Journal of Chromatography, 495 (1989) 61–70
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4927

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SOME POLAR PHOSPHOLIPIDS IN SERUM

JIŘÍ BOSWART

Laboratory for Functional Diagnostics in Childhood and Adolescence, Faculty of Medicine, Charles University, Ke Karlovu 2, 120 00 Prague 2 (Czechoslovakia)

THOMAS SCHMIDT

Department of Chemistry, Karl-Marx University, Talstrasse 35, 7010 Leipzig (G D R)

PAVEL KOSTIUK

Laboratory for Functional Diagnostics in Childhood and Adolescence, Faculty of Medicine, Charles University, Ke Karlovu 2, 120 00 Prague 2 (Czechoslovakia)

and

VĚRA PACÁKOVÁ* and KAREL ŠTULÍK

Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)

(First received April 26th, 1989, revised manuscript received June 22nd, 1989)

SUMMARY

The experimental conditions have been optimized for high-performance liquid chromatographic determination of phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) in serum The phospholipids are separated on a silica gel column, using a mobile phase of acetonitrile–methanol–water (100 10 18, v/v), with ultraviolet photometric detection at 200 nm The limit of detection was 0 2 μg (in 20 μl) for natural phospholipids and 2 5 μg for synthetic phospholipids, the relative standard deviation was ca 5% An alternative detection is tensammetry at a mercury electrode, at a potential of −1 8 V, with an a c current frequency of 60 Hz and an amplitude of 20 mV The tensammetric detection has an advantage in its independence of the structure of the phospholipids In measurements without a column (flow-injection analysis), the tensammetric detection also yields a somewhat lower limit of detection than photometry (0 15 μg per 20 μl), but this value increases more than ten times in chromatographic detection The precision is poorer and is more susceptible to interferences The method was applied to the determination of the above substances in the blood of obese children, as a function of physical stress and spa treatment It was shown that physical exercise causes a decrease in the contents of

0378-4347/89/$03 50

PE and PC in the patients On the other hand, the spa treatment has no pronounced effect on the phospholipid content in the blood

INTRODUCTION

The analysis of serum lipids is of considerable interest in biology and medicine and often represents a formidable task for the analyst [1] It is usually necessary to separate the sample into classes of lipids and only then to resolve and identify the individual components The application of gas chromatography (GC) for this purpose is complicated by the high polarity of lipids and thus thin-layer chromatography (TLC) has been primarily used for routine analyses, however, the latter method suffers from a poorer reproducibility The combination of TLC with GC has been found to give good results [2] Recently, high-performance liquid chromatography (HPLC) has gained importance in this field [3]

Polar stationary phases are required for the HPLC separation of lipids into classes Silica gel has been primarily used (see, e g , refs 4–6), but the application of diol-modified [7] and NH_2-modified [8] phases and chemically bonded ion exchangers [9] has also been described Non-polar, e.g., octadecylsilane, phases require derivatization of the analytes for class separation [10] but can be used for direct separation within a class [11,12]

Direct detection of lipids separated by HPLC is difficult, as they absorb radiation only at low wavelengths (195–215 nm), with low absorption coefficients, this necessitates the use of high-quality photometric detectors and careful selection of high-purity solvents A refractive index detector can be used [6,12], but its sensitivity is poor. Flame ionization detection [11] and infrared absorption detection [13] have limited application Liquid chromatography–mass spectrometry (LC–MS) yields good results [14], but is costly Some classes of lipids can be converted into derivatives that strongly absorb UV radiation [15] or fluoresce [16], but these methods suffer from the well known drawbacks of derivatization procedures

The present paper describes results obtained in connection with a clinical study of metabolism in obese children It was required to monitor the contents of some phospholipids, namely phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, in blood, as a function of physical stress and the diet We have optimized a separation system with silica gel as the stationary phase, using direct UV photometric detection In an effort to develop an alternative method of detection, we tested tensammetric measurement which has not yet been employed in the detection of lipids

EXPERIMENTAL

Apparatus

The Gilson gradient analytical system (Villiers-le-Bel, France) was used for chromatographic measurements in the isocratic mode The samples were injected by a Rheodyne Model 7125 valve with a 20-μl loop A Separon SGX glass column, 150 mm$\times$3.3 mm I.D., packed with silica gel, 5 μm (Tessek, Prague, Czechoslovakia), was employed

The UV detection was carried out at 200 nm. The measurements were performed at laboratory temperature, at mobile phase flow-rates of 0 5 and 0 8 ml min^{-1}

