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DENSITOMETRIC QUANTIFICATION OF INDIVIDUAL PHOSPHOLIPIDS

IMPROVEMENT AND EVALUATION OF A METHOD USING MOLYBDENUM BLUE REAGENT FOR DETECTION

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SUMMARY

A densitometric method for simultaneous quantification of individual phospholipids based on visualization with molybdenum blue reagent is presented Previously reported problems concerning colour instability have been solved The method is specific for phosphorus-containing compounds and independent of the degree of fatty acid unsaturation The molar absorptivities of most analysed phospholipids do not differ more than 10% from that for phosphatidylcholine from egg yolk For cases of greater deviation (sphingomyelin, lysophosphatidylcholine and phosphatidylserine), analysis is accomplished by use of appropriate standard mixtures The between-series coefficient of variation is ca 9% The method is compared to a preparative thin-layer chormatographic assay and another commonly used densitometric method

INTRODUCTION

An accurate method for simultaneous analysis of different phospholipids is important in studies of biological material, especially in membrane biochemistry A commonly used method for determination of phospholipid concentrations includes phosphorus analysis of lipids isolated by preparative thin-layer chromatography (TLC) [1- 3] This procedure is time-consuming and insufficient when limited amounts of material are available, e.g in studies on cell cultures Possibilities of more rapid and sensitive methods arise when the lipids are separated on high-performance thin-layer chromatographic (HPTLC) plates, visualized by a detection reagent and quantified by densitometry.

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There are several reports on detection reagents for lipids Most of them utilize the charring reaction, which gives a general reaction with all lipid classes This involves detection of fatty acids, glycolipids and steroids, of which some have R_F values similar to phospholipids, thereby resulting in a complex pattern on the HPTLC plate. The colour development of these methods is dependent on weight and degree of saturation of the lipids. Examples of these reagents are copper acetate–phosphoric acid [4], sulphuric acid [5] and sulphuric acid–dichromate [6] Other reagents such as phosphomolybdic acid in ethanol [7], which involve a heating step, also colour all lipids. Fluorimetric methods for detection of lipids have also been reported, and they are often more sensitive. These reagents are, however, either general for all lipids [8] or react only with polyunsaturated lipids [9] A few comparisons between different reagents have been performed. Sherma and Bennett [10] reported that phosphomolybdic acid and copper acetate reagents were the most suitable reagents for densitometry of phospholipids However, neither of these reagents detected dipalmitoylphosphatidylcholine (PCdp) Spillman et al [11] examined the effect of phospholipid saturation on detection using various reagents In contrast to the previously mentioned study, they reported phosphomolybdic acid to be independent of fatty acid saturation, possibly because the reaction was performed at a higher temperature Among seven reagents, they also showed that 8-anilino-1-naphthalene sulphonate (ANS) and bromothymol blue are insensitive to varying degrees of saturation However, these reagents also stain all lipids indiscriminately.

The fatty acid composition of biological phospholipids varies to a great extent between subclasses and different organs [2, 12, 13] and variations can also be induced under certain pathological conditions [14, 15] To avoid false differences in phospholipid concentrations owing to fatty acid variations, it is important to have access to a method for quantification of phospholipids that is independent of the number of double bonds It would therefore be advantageous to use a reagent that reacts with the phosphate group in the lipid and does not require a heating step, thereby being specific for phospholipids. Ammonium molybdate–perchloric acid reagent [7] is specific for phosphate but is rather insensitive and reacts slowly at room temperature [16] However, the molybdenum blue reagent reported by Dittmer and Lester [16] has a higher reaction rate without heating. This reagent produced a coloured background on the TLC plates A modification of this procedure leading to a more uniform wetting of the plates and a reduction of background colour has been described [17] However, these authors reported that the reagent was not stable enough on silica gel plates and, therefore, was unsuitable for densitometry

The aim of this study was to develop a standardized procedure for detection, using molybdenum blue reagent, suitable for reflectance densitometry The final method was evaluated with respect to the molar absorptivity of different phospholipid subclasses and phospholipids with different fatty acid compositions. Accuracy and precision of the method were determined on biological material comprising rat brain extracts. Furthermore, comparisons were performed using another common spray reagent and a preparative TLC method with phosphate assay

