Journal of Chromatography, 507 (1990) 339–349
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1699

Separation and determination of molecular species of phosphatidylcholine in biological samples by high-performance liquid chromatography

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SUMMARY

A method for determining the molecular species composition of phosphatidylcholine in biological samples by reversed-phase high-performance liquid chromatography with dual-wavelength ultraviolet detection is described. The optimum compromise between analysis time and chromatographic resolution under isocratic and isothermal conditions (0.8 ml/min and 32°C, respectively) was obtained with the mobile phase methanol–ethanol (6:4, v/v) containing 20 m*M* choline chloride–water–acetonitrile (90:7:3, v/v/v). The problems of quantification at 205 nm, due to large differences in the detector response with the degree of unsaturation, were resolved by using the appropriate calibration factors chosen with the ratio of absorbances at 205 and 215 nm. The proposed procedure gave results in good agreement with fatty acid composition in samples of rat bile, liver, liver mitochondria and microsomes determined by gas–liquid chromatography.

INTRODUCTION

The determination of phosphatidylcholine (PC) molecular species is important in lipid micellar biochemistry and in studies of membrane function. However, both the fractionation and quantification of PC still present problems if complex mixtures of molecular species, such as those found in biological samples, must be analysed.

The separation of PC molecular species is typically achieved by reversed-phase high-performance liquid chromatography (RP-HPLC) of either unmodified PC molecules[1–6] or enzymatic hydrolysis products of PC (*e.g.*, phosphatidic acid[7,8], or diacylglycerol[9–12]). In the last instance the hydrolysis product of PC can be derivatized with a strong UV chromophore, which offers some advantages in terms of resolution and quantification. On the other hand, this approach requires some additional steps in the analytical procedure and may be unsuitable for applications in which PC must be recovered from column eluates or in which PC isotopically labelled in the choline must be analysed[13].

The chromatography of intact PC molecules does not have these limitations but

0021-9673/90/$03.50

UV detection in the 200-nm range creates problems for quantification. At that wavelength, the detector response is a function of the number of double bonds and is not the same for the various PC molecular species[3], which causes serious problems for the analysis of biological samples in which each chromatographic peak may contain different molecular species of PC. In a previous paper[5], we reported a method for biliary PC which used calibration factors for each peak to alleviate this problem. These were determined with a standard bile PC and were based on an exact knowledge of the elution profile of the molecular species in this particular type of sample.

In this paper, we report changes to the mobile-phase composition that give results comparable to our previous findings without the need for high column temperatures or a flow programme.

EXPERIMENTAL

Palmitoyloleoyl-, palmitoyllinoleoyl- and palmitoylarachidonoyl-PC were purchased from Sigma (St. Louis, MO, U.S.A.). Oleoyllinoleoyl-, dilinoleoyl- and linoleoyllinolenyl-PC were isolated by preparative HPLC from purified soy lecithin, as previously described[5]. Analogously, palmitoyldocosahexanoyl-, palmitoyldocosapentanoyl-, stearoylarachidonoyl-, stearoyllinoleoyl- and stearoyloleoyl-PC were isolated from purified egg lecithin.

Lipid extracts from samples of whole liver, liver microsomes, liver mitochondria and the bile of male Wistar rats on a standard laboratory diet were prepared with chloroform–methanol (2:1, v/v), according to Folch *et al.*[14]. PC was isolated from lipid extracts by thin-layer chromatography (TLC) with the solvent system chloroform –methanol–acetic acid–water (65:25:15:4, v/v). The TLC scrapings were dissolved in a small volume of mobile phase. Silica gel particles were subsequently removed by centrifugation and an aliquot of 20–50 μl of the sample was injected into the column.

