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QUANTITATIVE DETERMINATION OF NATURAL DIPALMITOYL LECITHIN WITH DIMYRISTOYL LECITHIN AS INTERNAL STANDARD BY CAPILLARY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative determination of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine from dog lungs by means of the corresponding diacyl glycerol trimethylsilyl ether derivative with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as internal standard was carried out by gas–liquid chromatography with glass capillary columns. The individual diacyl glycerol species were identified by using a combination of gas–liquid chromatography and mass spectrometry. In a total of 32 determinations percent deviations from the mean value between ± 2.0 and ± 4.4% were achieved. By using glass capillary columns instead of conventional columns the time required for an analysis was reduced to *ca.* 30% and separation was considerably improved. Thus the diacyl glycerol species from PC-32 were separated according to both the 1,2 and 1,3 positional isomerism and the degree of saturation of the acyl radicals.

INTRODUCTION

In several diseases such as the respiratory distress syndrome (RDS) of the newborn[1–4] or the adult RDS —the socalled shock lung[5,6]— the lung disfunction is at least in part the result of an impairment of the lung surfactant system. Dipalmitoyl lecithin (DPPC) is generally accepted as the major active component of the pulmonary surfactant[4,7–9]. There is strong evidence that the reduction of DPPC is characteristic of these pulmonary infections. A treatment resulting in an increase in the phospholipid synthesis requires a quantitative determination of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) which has to be as exact as possible and which can be carried out in serial analyses.

The usual methods of determining disaturated phosphatidylcholine species comprise separating the corresponding diacyl glycerol acetates by argentation thin-layer chromatography (TLC)[10–13] or by adduction with mercury acetate or osmium tetroxide combined with column chromatography or TLC, respectively[9,14,15].

Gas–liquid chromatography (GLC) with conventional columns has been used to separate the 3-*sn*-phosphatidylcholine species from various biological sources by means of the corresponding diacyl glycerol acetates[16–19] or trimethylsilyl (TMS) ether derivatives[20].

Ogino *et al.*[21] characterized 3-*sn*-phosphatidylcholine species from fetal rat and rabbit lungs as TMS ether (PC_{30}–PC_{34}) by a combination of GLC and mass spectrometry (MS).

This paper describes a method for the quantitative determination of DPPC in lung tissue with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine as internal standard by GLC with glass capillary columns.

In accordance with the results of other authors, dimyristoyl lecithin could not be detected in natural lecithins[20,21]. This was shown in a series of samples without the internal standard. The use of other internal standards, such as dialkyl or 1-acyl-2-alkyl-*sn*-glycero-3-phosphocholine, has not proved possible as it is well established that plasmanylcholine also occurs in nearly all natural sources of phospholipids. As considerably better separation was achieved with glass capillary than with conventional columns, it was possible to separate the DPPC from the other 3-*sn*-phosphatidylcholine species having the same number of carbon atoms. The individual components were identified by GLC–MS.

EXPERIMENTAL

Apparatus

A Carlo Erba Fractovap 2350 gas chromatograph equipped with a hydrogen flame detector in connection with a System 1 Integrator was used for the quantitative analyses of diacyl glycerols (Carlo Erba, Milan, Italy). The samples were analysed on a SE-30 column (12 m × 0.25 mm I.D.). The injection port and detector temperature were maintained at 350°. The oven temperature was programmed to rise from 24° to 300° at a rate of 4°/min. Hydrogen was used as carrier gas at a flow-rate of 40 cm/sec.

An Ultraturax (IKA-Werk, Staufen, G.F.R.), a Kontes Micro-Ultrasonic Cell Disruptor (Kontes, Vineland, N.J., U.S.A.) and a Camag Lonomat III (Camag, Muttenz, Switzerland) were used.

A direct coupling of the glass capillary column with a Varian MAT 311A mass spectrometer in connection with a Varian 166 spectrosystem was used for the identification of the individual diacyl glycerol species. The temperature of the interface was 350°, the pressure in the ion source was $5 \cdot 10^{-6}$ Torr at a helium flow-rate of 3 ml/min. At an electron energy of 70 eV and an electron current of 3 mA every 0.7 sec, a mass spectrum in the range 20–800 mass units was recorded and evaluated by means of the spectrosystem.

Procedure

The lungs of dogs were bled *in situ* with a physiological NaCl solution, were removed immediately afterwards and homogenized with an Ultraturax in 40 volumes of chloroform–methanol (2:1) and extracted overnight under nitrogen. The tissue lipid extracts were washed using the method of Folch *et al.*[22].

Thin-layer chromatography. Aliquots of samples were applied as bands with

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (100 μg) on previously cleaned TLC plates. The plates were developed in chloroform–methanol–water (60:35:5), dried and the 3-*sn*-phosphatidylcholine fraction was removed from the plates.

