diacylglycerols obtained from phosphatidylcholine of rat liver microsomes by phospholipase C treatment is reported.

MATERIALS AND METHODS

Chemicals

Dimethylformamide (DMF) was purified by distillation with octadecylisocyanate. α-Naphthylisocyanate was purified by distillation in vacuo. The standard compounds 1,2-dimyristoyl-, 1,3-dimyristoyl-, 1,2-dipalmitoyl-, 1,3-dipalmitoyl-, 1,2-distearoyl-, 1,3-distearoyl and 1,2-dioleoylglycerol were purchased from Applied Science Labs. (State College, PA, U.S.A.).

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Separation of diacylglycerols from phosphatidylcholine

Rat liver microsomes were extracted according to the procedure of Bligh and Dyer [8] and the lipid extract was separated by two-dimensional thin-layer chromatography with chloroform—methanol—conc. ammonia (130:50:10) in the first dimension and chloroform—acetone—methanol—acetic acid—water (60:80:20:20:10) in the second dimension. The phosphatidylcholine-containing spot was extracted with chloroform—methanol (1:4). The solvent was evaporated in a stream of nitrogen at 40°C and the phosphatidylcholine was treated with phospholipase C (*Clostridium welchii*) from Sigma (St. Louis, MO, U.S.A.) as described in ref. 9. The diacylglycerols were purified by thin-layer chromatography with hexane—diethyl ether—acetic acid (70:30:4) as solvent.

Derivatization procedure

Ten to fifty nmol of the standard mixtures of diacylglycerols or diacylglycerols from the biological source were solubilized in chloroform—methanol (2:1) and transferred into the reaction vials. After evaporation to dryness in a stream of nitrogen the diacylglycerols were dissolved in 100 μl of DMF. A 200-fold molar excess of α-naphthylisocyanate and a four-fold molar excess of 1,4-diazabicyclo(2,2,2)octane were added to this solution. The stoppered vial was heated at 85°C for 2 h. After cooling to room temperature, excess reagent was destroyed by addition of 10 μl of methanol. After 10 min the reaction mixture was centrifuged and aliquots of 10—40 μl from the clear supernatant were applied to the column.

HPLC separation

The HPLC separations were carried out on a Hewlett-Packard Model 1084 B high-performance liquid chromatograph equipped with a variable-wavelength detector (190—600 nm). For fluorescence detection a Fluorichrom (Varian, Los Altos, CA, U.S.A.) was used (excitation 280 nm, emission 360 nm).

A Hewlett-Packard chromatographic column (200 × 4.6 mm) was used which was packed either with LiChrosorb RP-8 (10 μm) or with RP-18 (5 μm) supplied by Merck (Darmstadt, F.R.G.). The solvent system for the separation on the RP-8 column was acetonitrile—water (83:17). After 5 min the composition of the solvent was changed to 87% acetonitrile and 13% water. For the separation of the complex mixture of diacylglycerols an RP-18 (5 μm) column was used. According to the preconditions the solvent system was in this case a mixture of acetonitrile (solvent A) and acetonitrile—water (80:20) (solvent B). A linear solvent gradient was run from 50% B to 20% B between 0 and 75 min which produced a linear gradient of water running from 10% to 4%. The flow-rate was in all experiments 1 ml/min and the column temperature was set at 60°C.

Gas—liquid chromatography

For gas chromatographic analysis of the separated peaks a Varian 2100 instrument with a flame-ionization detector and a data system CDS 101 was used. A coiled glass column (1.8 m × 2 mm I.D.) was packed with 10% EGSS-X on Gas Chrom Q 100—120 mesh. The separation was carried out at a temperature of 150°C for 9 min and then with a temperature programme up to 190°C (1°C/min). The carrier gas was nitrogen (flow-rate 20 ml/min).

RESULTS AND DISCUSSION

Various methods for the analysis of diacylglycerol species are described in the literature. The most common method for the species analysis based on argentation thin-layer chromatography [5, 7, 10, 11] allows the separation of the molecular species according to the degree of unsaturation, but such techniques are time-consuming and require much larger amounts of sample than are conveniently available from routine preparations of cell compartments or bioptic materials. Another method is the analysis of diacylglycerol species as their *tert.*-butyldimethylsilyl ethers by a combination of capillary gas chromatography and mass spectrometry [12]; this, however, is very expensive and time-consuming. Also a method for the analysis of these species using capillary gas chromatography alone has been published [13]. The analysis of low concentrations of diacylglycerols by HPLC using sensitive ultraviolet or fluorescence detection is only possible after derivatization. The separation of *p*-nitrobenzoyl derivatives of diacylglycerols has already been described [14]. The detection limit of this method was in the nanomole range for each species and only commercial standard compounds were investigated. The reaction of alcoholic hydroxyl groups with α-naphthylisocyanate forming urethanes was used for the analysis of alcohols and drugs containing hydroxyl groups [15, 16].