EXPERIMENTAL

Chemicals

The phospholipid standards used were purchased from Sigma (St. Louis, MO, U.S A) dipalmitoylphosphatidylcholine (PCdp, P-6769), dilinoleoylphosphatidylcholine (PCdl, P-7649), phosphatidylcholine from egg yolk (PCegg, P-5388), palmitoyllysophosphatidylcholine (L-5254), dipalmitoylphosphatidylglycerol (P-2892), cardiolipin from bovine heart (CL, C-5646), phosphatidylinositol from soybean (PI, P-0639), dipalmitoylphosphatidic acid (P-0902), sphingomyelin from bovine brain (SM, S-7004) and bovine phosphatidylethanolamine (PE, P-9137). Bovine phosphatidylserine (PS; 6589) was obtained from P -L Biochemicals (Milwaukee, MI, U S A)

All solvents were of analytical grade TLC plates (silica gel 60) and HPTLC plates (silica gel 60) were obtained from Merck (Darmstadt, F.R G). Sephadex G-25 and DEAE-Sepharose were obtained from Pharmacia (Uppsala, Sweden)

Biological samples

The biological samples analysed consisted of lipid extracts from rat brain. The tissue was homogenized in water and the lipids were extracted in the solvent system chloroform–methanol–water (4 8 3) [18]. The extract was purified from low-molecular-weight compounds by gel filtration chromatography on a Sephadex G-25 column [19] The lipids were separated into a neutral and an acidic fraction by ion-exchange chromatography on a DEAE-Sepharose column [20]. The lipids were solved in chloroform—methanol—water (60 30:4.5) when applied to the Sepharose column The neutral fraction was eluted with the same solvent and the acidic fraction was eluted with 0.05 *M* potassium acetate in methanol The acidic extract was purified from the salt on a Sephadex G-25 column [19].

Biochemical analysis

The concentrations of phospholipids in stock solutions of standards and in biological extracts were determined by a modification of the method described by Bartlett [21]

In addition to the molybdenum blue method described below, individual phospholipids were quantified with a preparative TLC procedure in the following way. The lipid extracts were applied to thin-layer plates by a constriction pipette. The neutral phospholipids were separated in the solvent system chloroform–methanol—water (65 25 4) and the acidic lipids were separated in chloroform–acetone–methanol–acetic acid–water (50 20.15 10.5) The lipids were detected with iodine vapour and the spots were scraped off when the colour had disappeared. The amount of phosphorus in each spot was assayed [21]

The molybdenum blue method was also compared to another densitometric method. The lipids were separated on HPTLC plates as described below The plates were sprayed with a 3% copper acetate solution in 8% aqueous phosphoric acid [4] and heated for 20 min at 140°C. The plates were scanned at 450 nm within 1 h when cooled to room temperature

Preparation of the molybdenum blue reagent

The reagent was prepared as described by Ryu and MacCoss [17] as follows A 24-g amount of molybdenum(VI) oxide (MoO_3) was added to 600 ml of 12 5 *M* sulphuric acid in a round flask The mixture was boiled carefully until the molybdenum oxide was dissolved. The solution (mixture I) was cooled to room temperature Powdered molybdenum (1.2 g) was added to 300 ml of mixture I. The mixture was boiled with a reflux condenser for 1 h (mixture II). The solution was cooled to room temperature and decanted from insoluble material Equal volumes of mixture I and II were mixed and filtrated. The filtrate was diluted with two volumes of water Acetic acid was added to make a final reagent/acetic acid ratio of 4 1 (v/v) The reagent was left for about one week before use The reagent was stable for at least six months

Sample application and chromatographic separation

Lipid solution (5 or 10 µl) was applied to the HPTLC plate in a 5-mm band with a constriction pipette The chromatography was run in a chromatography tank lined with filter paper The neutral phospholipids, PE and phosphatidylcholine (PC), were separated in the solvent system chloroform—methanol—water (65 25 4) The acidic lipids PI, PS, phosphatidylglycerol (PG), CL and phosphatidic acid (PA) were separated in chloroform- acetone—methanol—acetic acid—water (50 15 10 10 5). Separation of lysophosphatidylcholine (LPC), SM and PC was performed in the solvent chloroform—methanol—water (60 35 8)

Procedure for detection of the phospholipids using molybdenum blue reagent

After evaluation of varying aspects of the method as described in results, the final procedure was settled as follows The HPTLC plate was dipped in the molybdenum blue reagent for 5 s and then placed in a horizontal position for 5 min The plate was dipped for 25 s in distilled water and for 10 min in ethanol It was dried in a stream of cold air with a hairdryer until the plate was cloudy. To reduce the background noise of the densitometric measurement, it is important to dry the plate evenly It is also important to note that the background is coloured if the plate is getting too dry. The HPTLC plate was immediately covered with a clean glass plate and the edges were taped The plates were scanned within 15 min.