Enzymatic hydrolysis. The 1,2-diacyl-*sn*-glycerols of 3-*sn*-phosphatidylcholine were obtained by digestion with phospholipase C from *Bacillus cereus*. After removal from the TLC plates, the 3-*sn*-phosphatidylcholine samples were dried under nitrogen and 2.5 ml of the 0.1 *M* tris buffer, (pH 7.4, 0.03 *M* with respect to CaCl₂) was added[23,24]. After sonication for *ca*. 15 sec, 10 μg of phospholipase C and 2.5 ml of diethyl ether were added and the mixture was stirred. After 3 h, when the reaction was complete, the ether layer was separated and the water layer was re-extracted five times with 2.5 ml of ether. The pooled ether extracts were dried under nitrogen.

Silylation. The TMS ethers of 1,2-diacyl-*sn*-glycerols were prepared by reacting (at room temperature for 1 h) with pyridine–hexamethyldisilazane–chlorotrimethyl-silane (12:5:2)[20]. The resulting TMS ether solutions were used directly for subsequent analyses.

Reagents. Chloroform, methanol, pyridine, hexamethylsilazane, chlorotri-methylsilane and diethyl ether were obtained from Merck (Darmstadt, G.F.R.). Phospholipase C from *B. cereus* was obtained from Boehringer (Mannheim, G.F.R.). 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phos-phocholine, 1,2-dipalmitoyl-*sn*-glycerol, 1,3-dipalmitoyl-*sn*-glycerol, 1,2-dimyristoyl-*sn*-glycerol were obtained from Sigma (St. Louis, Mo., U.S.A.). 1-Palmityl-*sn*-glycerol, 1,3-dioleyl-*sn*-glycerol, 1-palmityl-3-oleyl-*sn*-glycerol and 1-stearoyl-3-oley-*sn*-glycerol were obtained from Analabs, a subsidiary of New England Nuclear (Boston, Mass., U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram of a mixture of standards of 1,2- and 1,3-diacyl glycerols. On non-polar silicone phases, GLC with glass capillary
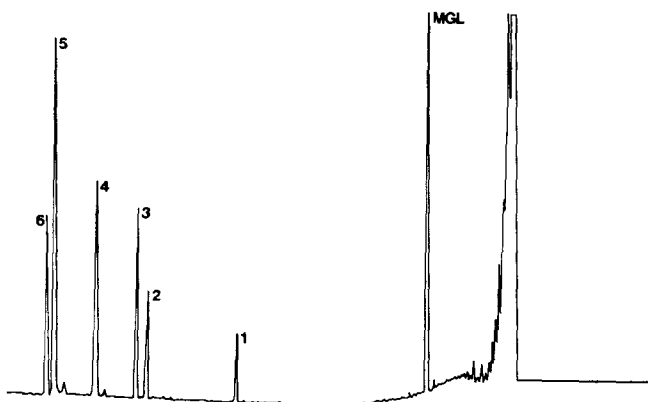

Fig. 1. GLC of reference 1,2- and 1,3-diacyl-*sn*-glycerols as TMS ethers. MGL = Palmitoyl-*sn*-glycerol; 1 = 1,3-dimyristoyl-*sn*-glycerol; 2 = 1,2-dipalmitoyl-*sn*-glycerol; 3 = 1,3-dipalmitoyl-*sn*-glycerol; 4 = 1-palmitoyl-3-oleoyl-*sn*-glycerol; 5 = 1,3-dioleoyl-*sn*-glycerol; 6 = 1-stearoyl-3-oleoyl-*sn*-glycerol. Column, 12-m SE-30, 150°–300°, programmed at 10°/min, carrier gas hydrogen.

columns permits the separation of the 3-*sn*-phosphatidylcholine species having the same number of carbon atoms according to both the 1,2 and 1,3 positional isomerism and the degree of saturation of the acyl radicals.

A good separation of the corresponding 1,2-diacyl glycerols from PC-32 was achieved on a 12-m SE-30 column, as illustrated in Fig. 2.
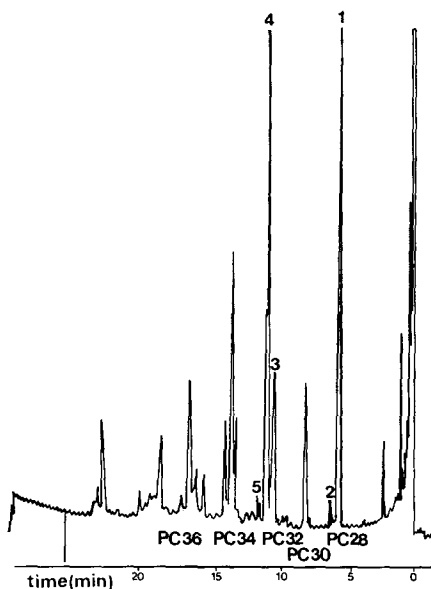