Our aim was the application of this derivatization procedure to 1,2-diacylglycerols. In order to establish the optimum conditions for derivatization, the influence of excess reagent, reaction time, temperature and the usefulness of 1,4-diazabicyclo(2,2,2)octane as catalyst were determined. For the formation of the naphthylurethanes of alcoholic compounds other than diacylglycerols a 1.5- or 30-fold molar excess of the reagent was found to be optimal [15, 16]. For the less reactive diacylglycerols we found that a 200-fold excess of the reagent was the optimal condition.

Using these conditions the diacylglycerol urethanes have sufficient stability after centrifugation and no acyl migration was observed. The reaction with *sn*-1,3-diacylglycerols shows no differences compared with the reaction of *sn*-1,2-diacylglycerols.

Determination of the detection limit of diacylglycerols as well as linearity and reproducibility of the derivatization were carried out with *sn*-1,2-dipalmitoylglycerol separated on an RP-8 (10 μm) column. The detection limit with ultraviolet detection at 290 nm was 100 pmol of diacylglycerol. With the fluorescence detector the detection limit was at least ten times lower. The linearity of the derivatization ranged from 0.2 to 50 nmol, which is in the range of biochemical interest. A comparison of the different standard compounds, dioleoylglycerol and dipalmitoylglycerol, showed that there was no preference for either one of the species in the reaction with α-naphthylisocyanate. Investigation of five identical samples of 1,2-dipalmitoylglycerol demonstrated the good reproducibility of our derivatization procedure (coefficient of variation 3.1%). To exclude differences in the yield of the reaction, the quantitation of biological mixtures was based on 1,2-distearoylglycerol as internal standard. This diacylglycerol can be used because it was not present in our biological samples.

As shown in Fig. 1 the mixture of standard compounds was completely separated with good peak shape under our conditions. As expected from the

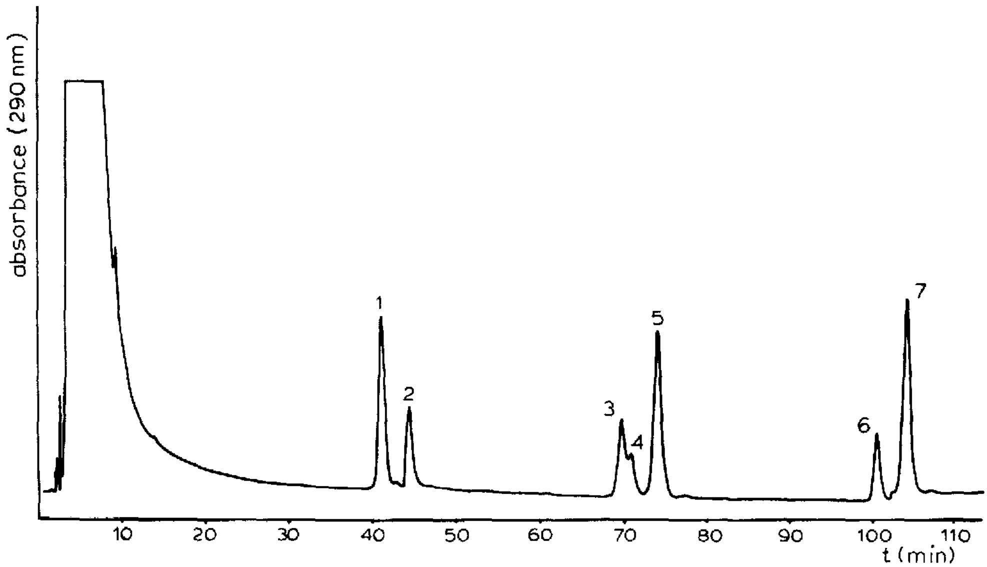

Fig. 1. HPLC separation of diacylglycerol naphthylurethane standards. 1 = 1,3-dimyristoyl-, 2 = 1,2-dimyristoyl-, 3 = 1,3-dipalmitoyl-, 4 = 1,2-dioleoyl-, 5 = 1,2-dipalmitoyl-, 6 = 1,3-distearoyl-, 7 = 1,2-distearoylglycerol napthylurethane.