Tensammetric detection on a mercury electrode was studied using an EDLC detector with a static mercury drop electrode (Laboratorní Přístroje, Prague, Czechoslovakia) and an OH-105 a.c polarograph (Radelkis, Budapest, Hungary) The electrode potentials are referred to a saturated Ag/AgCl reference electrode The a.c frequency was 60 Hz and the voltage peak-to-peak amplitude was 20 mV For tensammetric measurements the mobile phase was deaerated by passage of helium

Chemicals

The standard substances of 3-*sn*-phosphatidylcholine (PC), 3-*sn*-phosphatidylethanolamine (PE), 3-*sn*-phosphatidylserine (PS), N-acyl-4-sphingenyl-1-O-phosphocholine (sphingomyelin, SM), 1-palmitoyl-*rac*-glycero-3-phosphocholine (LPC), 1-palmitoyl-*rac*-glycero-2-phosphoethanolamine (LPE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine were obtained from Serva (Heidelberg, F R G.) Acetonitrile (HPLC grade) was supplied by Aldrich (Milwaukee, WI, U.S A) and methanol and chloroform (reagent grade) were supplied by Lachema (Brno, Czechoslovakia) The chloroform was dried and purified by rectification All other substances were reagent-grade products from Lachema

Sample preparation

A procedure common in analyses of lipids [17] was employed for the sample treatment. The serum obtained from the whole blood samples was immediately frozen to $-20°C$. Prior to analysis, 2 ml of the serum were mixed with 20 ml of the fresh extraction mixture, chloroform–methanol (2 1, v/v) and vigorously shaken for 20 min. The mixture was then filtered, the liquid transferred to a separatory funnel and the filter washed with three 4-ml portions of the extraction mixture, the washings were added to the filtrate in the funnel To the filtrate, 7 ml of a 5% aqueous solution of sodium chloride were added and the mixture allowed to stand for 3 h at laboratory temperature To speed up the phase separation, centrifugation can be used The bottom layer was transferred to a flask, another 10 ml of the extraction mixture were added to the

upper layer remaining in the funnel, allowed to stand for 1 h, the bottom layer was added to the contents of the flask and, finally, the solution of the lipids in the flask was evaporated to dryness at 60°C, under a stream of nitrogen

The dry lipid sample thus obtained was dissolved in 0 5 ml of chloroform. The solution was applied to a precoated Merck plate (silica gel $H + HF_{254+366}$, 1 1, w/w, 250 μm) that was preactivated for 30 min at 100°C The plate was developed with *n*-heptane–diethyl ether–acetic acid (85 15 1, v/v) and the phospholipid spot detected by UV fluorescence was mechanically removed and transferred to a column containing 1 ml of Florosil (60–100 mesh)–silica gel (70/325) (Merck, Darmstadt, F R G.) (1 1, w/w). The phospholipids were eluted by three 2-ml portions of chloroform–methanol (1 1, v/v) and the eluate was evaporated to dryness at 60°C under a stream of nitrogen The residue was dissolved in 5 ml of methanol and an aliquot was injected into the HPLC system

RESULTS AND DISCUSSION

Separation

Two mobile phases have been recommended for the separation of phospholipids on silica gel and UV photometric detection, a mixture of hexane–isopropanol–water [4,18] and acetonitrile–methanol–water [5,7,9] As the former mixture led to slow separations, we selected the latter combination of solvents and optimized the composition for the column used The optimum mobile phase was found to be acetonitrile–methanol–water (100 10 18, v/v) An example of the separation of the phospholipid standards under these conditions is given in Fig 1 and the corresponding capacity factors are listed in Table I With increasing content of water the capacity factors generally decrease and the peaks become sharper

The individual classes of phospholipids are adequately resolved, lysophospholipids are always eluted after the corresponding diacylphospholipids Neutral lipids, such as triglycerides, cholesterol and cholesterol ester, are always eluted with the solvent front in strongly tailing peaks If they are present at high concentrations, the peak tails interfere in the determination of phospholipids, this is the case in analyses of blood and thus it is necessary to remove them from the sample (see the TLC procedure in Experimental) The separation of PS from the solvent front can probably be improved by gradient elution, however, as the UV photometric detection requires the use of an extremely low wavelength, gradient elution brings serious problems and thus was not attempted in this work The differences in the resolution of PC and SM in Figs 1 and 2 are probably caused by differences in the fatty acid composition between the standards and the samples We did not determine this composition, as we felt it unimportant for the present analysis