Fig. 2. GLC of the corresponding diacyl glycerols as TMS ethers from dog lung 3-*sn*-phosphatidyl-choline. 1 = 1,2-Dimyristoyl-*sn*-glycerol; 2 = 1,3-dimyristoyl-*sn*-glycerol; 3 = 1-palmitoyl-2-palmi-toleyl-*sn*-glycerol; 4 = 1,2-dipalmitoyl-*sn*-glycerol; 5 = 1,3-dipalmitoyl-*sn*-glycerol. Column, 12-m SE-30, 240°–300° programmed at 4°/min, carrier gas hydrogen.

Quantitative determinations

The reproducibility of the quantitative determination of dipalmitoyl lecithin with dimyristoyl lecithin as internal standard was confirmed with eight different lung samples in duplicated tests. Each individual sample was analysed twice. The greatest deviation from the mean in these duplicated tests was 4.4%, and the average repro-ducibility achieved was $\pm$ 3% (Table I).

GLC method with glass capillary columns used as described with non-polar silicone phases (SE-30 and OV-1) can bring about a separation sufficient to carry out a quantitative determination of the 1,2-dipalmitoyl-*sn*-glycerol. By use of the mercury adduction method or argentation TLC, the 3-*sn*-phosphatidylcholine species can be separated according to the number of double bonds but not according to the number of carbon atoms. The saturated 3-*sn*-phosphatidylcholine species isolated by these methods contain other fatty acids in addition to palmitic acid, in particular myristic acid and stearic acid.

The following values for the percentage of palmitic acid in the fatty acids of the disaturated phosphatidylcholine of the lung have been obtained: 67.7 (ref. 14), 80–98 (ref. 10), 95 (ref. 7) and 73 (ref. 15). Henderson and Hackett[25] found that 90%

TABLE I

RELATIVE STANDARD DEVIATION OF EIGHT DIFFERENT LUNG SAMPLES IN DOUBLE TESTS (A, B); EACH INDIVIDUAL SAMPLE WAS ANALYSED TWICE (a, b VALUES)

D = percent deviation.

Sample	DPL (mg/g dry weight)		D (%)	D (A + B) (%)
	(a)	(b)		
1 A	7.00	6.70	±2.2	±2.7
B	7.07	6.85	±1.6	
2 A	5.59	5.42	±1.6	±3.4
B	5.80	5.66	±1.4	
3 A	4.54	4.72	±2.0	±2.0
B	4.72	4.60	±1.3	
4 A	5.17	4.90	±2.6	±4.4
B	5.35	5.12	±2.2	
5 A	4.41	4.08	±3.9	±3.9
B	4.28	4.08	±2.4	
6 A	6.86	6.86	±0.0	±3.3
B	7.33	7.33	±0.0	
7 A	9.85	10.10	±1.3	±2.2
B	10.02	9.69	±1.7	
8 A	9.62	10.12	±2.6	±2.6
B	9.72	10.12	±2.0	

of the disaturated fraction was present as 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine. In addition to the varying percentage of DPPC in the disaturated fraction the quantitative evaluation by densitometry or by measuring the fluorescence on different plates results in a greater standard deviation[26] than the deviation to be expected in a quantitative determination according to the present method.

A more precise determination of DPPC is possible by GLC determination of the composition of the fatty acids (after digestion with phospholipase A) in the α- and β-positions of the 3-sn-phosphatidylcholine. When comparing the determination of the diacyl-sn-glycerol species by GLC with glass capillary columns and the determination of the fatty acid composition in the α- and β-positions of the 3-sn-phosphatidylcholine, the times required for isolating the 3-sn-phosphatidylcholine and for enzymatic digestion are approximately the same, whereas the analysis according to the method described in the present paper is considerably shorter than the determination of the fatty acid composition in the α- and β-positions of the 3-sn-phosphatidylcholine. Since 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine seems to be present in quantitatively significant amounts, the percentage of the palmitic acid in the fatty acids of the β-position cannot be directly correlated with DPPC.

Myher and Kuksis[20] separated natural diacyl-sn-glycerols by GLC with packed columns containing 3% Silar 5-CP on Gas-Chrom Q. The long time required for the analysis (ca. 100 min as compared to 20 min according to the method described in this paper) results in wider peaks and thus renders the quantitative evaluation more difficult[27].

The mass spectra of some 1,2- and 1,3-diacyl-sn-glycerols have already been described[21,28]. In principle, the identification of the 1,2- and 1,3-positional isomerism

is possible owing to the differences in the mass spectra. The most important information disclosed by the mass spectrum are: the molecular weight (by means of the $M-15$ ion), the identification of the individual fatty acids by the RCO and $RCO+74$ ions, respectively, as well as the identification of the 1,3-derivatives by the preferred formation of the $M-RCOOCH_2$ ion[28].

