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Improved determination of individual molecular species of phosphatidylcholine in biological samples by high-performance liquid chromatography with internal standards

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ABSTRACT

Phosphatidylcholine isolated from samples of bile, liver and plasma was converted into 1,2-diradylglycerobenzoate molecular species by hydrolysis with phospholipase C and reaction with benzoic anhydride. Up to seventeen molecular species were separated and determined by reversed-phase high-performance liquid chromatography with detection at 230 nm. The major improvement introduced here was the use of distearoylphosphatidylcholine as the internal standard, which corrected the results for incomplete hydrolysis and benzoylation. Other improvements concerned the clean-up of benzoyl derivatives and the chromatographic separation. The analytical results obtained were validated by comparison with the results of either lipid phosphorus or gas chromatographic determinations.

INTRODUCTION

There are three different approaches to the high-performance liquid chromatographic (HPLC) determination of the molecular species profile of biologically relevant phospholipids, namely the separation of intact phospholipids, both unmodified [1–8] and modified [9–12], and UV-absorbing derivatives of partially hydrolysed phospholipids (*i.e.*, the 1,2-diradylglycerols) [13–19].

The determination of unmodified intact phospholipids, in spite of its simplicity, has not gained wide acceptance because of problems relating to the detection of the molecular species with a low degree of acyl unsaturation, as pointed out in previous reports on the determination of molecular species in bile phosphatidylcholine [7,8].

The determination of intact phospholipids based on postcolumn fluorescence derivatization with 1,6-diphenyl-1,3,5-hexatriene is attractive [12]. However, a particular apparatus is required and the determination is likely to be affected by molecular species-related changes in interaction between the fluorescent probe and the phosphatidylcholine micelles formed by the water enrichment due to the post-column flow. Consequently, methods in which the phospholipids are converted into diradylglycerols tagged with a UV chromophore have been often preferred in practical applications [20,21], in spite of the more complex and critical analytical procedure required. Further, it is claimed that the UV-tagged diradylglycerol subclasses (*i.e.*, the alk-1-enylacyl, alkylacyl and diacyl species) are more easily separated by thin-layer chromatography (TLC) and determined [15] than the diradylglycerol acetates described by Nakagawa and Horrocks [14].

In this study, we introduced various improvements to existing methods for the determination of the diradylglycerobenzoates derived from biological phosphatidylcholines. The major improvement was the introduction of an internal standard (the distearoylphosphatidylcholine) that gave quantitative results corrected for partial hydrolysis and benzoylation. Other improvements concerned the pro-

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cedure of derivative purification and the chromatographic conditions used for obtaining a chromatographic profile of the molecular species well suited for the analysis of bile, plasma and liver phosphatidylcholines.

EXPERIMENTAL

The molecular species of phosphatidylcholine, dipalmitoyl (16:0–16:0), distearoyl (18:0–18:0), palmitoyloleoyl (16:0–18:1), palmitoyllinoleoyl (16:0–18:2) and palmitoylarachidonoyl (16:0–20:4), the diarachidine and the phospholipase C (from *Bacillus cereus*) were purchased from Sigma (St. Louis, MO, USA). The species oleoyllinoleoyl (18:1–18:2), stearoyllinoleoyl (18:0–18:2) and stearoyl arachidonoyl (18:0–20:4) were isolated by preparative column chromatography from purified soy and egg lecithins as described previously [7]. The 1,2-diarachidine isomer was isolated from diarachidine using borate-impregnated TLC plates as described by Christie [22]. Methylheptadecanoate and silica gel 60 TLC plates were purchased from E. Merck (Darmstadt, Germany) and benzoic anhydride and 4-pyirrolidinopyridine from Fluka (Buchs, Switzerland). Other chemicals of analytical-reagent grade and solvents of HPLC grade were purchased from Carlo Erba (Milan, Italy).

Bile samples were mixed with isopropanol (1:5, v/v) and centrifuged to remove proteins. Phosphatidylcholine was isolated from this alcoholic solution by TLC with the solvent system chloroform–methanol–acetic acid–water (65:25:15:4, v/v). The band of phosphatidylcholine was scraped from the silica gel and eluted with 10 ml of chloroform–methanol–water (60:40:6, v/v/v).

Lipid extracts from liver and plasma samples were prepared with chloroform–methanol (2:1, v/v) according to Folch *et al.* [23]. Phosphatidylcholine was isolated by TLC as described above.