The HPLC apparatus, manufactured by Gilson Medical Electronics (Middleton, WI, U.S.A.), was equipped with a dual-wavelength detector (settings 205 and 215 nm, 0.1 a.u.f.s.) and a dual-channel integrator. The column used in all the experiments was 5-μm Spherisorb ODS-2, 25 cm x 4.6 mm I.D. (Phase Separations, Queensferry, Clwyd, U.K.) which was kept at 32 ± 0.1°C with a Clar-055 electronic thermostat (Violet, Rome, Italy). The mobile phases were prepared by mixing the ion-pair solution [IPS; methanol–ethanol (6:4, v/v) containing 20 m*M* choline chloride] with acetonitrile and water in various proportions. The best analytical result was obtained with IPS–water–acetonitrile (90:7:3, v/v/v) at a flow-rate of 0.8 ml/min.

The total fatty acid (FA) distribution in biological samples and FA quantification in the isolated molecular species were obtained by gas–liquid chromatography (GLC) as described previously[15].

RESULTS AND DISCUSSION

In a previous paper[5], we described the separation of the major molecular species of biliary PC with a mobile phase containing choline chloride, as described by Patton *et al.*[3]. We obtained a three-fold reduction in the time required for the chromatographic separation by using a particular type of column under unusual condi-

tions of high temperature and programmed flow-rate. In this study, we attempted to obtain similar results by using a common reversed-phase column under normal analytical conditions.

We reduced the analysis time by increasing the strength of the eluent, using a methanol–ethanol mixture instead of pure methanol as the mobile phase. Fig. 1 shows the effect of this replacement in our previous[5] mobile phase [methanol containing 20 m*M* choline chloride–water–acetonitrile (90:8:3, v/v/v)] on the retention times of palmitoyllinoleoyl- and palmitoylarachidonoyl-PC molecular species. The results indicate that retention times of 16–17 min can be obtained with a methanol to ethanol ratio of 1.5. Consequently, an analysis time of *ca.* 30 min can be obtained with biological samples, because the above-mentioned species are eluted in the middle of the major PC natural species.

This modification required some further adjustments in the proportions of acetonitrile and water in order to maintaining both the transparency of the mobile phase and the resolution of some critical pairs. This last aspect was studied using a mixture of five molecular species of PC which form a cluster of peaks in the chromatograms of biological samples. This mixture was chromatographed with different water contents in the mobile phase. Fig. 2 shows the significant effect of the water content on the resolution of palmitoyllinoleoyl- and oleoyllinoleoyl-PC and on the length of the analysis time for three of these conditions. The results of these experiments, summarized in Table I, demonstrate that the loss of resolution between palmitoyllinoleoyl-

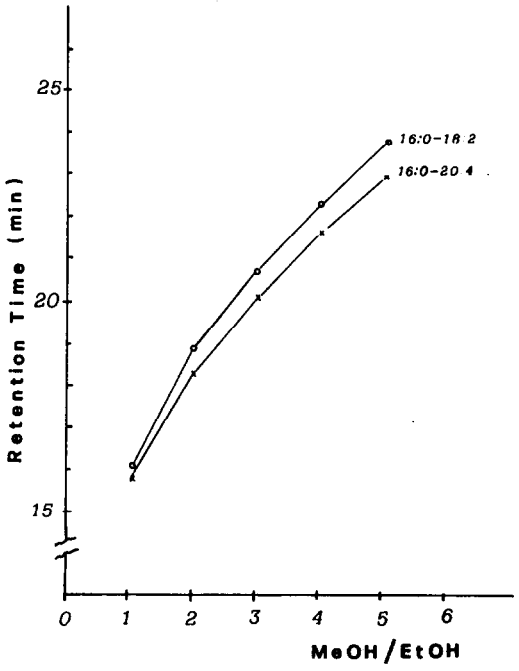

Fig. 1. Changes in retention times of palmitoyllinoleoyl-PC (16:0–18:2) and palmitoylarachidonoyl-PC (16:0–20:4) with increase in methanol to ethanol ratio in the mobile phase. Mobile phases: methanol (MeOH)–ethanol (EtOH) mixtures containing 20 *M* choline chloride–water–acetonitrile (90:8:3, v/v/v).