The use of *tert*.-butyl-dimethylsilyl ethers of diacyl glycerols as reported by Myher *et al.*[29] enhances the ion intensities in higher mass ranges, thus making these derivatives more sensitive for characterization. However, for routine analytical use, more time consuming steps are necessary.
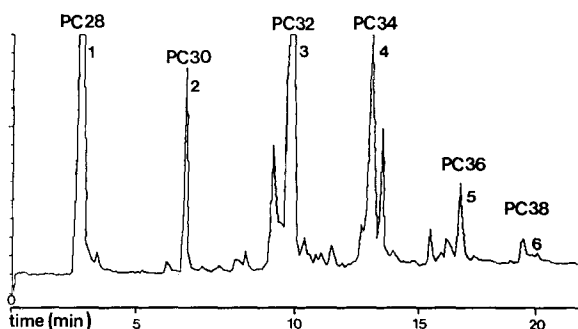
Fig. 3. A portion of the computer-reconstructed sum plot in the range from PC-28 to PC-38 recorded by GLC–MS (TMS derivatives).

Fig. 4. Main molecular species of PC-30–PC-34 of dog lung 3-*sn*-phosphatidylcholine.
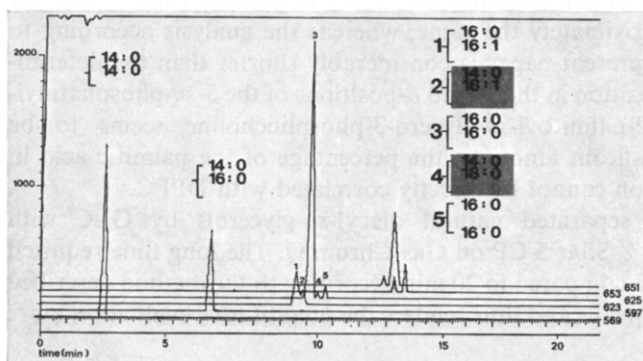

Fig. 5. $M^+ - 15$ ion profiles of dog lung 3-*sn*-phosphatidylcholine of the sum plot from Fig. 3. PC-28 (dimyristoyl-*sn*-glycerol) TMS derivative was used as internal standard.

Fig. 3 shows the sum plot in the range from PC-28 to PC-38 reconstructed by the computer which is correlatable to the gas chromatogram. The different PC-32 species to be expected in principle are shown in Fig. 4.

The ion profiles of the M—15 ions in Fig. 5 show that by means of the capillary column not only the mono-unsaturated 1,2- and 1,3-PC-32 species but also the corresponding saturated 1,3-isomers may be separated from the saturated 1,2-species (which corresponds to DPPC). It also seems to be possible to separate 1-myristoyl-2 stearoyl species from the 1,2-dipalmitoyl species.

In addition, the formation of undesired 1,3-isomers may be suppressed to a large extent by using HMDS–TMS–pyridine as silylating reagent. The mass spectrum of the 1,2-dipalmitoyl-*sn*-glycerol shown in Fig. 6 correlates clearly with the one cited in literature which was recorded with direct inlet[28]. This confirms the correct identification of the peaks. The identification of the PC-34–PC-38 species will be published elsewhere.
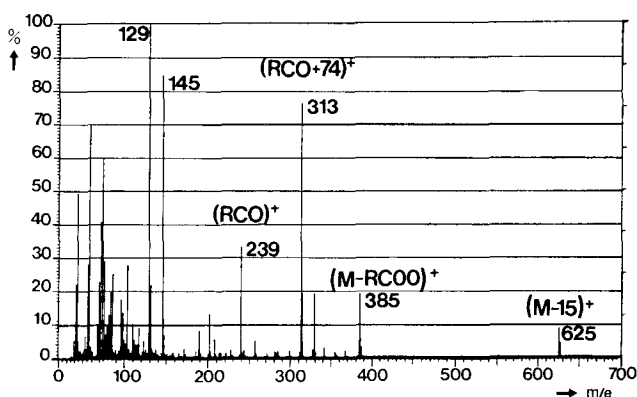

Fig. 6. Mass spectrum of 1,2-dipalmitoyl-*sn*-glycerol TMS ether obtained by GLC–MS from dog lung 3-*sn*-phosphatidylcholine.

CONCLUSION

The internal standard method described proved to be reliable in determining DPPC in lung tissue. Compared with other methods, the determination by GLC with glass capillary columns permits an exact direct determination of DPPC with a mean percent deviation from the mean of $\pm$ 3%, the time required for the GLC analysis being as short as *ca.* 20 min.

ACKNOWLEDGEMENTS

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