Densitometric measurements on HPTLC plates

A CAMAG TLC scanner II was used for the analysis The measurements were performed in the reflectance mode at 700 nm The slit width was 0 4 mm and the slit length 10 mm The scanning rate was 0.5 mm/s The resulting signal was evaluated by a Spectra Physics 4270 integrating unit

Statistical methods

The within-series variation was calculated from the standard deviation and was described as the coefficient of variation (C V., %). The C.V. for between-series variation was calculated from $s = \sqrt{\Sigma (X_{i1} - X_{i2})^2 / 2n}$, where $X_{i1} - X_{i2}$ is the difference between double samples and n is the number of double samples

RESULTS

Colour reaction of phospholipids

Application of the reagent to the plates was performed by dipping the plates for 5 s in reagent instead of staining in order to get an even spread over the plate The optimal reaction time was determined as 5 min At this time, the colour of the spots had reached a maximal intensity which, after a few minutes, started to decrease Then the plates were dipped in distilled water for 25 s, which was found to be the time needed for excess of reagent to disappear without loss of lipid colour Since some of the phospholipids were found to fade in the presence of water, the plates were then dipped for 10 s in ethanol and dried in a stream of cold air. It was of great importance that the plates were evenly dried in a reproducible way (see Experimental) The plate was covered with a clean glass plate immediately after drying, since this was found to prevent colouration of the background and fading of the spots Phospholipids were coloured blue with the reagent but other lipids, e.g. cerebroside, sulphoglycosylsphingolipid, cholesterol and free fatty acids, could not be detected (Fig 1).

The absorbance spectra were recorded and found to be the same for all phospholipids analysed The absorbance maxima were situated between 700 and 725 nm (Fig. 2) All measurements in this study were performed at 700 nm.

The stability of the colour was examined by scanning one plate with different lipids at different times after reaction (Fig. 3). PC, PE, CL and LPC

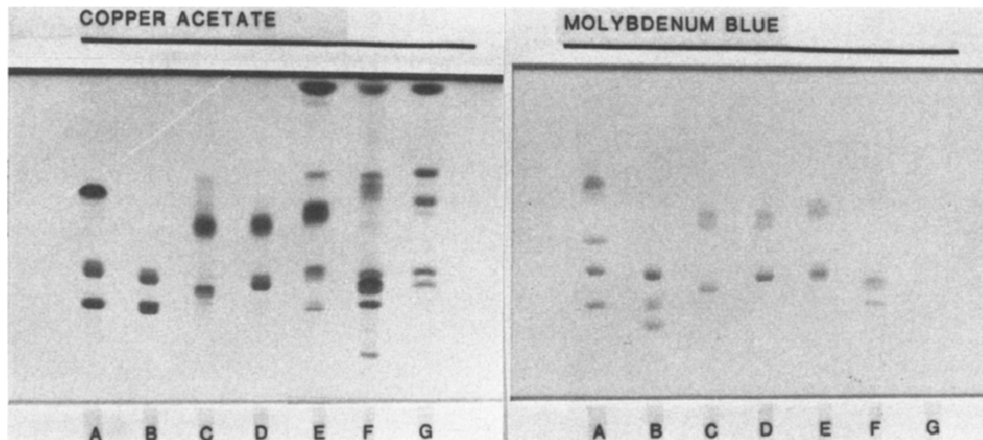

Fig 1 Comparison between detection with molybdenum blue and copper acetate reagents The lipid mixtures contained (with increasing R_F value) (A) PI, PC, PG, CL and PA, (B) LPC, SM and PC, (C) PS and PE, (D) PC and PE, (E) neutral fraction of rat brain extract, (F) acidic fraction of rat brain extract, (G) sulphoglycosylsphingolipids (two bands for hydroxy and non-hydroxy form), cerebrosides (two bands for hydroxy- and non-hydroxy form) and cholesterol The separation was performed on HPTLC plates in the solvent system chloroform—acetone—methanol—acetic acid—water (50 15 10 10 5) All phospholipids were visualized by the molybdenum blue reagent, while, for example, cerebroside and cholesterol in E and G and sulphoglycosylsphingolipid in F and G could not be detected On the other hand, the copper acetate reagent colours all lipids except those with only saturated fatty acids, e g dipalmitoyl-PG and dipalmitoyl-PA in A and palmitoyl-LPC in B