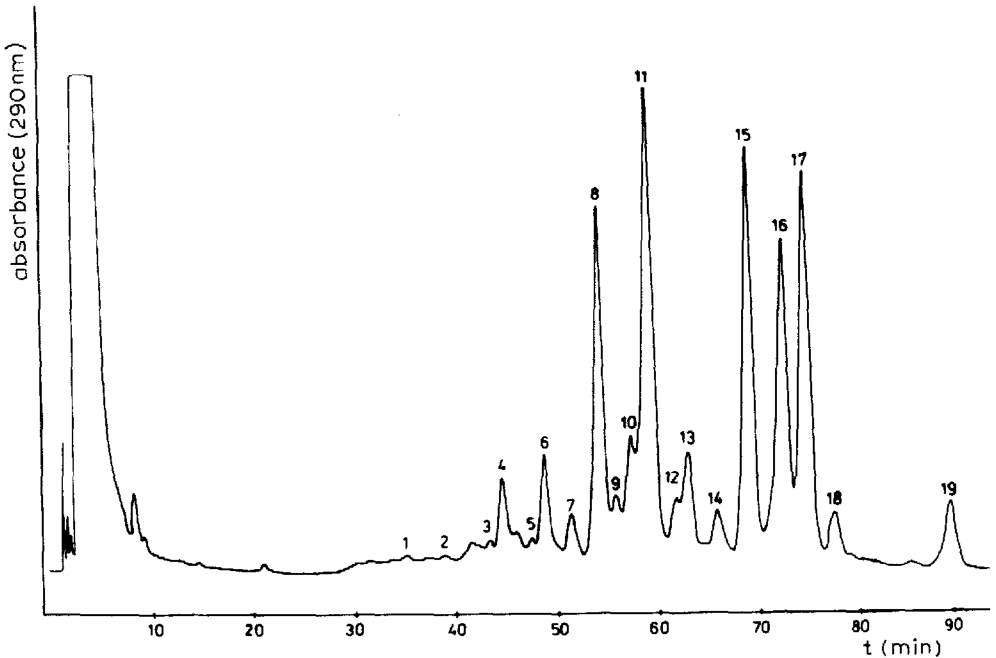

Fig. 2. HPLC separation of diacylglycerol naphthylurethanes from phosphatidylcholine of rat liver microsomes. Peaks are numered in sequence of elution time and are listed in Table I.

literature, the unsaturated diacylglycerols were eluted earlier than the saturated compounds with the same carbon number. In each case the 1,3-diacylglycerols were eluted before the 1,2-isomers. We applied our method to the analysis of a biological pattern of diacylglycerols using phosphatidylcholine from rat liver microsomes hydrolysed by phospholipase C. The species pattern of phospha-

tidylcholine from this source is well known by species separation with other analytical techniques. Identification of the single peaks of the separation pattern of biological diacylglycerols requires a second analytical method because only a few standard compounds were available. For a correct identification of the HPLC-separated species we collected each peak from at least five runs and determined the fatty acid composition of the single peaks by gas chromatographic analysis. As shown in Fig. 2, the diacylglycerol moieties of phosphatidylcholine from rat liver microsomes were fractionated into nineteen peaks related to 24 molecular species. This number was much higher than that obtained by conventional techniques such as argentation thin-layer chromatography. The distribution of the molecular species of phosphatidylcholine in rat liver microsomes is listed in Table I. As shown by gas chromatography not all peaks represent single diacylglycerol species. The major components were 16:0—20:4, 16:0—18:2, 16:0—18:1, 18:0—20:4 and 18:0—18:2. They represent nearly 70% of the detected peaks. As shown by comparison with standard compounds and by gas chromatographic fatty acid analysis, peaks 7, 9 and 14 represent 1,3-diacylglycerol species which were formed during the isolation of diacylglycerols. Comparison of our results with previously reported values [17, 18] shows a good agreement in the distribution of the major components, whereas Patton et al. [19] described a surprisingly low amount of the 16:0—18:1 species with 1.4% in phosphatidylcholine from rat liver.