Phosphatidylcholine was converted into the corresponding diradylglycerol by enzymatic hydrolysis with phospholipase C as described previously [24]. Briefly, the sample of bile phosphatidylcholine was mixed with 100 μg of distearoylphosphatidylcholine. The solvent was evaporated under nitrogen and the residue was dissolved in 0.5 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (pH 7.3) containing 0.4 m*M* zinc chloride, 1 m*M* mercaptoethanol and 20 units of phospholipase C (from *B. cereus*). The mixture was maintained under continuous stirring in a shaking water-bath for 3 h at 37°C. The diradylglycerol was extracted three times with 2-ml portions of diethyl ether. The extracts were combined and mixed with 100 μg of 1,2-diarachidine and the solvent was evaporated under nitrogen. The benzoate derivative of diradylglycerol molecular species was prepared by a modification of the method described by Blank *et al.* [19]. Briefly, 0.3 ml of reaction mixture (10 mg of benzoic anhydride and 25 μg of 4-pyrrolidinopyridine dissolved in 1 ml of anhydrous benzene) was added to the sample and allowed to stand for 90 min at room temperature. The reaction was stopped by adding 1 ml of concentrated ammonia solution. The benzoate derivative was extracted with three 2 ml portions of *n*-hexane to eliminate the excess of benzoic acid. The pooled extracts were passed through a cartdridge filled with 1.5 g of diatomaceous earth (Extrelut, E. Merck), which was wetted prior to use with 2 ml of 1 *M* hydrochloric acid. The cartridge was washed with 3 ml of *n*-hexane. The *n*-hexane was collected and evaporated under nitrogen. The residue was dissolved in 100 μl of the mobile phase for HPLC.

HPLC analysis was performed with an apparatus produced by Gilson Medical Electronics (Middleton, WI, USA), equipped with an UV detector set at 230 nm. The column used was a Spherisorb ODS-2, 5 μm (250 × 4.6 mm I.D.) (Phase Separations, Queensferry, Clwyd, UK), maintained at 38 ± 0.1°C by an electronic thermostat. The mobile phases tested during the study contained methanol, ethanol, isopropanol, water and acetonitrile in different proportions. For bile samples the best results were obtained with methanol–acetonitrile–ethanol–water (29:21:47:2.8, v/v) at a flow-rate of 1 ml/min.

The relative retention times (RRT) of molecular species contained in mixtures of authentic standards were calculated by dividing the retention time of each peak by that of the peak given by 1,2-diarachidine benzoate. A linear graph was constructed between log RRT and the total number of hydrophobic carbon atoms in the fatty acid side-chains of the disaturated molecular species of diacylglycerobenzoate tested [5]. The total hydrophobic carbon number (HCN) for the unsaturated molecular species was calculated by interpolation of the linear

graph using its log RRT value. The unsaturated species showed, in comparison with their saturated parent compounds, a reduction in the HCN values that were used for the prediction of the elution sequence of molecular species [5,7].

The identity of unknown peaks and the validation of the results obtained by the HPLC determination were made by the gaschromatographic (GC) determination of the fatty acid methyl esters prepared from either phosphatidylcholine or diradylglycerobenzoate fractions, as described previously [7].

RESULTS AND DISCUSSION

The HPLC of molecular species of diradylglycerobenzoates derived from biological phosphatidylcholines suffered from both qualitative and quantitative problems due to the complexity of natural mixtures and the analytical procedures applied, respectively.

The study of the qualitative problems started from an attempt to separate the complex mixture of 1,2-diradylglycerobenzoates derived from bile phosphatidylcholine in a manner comparable to