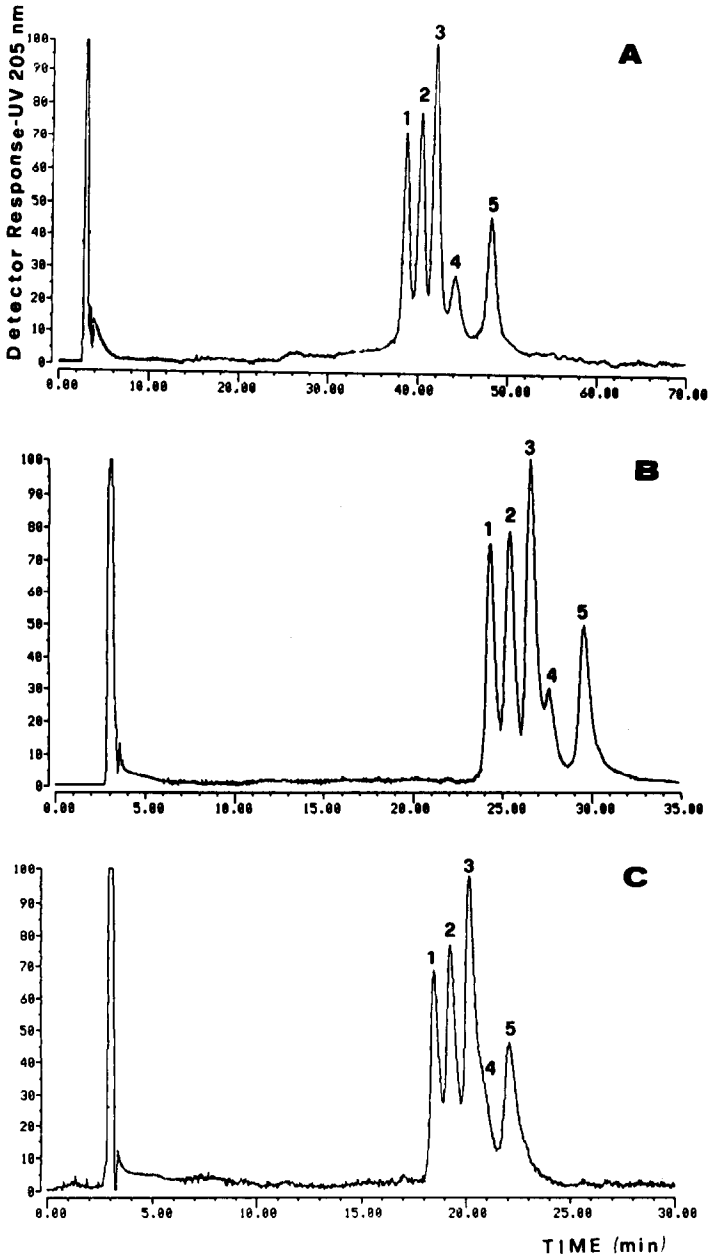

Fig. 2. Chromatography of five molecular species of PC that form a cluster of peaks in biological samples with different water contents in the mobile phase. Mobile phases: methanol–ethanol (6:4, v/v) containing 20 m*M* choline chloride–water–acetonitrile (90:*X*:3, v/v/v); *X* = (A) 9, (B) 7 and (C) 5.6. Peaks (1) 16:0–22:6; (2) 16:0–20:4; (3) 16:0–18:2; (4) 18:1–18:2; (5) 16:0–22:5.

TABLE I

EFFECT OF WATER CONTENT IN THE MOBILE PHASE ON THE NUMBER OF THEORETICAL PLATES (*N*), CAPACITY FACTOR (*k'*) AND MOBILITY OF FIVE MOLECULAR SPECIES OF PC.

Peak numbers and mobile phase compositions (A, B and C) correspond to those in Fig. 2.