Under the separation conditions used, PC and SM yield two peaks, corre-

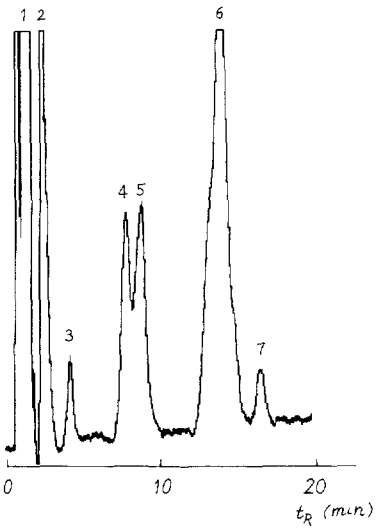

Fig 1 Chromatogram of a mixture of phospholipid standards Peaks 1=PS (4 2 μg), 2=PE (6 1μg), 3=LPE (2 5 μg), 4 and 5=PC (3 1 μg), 6=SM (6 3 μg), 7=LPC (3 0 μg) Stationary phase Separon SGX 5 μm Mobile phase acetonitrile-methanol-water (100 10 18) Flow-rate 0 8 ml min^{-1} UV photometric detection at 200 nm

TABLE I

CAPACITY FACTORS OF PHOSPHOLIPIDS

For the experimental conditions see Fig 1 The values in parentheses correspond to the extra peaks due to separation within the class

Phospholipid	Capacity factor
PS	0 5
PE	2 0
LPE	4 2
PC	8 8 (10 1)
SM	14 0 (16 7)
LPC	19 7

sponding to fractions of different molecular weights (Fig 1, see also the chromatogram of a blood sample in Fig 2) The peaks preceding peak 1 in Fig 2 correspond to the residue of neutral lipids, as the TLC procedures never remove neutral lipids quantitatively

Detection

The sensitivity of the UV photometric detection increases with decreasing wavelength, as the absorption maxima of the analytes are located below 200 nm However, the solvents used do not permit measurement at wavelengths

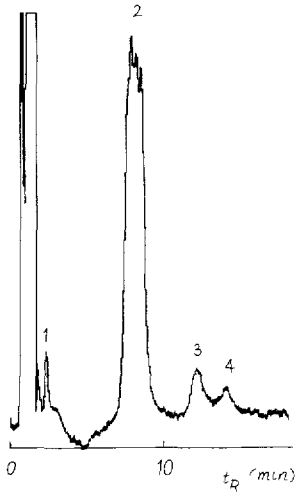

Fig 2 Chromatogram of a mixture of phospholipids in serum Peaks 1 = PE (103 μg/ml), 2 = PC (896 μg/ml), 3 and 4 = SM (144 μg/ml) For the experimental conditions see Fig 1

lower than 200 nm At this value, the limit of detection is ca 0 2 μg of the analyte in the injected volume for natural compounds, while for artificial preparations (LPC) the limit of detection is substantially higher, ca 2 5 μg in the injected volume The reason for this is a substantially smaller number of isolated double bonds in the artificial substances [7]

The fact that the sensitivity of UV photometric detection depends on the degree of unsaturation of the analyte complicates quantitation Nevertheless, the peak area can be correlated with the amount of the analyte We have found that the calibration graphs for the natural substances are linear within a concentration range from 0 2 to 15 μg, with a coefficient of correlation of 0 9967 The relative standard deviation of the peak area for PE and PC is 5% for an amount of 6 μg and sixteen parallel measurements

As an alternative detection method, tensammetry at a mercury electrode was examined The experiments were carried out in the same mobile phase as above, but lithium perchlorate was added to a concentration of 0 05 mol l^{-1}

Batch experiments at a hanging mercury drop electrode in this solution have shown that the potential regions of strong adsorption of the analytes on the electrode surface are from -0 2 to -0 7 V and above -1 5 V; the tensammetric signal in the latter region is much greater (see also refs 19–21) The optimum potential for tensammetric detection under the given conditions is -1 8 V (Fig 3) In measurements without a column, the signal increases linearly with increasing amplitude of the a c signal and with increasing surface area of the mercury drop electrode It is independent of the mobile phase flow-rate at values below ca 0 55 ml min^{-1} and at higher flow-rates it increases linearly