TABLE I

DISTRIBUTION OF MOLECULAR SPECIES IN PHOSPHATIDYLCHOLINE FROM RAT LIVER MICROSOMES

Peak number*	Molecular species	Percentage composition
1	14:0—22:6	0.16
2	14:0—20:4	0.21
3	16:1—18:2	0.55
4	16:0—20:5, 18:2—18:2	2.5
5	15:0—18:2	0.55
6	16:0—22:6, 16:1—18:1	3.3
7**	16:0—20:4	1.5
8	16:0—20:4	11.3
9**	16:0—18:2	1.8
10	16:0—16:1	3.7
11	16:0—18:2, 18:0—20:5, 16:0—22:5, 18:1—18:2	19.1
12	16:0—20:3	1.8
13	18:0—22:6	3.8
14**	18:0—20:4	2.0
15	18:0—20:4	14.4
16***	16:0—18:1	13.9
17	18:0—18:2	14.3
18	18:0—20:3	2.0
19	18:0—18:1	3.0

*See Fig. 1.

**These peaks represent 1,3-diacylglycerol species.

***1,2-Dipalmitoylglycerol overlaps with this peak.

In agreement with the results of other groups [17, 18] we found in the fatty acid pattern of phosphatidylcholine from rat liver microsomes 8.5% of the oleic acid. We found also this percentage of 8.5% of the 18:1 in the fatty acid pattern of the diacylglycerols produced by phospholipase C treatment of the phosphatidylcholine from rat liver microsomes. The methods described in the literature to determine the species composition of phospholipids by HPLC were based on the quantitation of the peaks by analysis of the phosphorus content of each peak [18, 19]. The sensitivity of this method is limited by the sensitivity of the phosphate determination. This indirect way of quantitation was necessary because the absorption at 206 nm varied with the degree of unsaturation in the single species and their peak areas as a basis of quantitation were not comparable. We believe that because of the derivatization the importance of our method is the direct measurement of the species by ultraviolet absorption or fluorescence. This is combined with an increase in sensitivity to the picomole range.

Moreover, using radioactively labelled precursors, our method allows the measurement of the biosynthesis of diacylglycerols or of phospholipids which can be converted to the diacylglycerols by collection of the single peaks and determination of their radioactivity.

REFERENCES

1 O. Renkonen, Adv. Lipid Res., 5 (1967) 329.
2 A. Kuksis, L. Marai, W.L. Breckenridge, D.A. Gornall and O. Stachnykl, Can. J. Physiol. Pharmacol.,46 (1968) 511.
3 E.P. Kennedy, Ann. Rev. Biochem., 26 (1957) 119.
4 H. Van Den Bosch, Ann. Rev. Biochem., 43 (1974) 243.
5 R. Roberti, L. Binaglia and G. Porcellati, J. Lipid Res., 21 (1980) 449.
6 C.J. Meade and J. Mertin, Adv. Lipid Res., 16 (1978) 127.
7 K. Ishidate and P.A. Weinhold, Biochim. Biophys. Acta, 664 (1981) 133.
8 E.G. Bligh and W.J. Dyer, Can. J. Biochem., 37 (1959) 911.
9 O. Renkonen, J. Amer. Oil Chem. Soc., 42 (1965) 298.
10 A. Kuksis and L. Marai, Lipids, 2 (1967) 217.
11 G.A.E. Arvidson, Eur. J. Biochem., 4 (1968) 478.
12 J.J. Myher, A. Kuksis, L. Marai and S.K.F. Yeung, Anal. Chem., 50 (1978) 557.
13 J.J. Myher and A. Kuksis, Can. J. Biochem., 60 (1982) 638.
14 M. Batley, N.H. Packer and J.W. Redmond, J. Chromatogr., 198 (1980) 520.
15 R. Wintersteiger, Z. Anal. Chem., 309 (1981) 201.
16 R. Wintersteiger, C. Wenninger-Weinzierl and W. Pacha, J. Chromatogr., 237 (1982) 399.
17 J.L. James, G.A. Clawson, C.H. Chan and E.A. Smuckler, Lipids, 16 (1981) 541.
18 M. Smith and P.B. Jungalawala, J. Lipid Res., 22 (1981) 697.
19 G.M. Patton, J.M. Fasulo and S.J. Robins, J. Lipid Res., 23 (1982) 190.