Peak No.	*Molecular species*	*Mobile phase A*			*Mobile phase B*			*Mobile phase C*		
		N	*k'*	*RRT*[a]	*N*	*k'*	*RRT*[a]	*N*	*k'*	*RRT*[a]
1	16:0–22:6	3270	15.71	1.00	4500	8.10	1.00	5200	6.02	1.00
2	16:0–20:4	3230	16.48	1.05	4200	8.50	1.04	5150	6.28	1.04
3	16:0–18:2	2500	16.88	1.07	4100	8.93	1.09	5100	6.65	1.10
4	18:1–18:2	2200	18.23	1.15	4000	9.31	1.13	4800	6.90	1.12
5	16:0–22:5	1900	19.94	1.25	3980	10.04	1.21	4500	7.37	1.19

[a] RRT (relative retention time) was calculated by dividing the retention time of each molecular species by the retention time of the 16:0–22:6 species (39.2, 24.4 and 18.8 min with mobile phase A, B and C, respectively).

and oleoyllinoleoyl-PC, observed with a low water content, is attributable to a decrease in selectivity, but not in column efficiency. Consequently, a mobile phase that gave a good compromise between resolution and analysis time was used throughout the study.

TABLE II

VALUES OF ABSORBANCE RATIO (AR) 205/215 nm, RELATIVE CALIBRATION FACTOR (RCF) AND RELATIVE RETENTION TIME (RRT) OBTAINED WITH PHOSPHATIDYLCHOLINE STANDARDS.

Means and standard deviations (S.D.) obtained from five replicate analyses of each molecular species.

Peak No.	*Molecular species*	*Double bonds*	*AR 205/215 nm (mean ± S.D.)*	*RCF*[a] *(mean ± S.D.)*	*RRT*[b] *(mean ± S.D.)*
1	18:2–18:3	5	3.59 ± 0.032	0.09 ± 0.005	0.761 ± 0.001
2	18:2–18:2	4	4.57 ± 0.039	0.13 ± 0.005	0.933 ± 0.002
3	16:0–22:6	6	1.55 ± 0.028	0.07 ± 0.004	1.000
4	16:0–20:4	4	3.48 ± 0.042	0.12 ± 0.007	1.043 ± 0.008
5	16:0–18:2	2	5.70 ± 0.029	0.27 ± 0.002	1.089 ± 0.001
6	18:1–18:2	3	5.19 ± 0.039	0.15 ± 0.004	1.180 ± 0.007
7	16:0–22:5	5	2.32 ± 0.015	0.08 ± 0.004	1.230 ± 0.008
8	16:0–18:1	1	6.22 ± 0.051	1.00	1.328 ± 0.005
9	18:0–20:4	4	3.46 ± 0.034	0.11 ± 0.006	1.478 ± 0.013
10	18:0–18:2	2	5.72 ± 0.028	0.30 ± 0.005	1.554 ± 0.011
11	18:0–18:1	1	6.32 ± 0.039	1.04 ± 0.007	1.811 ± 0.012

[a] RCF was calculated by dividing the calibration factor of each species by the calibration factor of 16:0–18:1-PC.

[b] RRT was calculated by dividing the retention time of each species by the retention time of 16:0–22:6-PC.