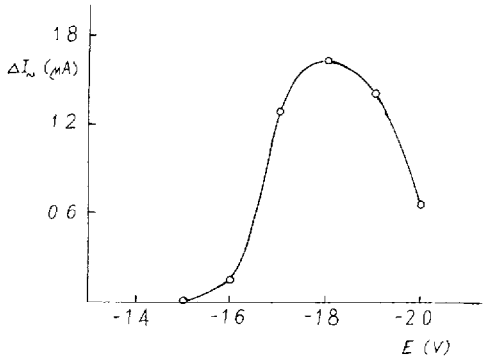

Fig 3 Dependence of the a c current signal on the electrode potential (60 Hz, pulse amplitude 20 mV) for 8 7 μg natural PC, mobile phase flow-rate 0 8 ml min^{-1}

TABLE II

PARAMETERS OF CALIBRATION PLOTS FOR VARIOUS PHOSPHOLIPIDS

Tensammetric detection, -1 8 V, 60 Hz, pulse amplitude 20 mV

Phospholipid	Linear dynamic range (μg)	Slope (μA/μg)	Correlation coefficient
Natural PC	0 4–12 5	0 1951	0 9998
Synthetic PC	0 4–7 5	0 1831	0 9996
PE	0 4–10 0	0 2008	0 9979
SM	0 4–5 0	0 1695	0 9998
	0 4–10 0	0 1721	0 9993

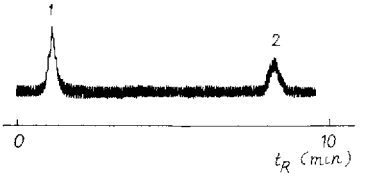

Fig 4 Chromatogram of PS and PC with tensammetric detection For the separation conditions see Fig 1, $LiClO_4$ was added to the mobile phase to a concentration of 0 05 mol l^{-1} Dropping mercury electrode potential -1 8 V (Ag/AgCl), drop time 2 s, a c frequency 60 Hz, amplitude 20 mV Peaks 1 = PS (15 μg), 2 = PC (20 μg)

with the square root of the flow-rate, this is in agreement with the general theoretical predictions and experimental observations of De Jong et al [22]

The parameters of the calibration plots at -1 8 V and a flow-rate of 0 8 ml min^{-1} are summarized in Table II It is interesting that the slopes of these plots are very similar, i e , the detection does not differentiate among the analytes according to the degree of unsaturation, this is an advantage over the UV photometric detection The limit of detection for the analytes is 0 4 μg per

TABLE III

SERUM PHOSPHOLIPID ANALYSIS

Patient No	Phospholipid	Concentration (μmol/l)		
		Before spa treatment		After spa treatment
		At rest	After physical stress	
Boys				
1	PE	17 39	13 9	12 81
	PC	1388 9	1036 3	1492 0
	SM	299 4	332 2	528 0
2	PE	23 91	17 97	13 59
	PC	1811 1	1453 3	1675 9
	SM	706 4	431 5	739 8
3	PE	12 37	13 70	16 80
	PC	1451 2	1288 3	1030 7
	SM	230 2	205 8	240 5
4	PE	17 90	15 20	14 13
	PC	1260 5	1244 1	1305 0
	SM	171 8	150 4	182 4
Mean	PE	17 89	15 19	14 33
S D		3 0	1 97	1 73
R S D (%)		16 8	12 95	12 1
Mean	PC	1477 9	1255 5	1375 9
S D		235 9	171 6	275 5
R S D (%)		16 0	13 7	20 0
Mean	SM	303 7	284 9	372 4
S D		242 0	126 0	259 9
R S D		68 8	45 2	61 5
Girls				
1	PE	14 87	12 50	16 91
	PC	1450 6	1019 7	2424 7
	SM	194 8	397 8	456 1
2	PE	16 51	14 75	10 87
	PC	1378 7	1027 8	1053 3
	SM	536 0	550 5	601 6
3	PE	15 04	14 87	11 61
	PC	1587 0	1380 2	1251 9
	SM	537 1	369 0	307 6
4	PE	11 31	10 92	11 39
	PC	1510 8	1506 0	1616 0
	SM	183 6	164 0	285 0

TABLE III (continued)

Patient No	Phospholipid	Concentration (μmol/l)		
		Before spa treatment		After spa treatment
		At rest	After physical stress	
5	PE	15 5	14 7	12 34
	PC	1913 6	1900 0	1597 5
	SM	184 1	114 4	139 0
6	PE	13 4	11 6	11 5
	PC	1464 2	1250 3	1219 7
	SM	302 9	126 6	143 5
Mean	PE	14 47	13 22	12 44
S D		1 82	1 77	2 24
R S D (%)		12 6	13 4	18 0
Mean	PC	1550 8	1347 3	1527 2
S D		190 6	331 8	492 6
R S D (%)		12 3	24 6	32 2
Mean	SM	323 1	287 1	322 1
S D		171 4	178 4	180 6
R S D (%)		53 0	62 1	56 1

volume injected (20 μl) The relative standard deviation (8 7 μg natural PC, twenty injections, a new mercury drop for each injection) is 5 0%, without drop renewal, the relative standard deviation increases to 7 0% The day-to-day reproducibility is even poorer, ca 9 0% The signal decreases on repeated injections with the same mercury drop and thus a new mercury drop should be used in each measurement