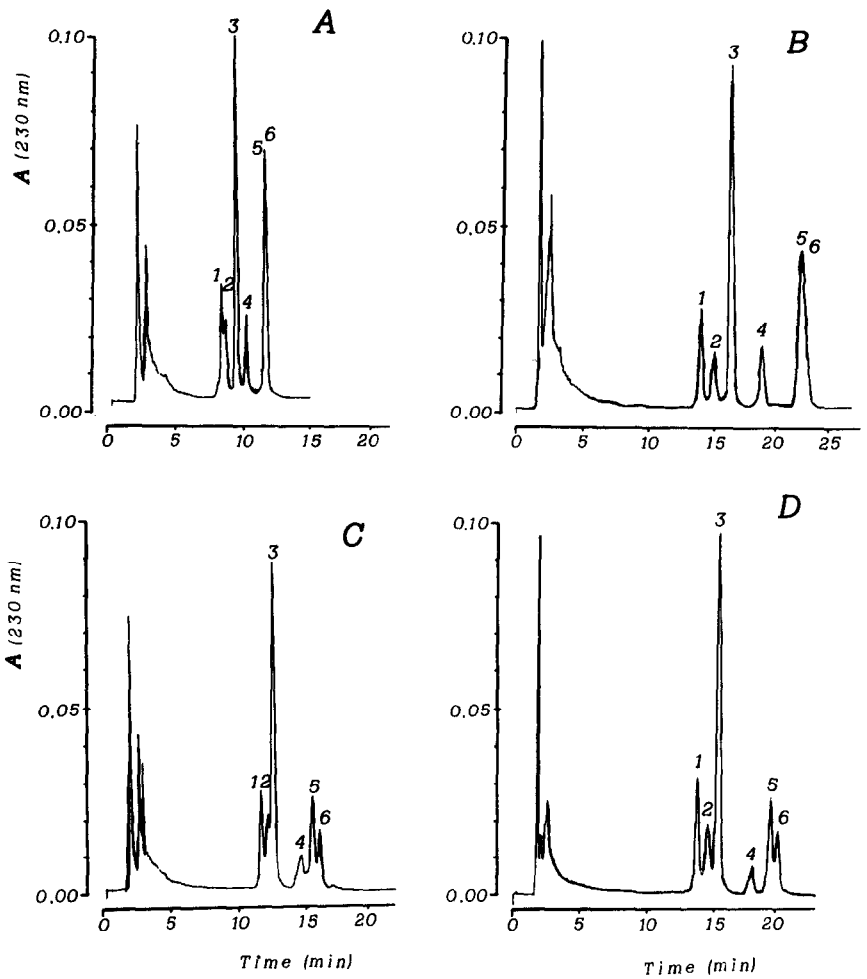

Fig. 1. Chromatographic separations of the six molecular species of diradylglycerobenzoates derived from the major molecular species of bile phosphatidylcholine with four different mobile phases: (A) acetonitrile–isopropanol (80:20, v/v); (B) acetonitrile–methanol–ethanol (70:12:18, v/v/v); (C) acetonitrile–methanol–ethanol (15:34:51, v/v/v) with 2.8% of water added; (D) acetonitrile–methanol–ethanol (30:28:42, v/v/v) with 3.0% of water added. Peaks: 1 = 16:0–20:4; 2 = 18:1–18:2; 3 = 16:0–18:2; 4 = 18:0–20:4; 5 = 16:0–18:1; 6 = 18:0–18:2.

that described previously for intact molecular species of human and rat bile [7,8]. This was done by studying the effects of modifications of the mobile phase described previously for the separation of 1,2-diradylglycerobenzoates derived from liver phospholipids [16] [acetonitrile–2-propanol (80:20, v/v] on the separation of the major molecular species of bile phosphatidylcholine. Some significant steps in the optimization process are illustrated in the four chromatograms shown in Fig. 1. This sequence showed that acetonitrile improved the resolution between 2-arachidonoyl and 2-linoleoyl species while water and methanol are required for the separation between the pairs of species 18:1–18:2/16:0–18:2 and 16:0–18:1/18:0–18:2.

HPLC under the final chromatographic conditions of the diradylglycerobenzoates derived from either human or rat bile phosphatidylcholines gave the results shown in Fig. 2. The same conditions also allowed good separations of liver and plasma phosphatidylcholines whose molecular species distributions were considered to be metabolically related to that of bile (Fig. 3).

The RRTs and the capacity factors (k') measured with standard mixtures of molecular species with different degrees of acyl unsaturation are reported