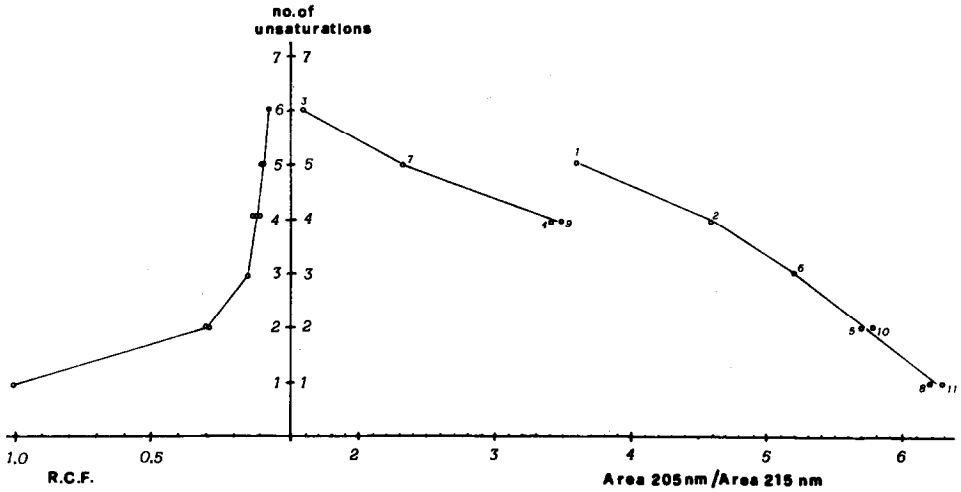

Fig. 3. Values of absorbance ratio (AR), peak-area ratio 205/215 nm and relative calibration factor (RCF) as a function of the degree of unsaturation of PC molecular species. These values were obtained with eleven pure molecular species of PC (see Table I).

TABLE III

MEAN VALUES OF ABSORBANCE RATIO (AR) 205/215 nm, OBTAINED FOR THE 21 PEAKS INTEGRATED IN SAMPLES OF PHOSPHATIDYLCHOLINE FROM RAT BILE, LIVER, LIVER MITOCHONDRIA AND LIVER MICROSOMES.

These values were used to calculate the relative calibration factors (RCF) and the degree of unsaturation by the curve shown in Fig. 3.

Peak No.	*Molecular species*	*AR 205/215 nm (mean ± S.D.)*	*RCF (mean ± S.D.)*	*No. of unsaturations*	
				Theoretical	Found
1	14:0–22:6	1.87 ± 0.87	0.058 ± 0.005	6	5.6
2	18:2–18:3	1.80 ± 0.20	0.058 ± 0.002	5	5.7
3	16:1–18:2	2.03 ± 0.30	0.063 ± 0.006	3	5.4
4	14:0–16:1	3.55 ± 0.69	0.089 ± 0.013	1	3.8
5	16:0–20:5	1.86 ± 0.35	0.058 ± 0.008	5	5.6
6	18:2–18:2	2.98 ± 0.37	0.078 ± 0.003	4	4.3
7	16:1–18:1 16:0–22:6	2.28 ± 0.21	0.065 ± 0.006	–	5.2
8	16:0–20:4	2.78 ± 0.14	0.073 ± 0.006	4	4.6
9	16:0–18:2	4.77 ± 0.39	0.134 ± 0.023	2	3.6
10	18:1–18:2	3.74 ± 0.47	0.091 ± 0.009	3	4.7
11	16:0–22:5	2.32 ± 0.42	0.068 ± 0.010	5	5.0
12	16:0–20:3	2.80 ± 0.61	0.073 ± 0.009	3	4.4
13	16:0–18:1	4.20 ± 0.57	0.098 ± 0.028	1	4.3
14	18:1–18:1	4.41 ± 0.72	0.098 ± 0.024	2	4.0
15	18:0–22:6	1.90 ± 0.13	0.059 ± 0.002	6	5.6
16	18:0–20:4	2.54 ± 0.20	0.073 ± 0.006	4	4.7
17	18:0–18:2	4.94 ± 0.40	0.165 ± 0.084	2	3.3
18	18:0–22:5	1.68 ± 0.12	0.055 ± 0.000	5	5.8
19	18:0–20:3	2.32 ± 0.16	0.068 ± 0.004	3	5.0
20	18:0–18:1	4.65 ± 0.50	0.100 ± 0.052	1	3.7
21	18:0–22:4	1.86 ± 0.13	0.058 ± 0.002	4	5.6