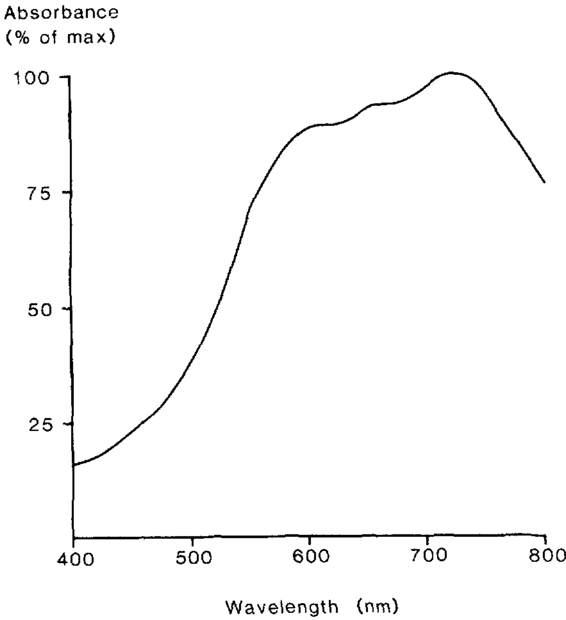

Fig 2 Absorbance spectrum of phospholipids recorded by densitometry after separation on a HPTLC plate and detection with molybdenum blue reagent

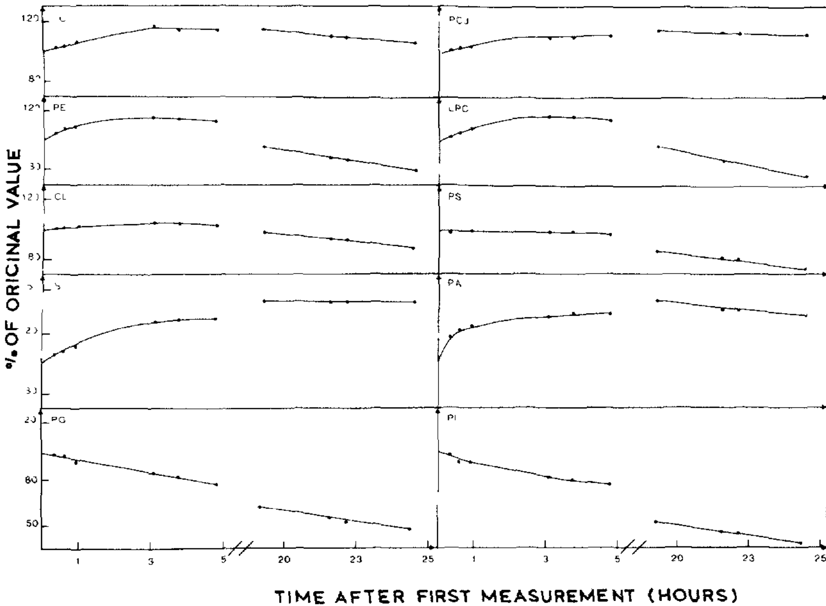

Fig 3 Colour intensity of phospholipid spots at different times after staining with molybdenum blue reagent The intensity is presented as a percentage of the original value, which is the absorbance measured 15 min after drying the HPTLC plate

increased slightly in intensity during the first 3 h. SM and PA increased by ca. 30% during this time, while PS was constant and PG and PI decreased slightly. Even after 20 h, several of the lipids had an absorbance differing by < 20%

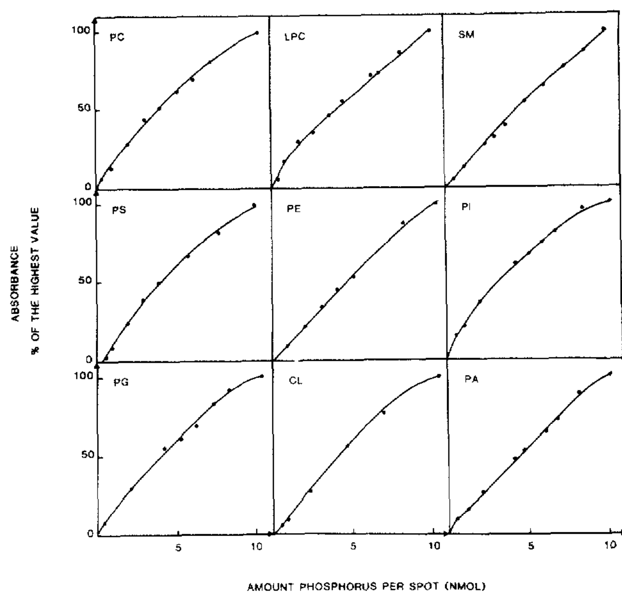

Fig 4 Standard curves of different phospholipids measured by densitometry after molybdenum blue staining

from the original value All measurements in this study were performed at the original time, which was 15 min after drying and covering the HPTLC plate with the glass plate

Standard curves of different phospholipids are presented in Fig. 4. According to these curves, a suitable amount of applied phosphorus for analysis is between 1 and 10 nmol.