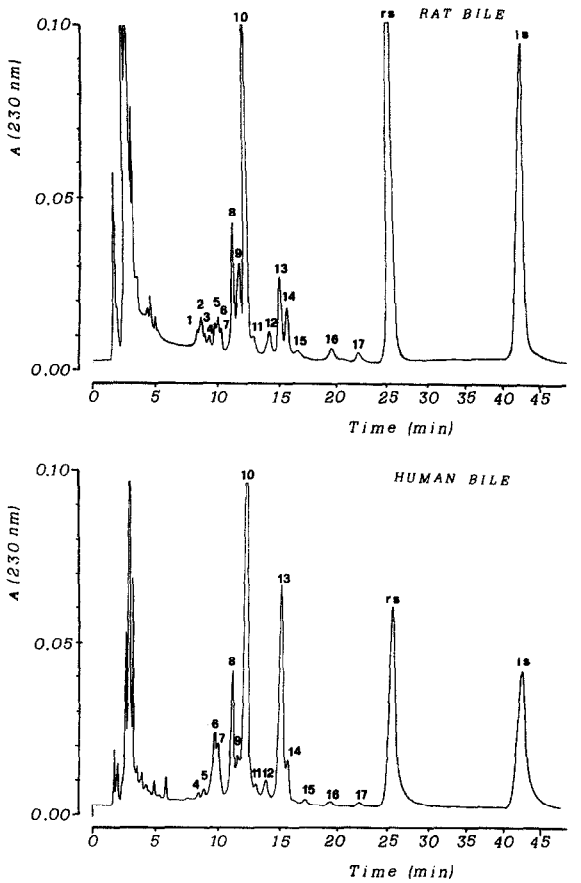

Fig. 2. HPLC separation of the molecular species of 1,2-diradylglycerobenzoates derived from phosphatidylcholine of rat bile and human bile under the analytical conditions described under Experimental. The identification of the molecular species corresponding to each peak is reported in Table II; rs is the peak relative to distearoylphosphatidylcholine and is that relative to 1,2-diarachidine.

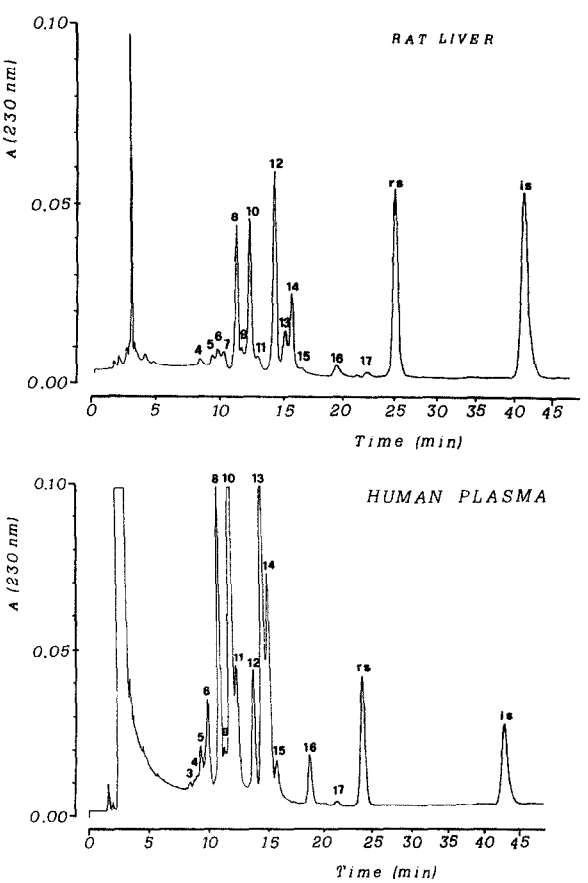

Fig. 3. HPLC separation of the molecular species of the 1,2-diradylglycerobenzoates derived from phosphatidylcholine of rat liver and human plasma under the analytical conditions described under Experimental. The identification of the molecular species corresponding to each peak is reported in Table II; rs is the peak relative to distearoylphosphatidylcholine and is that relative to 1,2-diarachidine.

TABLE I

CAPACITY FACTORS (k'), RELATIVE RETENTION TIMES (RRT) AND HYDROPHOBIC CARBON NUMBERS (HCN) OBTAINED WITH STANDARD MIXTURES OF PHOSPHATIDYLCHOLINE MOLECULAR SPECIES

Molecular species	Degree of unsaturation	k'	RRT[a]	HCN
14:0–14:0	0	5.395	0.220	28.00[b]
16:0–16:0	0	9.581	0.364	32.00[b]
18:0–18:0	0	16.674	0.608	36.00[b]
20:0–20:0	0	27.868	1.000	40.00[b]
16:0–18:1	1	9.450	0.362	31.96[c]
18:0–18:1	1	12.604	0.468	33.96[c]
16:0–18:2	2	7.516	0.295	30.39[c]
18:0–18:2	2	9.901	0.375	32.39[c]
18:1–18:2	3	7.170	0.283	30.02[c]
16:0–20:4	4	6.849	0.270	29.61[c]
18:0–20:4	4	8.844	0.341	31.61[c]

[a] RRT was calculated by dividing the retention time of each species by the retention time of the 20:0–20:0 species (41.8 ± 0.87 min).