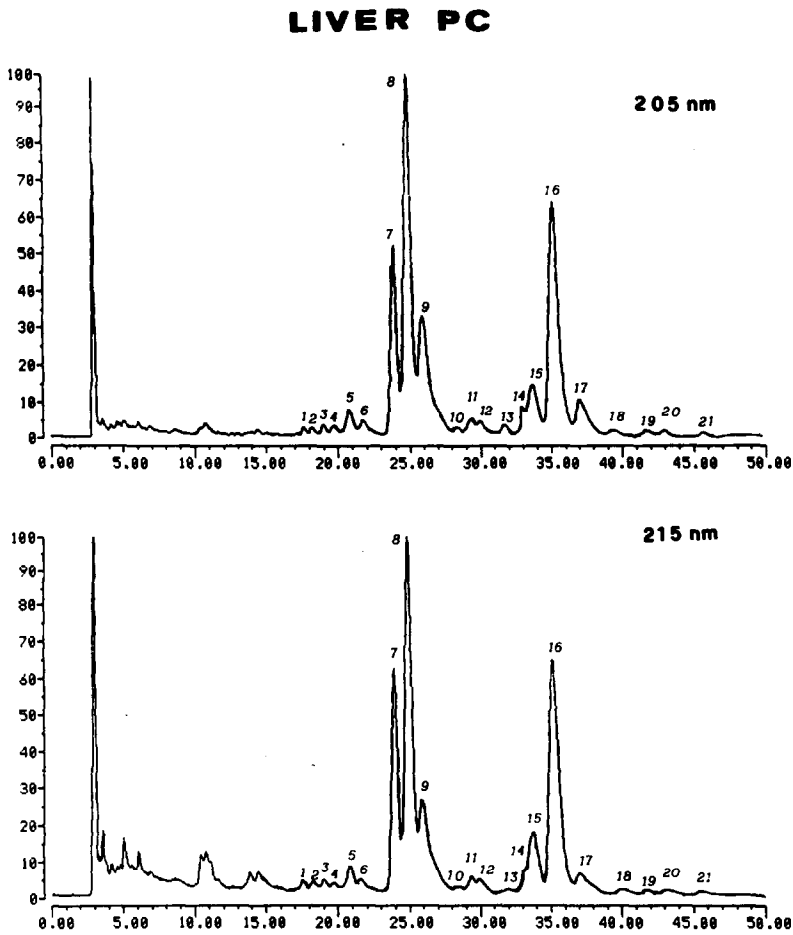

Fig. 4. Chromatograms of liver PC with UV detection at 205 and 215 nm. Peaks as in Table II. For HPLC conditions, see Experimental.

One of the major difficulties in analysing biological samples for intact molecular species of PC is that species with different degrees of unsaturation and, hence, with large difference in absorbance at the analytical wavelength (205 nm) may be not resolved[2]. This led us to investigate whether the absorbance ratio (AR) between the analytical wavelenght and another wavelength could be used for evaluating the average degree of unsaturation of the molecular species eluted in each band. This was made taking into account that UV absorption results primarily from the double bonds in the fatty acid moiety of PC[16] and that species with different degrees of unsaturation have distinct UV spectra[17]. The study was carried out with standard mixtures of eleven molecular species, by evaluating the ARs 205/210, 205/215 and 205/220 nm. The AR 205/215 nm represented a reasonable compromise between unsaturation-discriminating power and sensitivity and was adopted in the subsequent study. The AR 205/220 nm provided more variations with the degree of unsaturation.

However, at 220 nm the less unsaturated species were not integrated because of their lower absorptivity.

These standard mixtures were also used for determining the calibration factors (CF) used for correcting the peak areas at the analytical wavelength on the basis of the average degree of unsaturation of each peak. The CFs were calculated by dividing the molar amount of each molecular species by the area of the relative chromatographic peak.