In tensammetric measurements with a silica column, the sensitivity decreases (the limit of detection increases more than ten times), apparently due to mercury electrode passivation by organic compounds washed from the column (Fig 4) It can be concluded than tensammetric detection has an advantage over UV photometric detection in similar sensitivities for all the solutes, but has a somewhat poorer reproducibility and is more sensitive to the experimental conditions.

Application

The above method, involving TLC fractionation and HPLC determination with UV photometric detection, was applied to analyses of blood samples from ten obese patients, six girls and four boys of ages 13–14 years. The effect of physical stress was followed on the contents of PE, PC and SM in the blood Three days prior to the experiments the patients were fed a special diet with a controlled phospholipid content The experiment was carried out on a bicycle

ergometer, up to 90% of the maximum load Blood samples were collected in the morning at rest, after the ergometer experiment and six weeks later, after a reduction diet in a spa for obese patients The results are summarized in Table III.

It is seen that physical stress causes a decrease in the content of PE and PC in the blood of both boys and girls, on average by 15 1% PE and 15 05% PC for boys and 8 6% PE and 13.1% PC for girls. The change in the content of SM is ambiguous, on average there is a decrease (by 20.5% for boys and 11 2% for girls), but in individuals there may be both a decrease and an increase The spa treatment (diet, exercise) causes a decrease in the weight of the patients by ca. 10 kg, but the contents of the serum phospholipids studied are virtually unchanged The PE decreased slightly in the girls, but PC and SM did not change A decrease in PE and PC was found in the boys, but SM increased Hence the spa treatment had virtually no effect on the serum phospholipids contents

ACKNOWLEDGEMENT

The authors are grateful to Dr P Mareš of Lipid Metabolism Laboratory, Faculty of Medicine, Charles University, Prague, Czechoslovakia, for critical discussions.

REFERENCES

1 A Kuksis and J J Myher, J Chromatogr , 379 (1986) 57
2 P Mareš, M Ranný and J Sedláček, J Chromatogr , 275 (1983) 295
3 S L Smith, M Novotný, S A Moore and D L Felten, J Chromatogr , 221 (1980) 19
4 J R Yandrasitz, G Berry and S Segal, J Chromatogr , 225 (1981) 319
5 T L Kaduce, K C Norton and A A Spector, J Lipid Res , 24 (1983) 1398
6 A S Ritchie and M H Jee, J Chromatogr , 329 (1985) 273
7 W Kuhnz, B Zimmerman and H Nau, J Chromatogr , 344 (1985) 309
8 S Shi-Hua Cheng and A -Y Kou, J Chromatogr , 232 (1982) 237
9 R W Gross and B E Sobel, J Chromatogr , 197 (1980) 79
10 M Batley, N H Packer and J W Redmond, J Chromatogr , 198 (1980) 520
11 F C Phillips, W L Erdahl, J A Schmit and O S Privett, Lipids, 19 (1984) 880
12 G Sempore and J Bezard, J Chromatogr , 366 (1986) 261
13 K Payne-Wahl, G F Spencer, R D Plattner and R O Butterfield, J Chromatogr , 209 (1981) 61
14 A Kuksis, L Marai and J J Myher, J Chromatogr , 273 (1983) 43
15 M L Blank, M Robinson, V Fitzgerald and F Snyder, J Chromatogr , 298 (1984) 473
16 J Kruger, H Rabe, G Reichmann and B Rustow, J Chromatogr , 307 (1984) 387
17 P Mareš, personal communication
18 G M Patton, J M Fasulo and S J Robins, J Lipid Res , 23 (1982) 190
19 L Pospíšil, J Kůta, E Muller and H D Dorfler, J Electroanal Chem , 106 (1980) 359
20 E Muller and H D Dorfler, Bioelectrochem Bioenerg , 7 (1980) 459
21 L Pospíšil, E Muller, H Emons and H D Dorfler, J Electroanal Chem , 170 (1984) 319
22 H G de Jong, W Th Kok and P Bos, Anal Chim Acta, 155 (1983) 37