[b] Total number of hydrophobic carbon atoms in the fatty acyl side-chains.

[c] Calculated by the relationship HCN = 18.218 log RRT + 39.978, which was obtained by the linear regression of log RRT *vs.* the total number of hydrophobic carbon atoms in the fatty acyl side-chains of the disaturated molecular species.

in Table I. Table I also shows the HCN values for unsaturated molecular species as calculated by the linear regression of HCN *vs.* log RRT reported in the footnote.

The quantitative aspects of this method were studied without assumptions about the yield of either enzymatic hydrolysis or the benzoylation reaction. The objective evaluation of the quantitative results was realized by the addition of two analytical standards during the preparation of the diradylglycerobenzoate derivatives. Specifically, at the beginning of the procedure, to the phospholipid sample was added a known amount of the molecular species distearoylphosphatidylcholine that had the characteristics of an internal standard. This standard was virtually absent in the samples under consideration (below 0.2% of the molecular species distribution in many unspiked samples analysed) and gave a completely resolved chromatographic peak. The distearoylphosphatidylcholine produced an amount of 1,2-distearine related to the yield of enzymatic hydrolysis. However, its peak area was proportional to the amount of phosphatidylcholine converted into diacylglycerobenzoate throughout the procedure. To calculate the yield of the enzymatic hydrolysis, we added, after the hydrolysis a known amount of 1,2-diarachidine, a diacylglycerol species that could not be derived from the sample. Indeed, 1,2-diarachidine was used as an internal standard for calculating the amount of 1,2-distearine formed. This allowed us to verify that the yield of enzymatic hydrolysis, which had been reported to proceed near to completion (yield 98.6%) [25], was actually much lower (88.0 ± 6.7%, as determined in twelve separate experiments). This difference, which was probably explained by changes in the quality of the enzyme or the purity of the substrate, demonstrated the importance in the use of distearoylphosphatidylcholine as internal standard of taking into account the actual degree of hydrolysis. Another observation that emphasized the importance of the internal standard was derived from a comparison of replicate samples. In many instances, even if the amounts of phospholipids calculated with the internal standard (distearoylphosphatidylcholine) were in good agreement, the absolute areas of the peaks were clearly different. A possible explanation was provided by experiments with mixtures of pure molecular species of diacylglycerols. In these experiments, the yield of benzoylation, which had been reported to be quantitative [26], was found to be affected by the presence of moisture, alcohols and salts in the reaction medium (data not shown).

The possibility that differences in the degree of unsaturation of the molecular species affected the determination was ruled out by comparing the calibration graphs (peak area *vs.* amount of molecular species) of three species with different degrees of unsaturation (dipalmitoyl-, palmitoyloleoyl- and palmitoyllinoleoylphosphatidylcholine). These graphs were linear over a wide range of amounts and had similar slopes (data not shown). This was in agreement with the findings of Snyder and Kirkland [27], who reported similar molar absorptivity values for saturated and unsaturated diacylglycerol derivatives. These facts indicated that the peak areas did not need any specific correction for calculating either the distribution or amount of molecular species.

TABLE II

PERCENTAGE DISTRIBUTION OF MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE IN HUMAN BILE AND IN SAMPLES OF RAT BILE, LIVER AND PLASMA SAMPLES

The values reported represent the mean ± S.D. of three separate determinations on each sample.

No.	Molecular species	Human bile	Rat bile	Rat liver	Human plasma
1	16:1–20:4	n.d.[a]	0.7±0.24	n.d.	n.d.
2	16:1–16:1	n.d.	2.8±0.38	n.d.	n.d.
3	14:0–20:4	n.d.	0.7±0.17	n.d.	n.d.
4	16:1–18:2	0.5±0.12	0.7±0.18	n.d.	0.4±0.12
5	14:0–18:2	0.7±0.09	1.8±0.11	1.3±0.10	2.7±0.16
6	16:0–22:6 16:0–18:3	9.7±0.61	3.4±0.21	2.2±0.14	4.6±0.22
7	18:1–20:4	3.2±0.34	1.0±0.22	0.6±0.16	n.d.
8	16:0–20:4	9.9±0.57	10.1±0.79	17.1±1.01	12.7±0.86
9	18:1–18:2	3.8±0.25	8.1±0.51	1.3±0.16	0.5±0.12
10	16:0–18:2	44.1±2.88	46.9±3.23	19.9±1.28	28.1±1.43
11	16:0–20:3	0.5±0.09	0.3±0.10	1.1±0.10	8.4±0.72
12	18:0–20:4	1.4±0.12	4.1±0.18	29.8±1.52	6.3±0.42
13	16:0–18:1	20.6±1.06	11.3±0.77	8.3±0.46	18.9±0.95
14	18:0–18:2	3.7±0.14	4.6±0.21	14.3±0.77	12.1±0.99
15	18:0–20:3	0.3±0.10	0.3±0.10	0.6±0.10	1.8±0.10
16	18:0–18:1	0.8±0.12	1.5±0.20	2.6±0.18	3.7±0.24
17	20:0–18:2	0.6±0.10	1.7±0.12	1.1±0.08	n.d.