Table II shows the AR values at 205/215 nm and the CF, expressed as the CF relative (RCF) to palmitoyloleoyl-PC, obtained with standard PC species. These values are plotted in Fig. 3 as a function of the degree of unsaturation of the eleven pure molecular species used for detector calibration. The section of the graph that relates the degree of unsaturation to AR 205/215 nm, a relationship that is fundamental for calculating the RCF, is neither linear nor continuous. In fact, the species containing

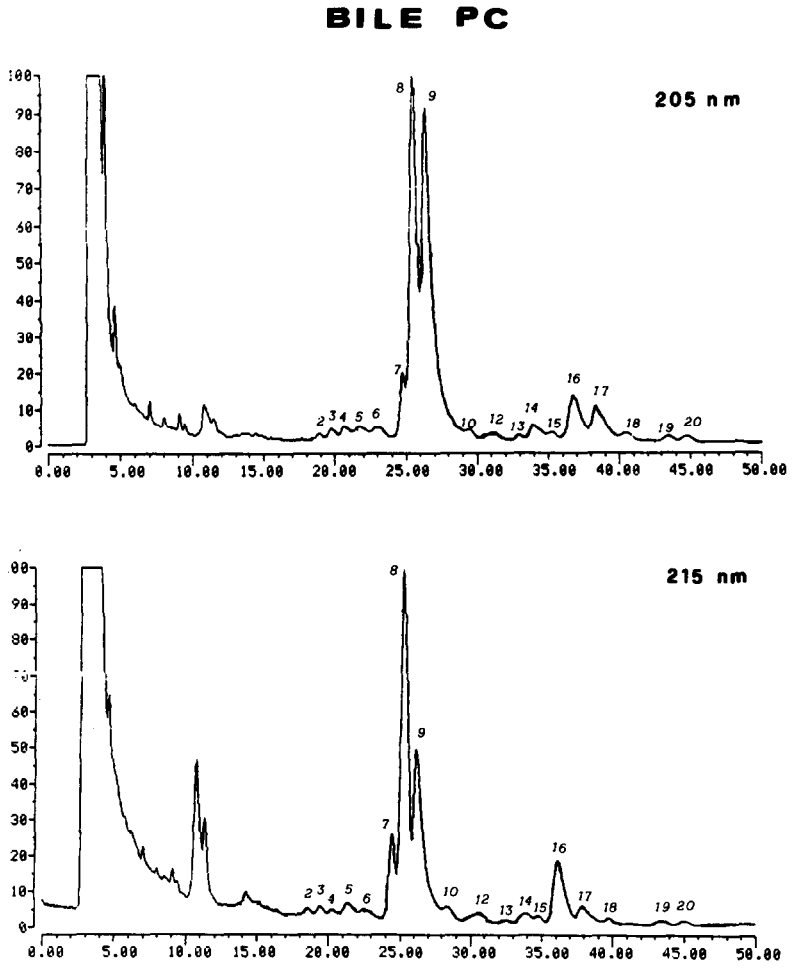

Fig. 5. Chromatograms of bile PC with UV detection at 205 and 215 nm. Peaks as in Table II. For HPLC conditions, see Experimental.

the more polyunsaturated fatty acids appear to lie on a curve separate from that for the species containing fatty acids with less than four double bonds. However, this does not create practical problems, because in biological samples there is an unequivocal correlation between AR and the degree of unsaturation and, hence, between AR and RCF.

The chromatographic pattern of the biological samples analysed in this study had a maximum of 21 peaks, which were identified both by their mobility and the GLC analysis of the species eluted with each peak. Figs. 4 and 5 show the chromatograms of liver and bile PC, obtained at 205 and 215 nm.

Table III reports the mean values of AR, RCF and degree of unsaturation found for the four different types of samples examined. It should be noted that the degree of unsaturation observed is generally higher than the theoretical value expected for the main molecular species in the band. This fact, which is particularly evident with monounsaturated species, indicates that minor polyunsaturated species contaminate other bands. In a case where it is known that a band is composed of two molecular species, *e.g.*, peak 7, it is possible to calculate the distribution of these species from the average degree of unsaturation.

TABLE IV

DISTRIBUTION OF MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE IN SAMPLES OF RAT LIVER, LIVER MICROSOMES, LIVER MITOCHONDRIA AND BILE OBTAINED WITH THE PROPOSED HPLC METHOD.

The values reported represent the means of three replicate analyses of each sample.

Peak No.	*Molecular species*	*Whole liver (%)*	*Liver microsomes (%)*	*Liver mitrochondria (%)*	*Bile (%)*
1	14:0–22:6	0.15	1.05	0.10	—
2	18:2–18:3	0.31	1.72	0.30	0.14
3	16:1–18:2	0.51	0.73	0.39	0.26
4	14:0–16:1	0.50	0.29	0.44	0.48
5	16:0–20:5	1.13	1.26	1.28	0.16
6	18:2–18:2	0.91	2.11	0.34	0.45
7	16:1–18:1	4.25	5.12	7.36	0.59
	16:0–22:6	3.89	3.72	5.98	1.55
8	16:0–20:4	19.61	21.06	24.42	16.59
9	16:0–18:2	25.40	23.13	21.79	57.73
10	18:1–18:2	0.61	0.86	0.36	0.63
11	16:0–22:5	1.91	1.65	1.18	—
12	16:0–20:3	1.16	0.53	1.01	0.39
13	16:0–18:1	0.67	0.57	0.43	0.30
14	18:1–18:1	5.63	1.85	1.27	1.49
15	18:0–22:6	3.68	2.47	3.29	0.30
16	18:0–20:4	17.50	17.57	21.96	3.48
17	18:0–18:2	10.20	12.72	7.18	14.46
18	18:0–22:5	0.13	0.22	0.10	0.12
19	18:0–20:3	1.07	0.96	0.29	0.13
20	18:0–18:1	0.61	0.23	0.49	0.21
21	18:0–22:4	0.16	0.18	0.07	—

TABLE V

COMPARISON OF TOTAL FATTY ACID DISTRIBUTIONS (%) DEDUCED FROM PC MOLECULAR SPECIES COMPOSITION AND GLC ANALYSIS

Fatty acid	*Microsomes*		*Mitochondria*		*Liver*		*Bile*	
	HPLC	*GLC*	*HPLC*	*GLC*	*HPLC*	*GLC*	*HPLC*	*GLC*
14:0	0.33	0.86	0.67	1.40	0.27	0.60	0.24	0.10
16:0	26.89	30.43	25.14	32.29	28.55	36.75	38.36	39.72
16:1	2.63	3.09	3.07	3.97	4.10	3.20	0.66	1.40
18:0	16.68	19.19	17.78	18.45	17.19	17.79	9.35	9.34
18:1	8.70	9.57	6.06	8.03	5.59	6.85	2.35	4.24
18:2	19.43	16.25	21.68	16.22	14.20	14.75	36.99	33.22
18:3	0.15	0.20	0.86	0.50	0.15	0.40	0.07	0.58
20:3	1.11	0.49	0.75	0.77	0.65	0.30	0.26	0.55
20:4	18.54	16.54	19.31	15.54	23.19	16.36	10.04	9.05
20:5	0.56	0.54	0.63	0.51	0.64	0.50	0.08	0.60
22:4	0.10	0.40	0.10	0.60	0.05	—	—	—
22:5	1.02	0.90	0.93	0.30	0.64	0.30	0.06	—
22:6	3.86	2.04	3.62	1.42	3.04	2.20	0.90	1.20

The results of the HPLC analysis of samples of PC, extracted from rat whole liver, liver microsomes, liver mitochondria and bile samples, are given in Table IV.

The total fatty acid compositions, deduced from HPLC analysis with dual-wavelength detection of these samples, are compared in Table V with the corresponding values of fatty acid composition determined by GLC. The agreement is generally good, except that differences greater than 5% may be observed for palmitic and polyunsaturated acids. This may be caused by the presence of disaturated species which are not detected unless present in large amounts and which may cause the underevaluation of palmitic acid and the overevaluation of polyunsaturated acids.

In conclusion, this method be suitable for the analysis of mixtures of highly unsaturated PC species of reasonably well characterized composition. It cannot be used to measure the more saturated species which present the greater analytical problems to UV detection methods in general.

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