[a] n.d. = < 0.2%.

Table II reports the distribution of molecular species obtained for samples of rat bile and liver and human bile and plasma by HPLC determination (run in triplicate). The average relative standard deviation (R.S.D.) was *ca.* 11%. However, if we excluded the contribution given by species which represented less than 1% of the distribution, the R.S. D. had a more acceptable value of 6%, without appreciable differences due to the type of sample.

The molecular species distributions determined

TABLE III

COMPARISON BETWEEN MEAN FATTY ACID DISTRIBUTION DEDUCED FROM MOLECULAR SPECIES COMPOSITION DETERMINED BY HPLC AND THAT FOUND BY GC ANALYSIS OF FATTY ACID METHYL ESTERS

Fatty acid	Rat bile		Human bile		Rat liver		Human plasma	
	HPLC	GC	HPLC	GC	HPLC	GC	HPLC	GC
14:0	1.2	1.1	0.4	0.4	0.6	1.2	1.4	1.0
16:0	36.3	36.9	42.4	44.2	24.3	21.9	36.3	36.3
16:1	3.4	2.9	0.2	1.2	n.d.[a]	0.5	0.2	0.8
18:0	5.3	6.0	3.1	3.6	23.5	22.9	11.9	13.2
18:1	11.0	10.9	14.3	15.8	6.4	7.2	11.5	13.5
18:2	31.9	32.4	26.3	24.7	18.8	17.3	21.9	19.9
18:3	0.8	1.0	2.4	0.5	0.6	0.4	1.1	0.9
20:0	0.8	0.2	0.3	0.3	0.5	0.3	n.d.	0.2
20:3	0.3	0.2	0.4	0.9	0.9	0.7	5.1	4.6
20:4	8.2	7.9	7.3	6.8	23.7	26.6	9.5	8.8
22:6	0.8	0.5	2.4	1.6	0.6	1.0	1.1	1.0

[a] n.d. = < 0.2%.

TABLE IV

COMPARISON BETWEEN QUANTITATIVE RESULTS FOR PHOSPHATIDYLCHOLINE BY HPLC (SUM OF ALL MOLECULAR SPECIES) AND GC OR LIPID PHOSPHORUS DETERMINATIONS

Results are reported as mean ± S.D. of three separate determinations on each sample.

Sample	Units	HPLC	GC	Lipid phosphorus
Human bile	mg/ml	4.0±0.25	3.9±0.22	3.8±0.23
Rat bile	mg/ml	0.4±0.03	0.4±0.02	0.4±0.02
Rat liver	mg/g	9.9±0.87	10.5±0.54	9.5±0.39
Human plasma	mg/ml	0.7±0.04	0.7±0.03	0.7±0.03

by the present HPLC method were validated by comparison between the fatty acyl composition deduced from the HPLC distribution and that found by GC determination of fatty acid methyl esters. The results of this comparison, shown in Table III, indicate that, for the major fatty acids, the difference between the theoretical and experimental proportions is 7.2% of the value found by GC. This value can be considered acceptable as it is only slightly higher than the average R.S.D. found in replicate analyses.

The quantitative results were validated by comparison of the amounts determined by HPLC with either GC or lipid phosphorus determinations. These results, reported in Table IV, did not show significant differences. The quantitative result was also verified by the use of standard mixtures of molecular species, which indicated agreement between the theoretical and found values to within ±5.5%.

The clean-up of the derivative with a diatomaceous earth cartridge also contributed to obtaining these results. This step eliminated any residue of reagents and gave chromatograms with a stable baseline and without spurious peaks.

In conclusion, the use of the analytical standard but also with modifications of both sample preparation and chromatographic separation of benzoate derivatives of diradylglycerobenzoate allowed a reliable determination of both the qualitative and quantitative compositions of molecular species of phosphatidylcholines derived from liver, bile and plasma extracts.

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