Influence of different fatty acid compositions

Analysis was performed on phosphatidylcholines with different degrees of saturation in their fatty acids. Amounts ranging from 1 to 10 nmol of PC from egg yolk, PC with two palmitic acids (PCdp) and PC with two linoleic acids (PCdl) were analysed. PCdl and PCdp were found to have the same absorbance/nmol, while PC from egg yolk had a 10% higher molar absorptivity

Comparison of different phospholipids

Mixtures of different phospholipids were applied on HPTLC plates in different amounts and analysed The absorbance value for PCegg was set to 100% and was thus used as a reference point. The value of each lipid at one amount of application was related to the value for PC at the same amount Fig. 5 presents the relative absorptivity of PI, PG, PA and CL CL and PA absorbed slightly higher and PI and PG slightly lower than PC. However, the values did not differ by more than 10% from PC at amounts ranging from 3 to 10 nmol Below 3 nmol, the values deviated largely and in this range there were also greater standard deviations Larger divergences from PC were obtained with SM, LPC and PS (Figs. 6 and 7) PE absorbed almost like PC (Fig. 7) but it is important to notice that the curves for this lipid, together with SM and LPC, were not parallel to that for PC

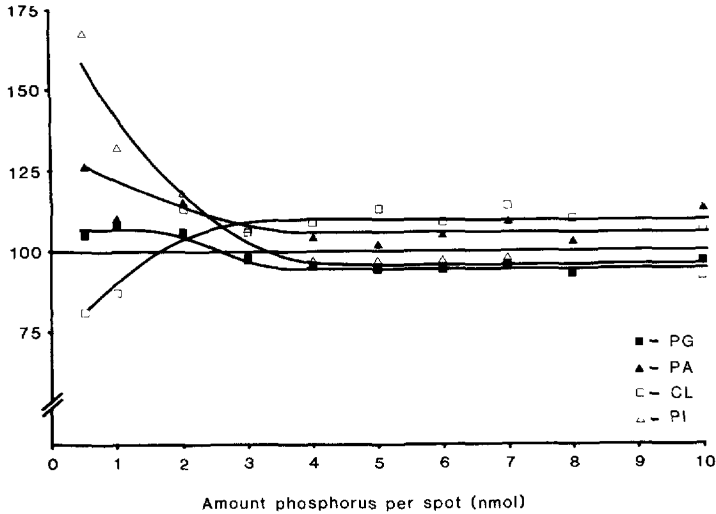

Fig 5 Comparison of molar absorbances of PC, PI, CL, PA and PG A dilution series of mixtures with equal amounts of the lipids was separated on HPTLC plates, coloured by molybdenum blue reagent and analysed by densitometry Each absorbance value was related to the value for PC in the same sample The absorbance for PC was set to 100%

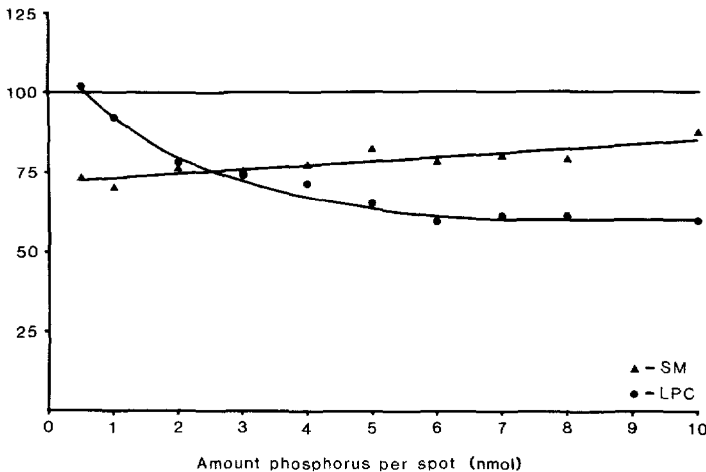

Fig 6 Comparison of molar absorbances of PC, SM and LPC The absorbance values of SM and LPC were related to PC as in Fig 5

Precision of the method and comparison with the preparative TLC method

Densitometric analysis was performed on lipid extracts from rat brain PC and PE were quantified in the neutral fraction with PCegg as standard lipid, and PI and PS were determined in the acidic fraction with PCdp as standard. Extract containing 11 nmol total lipid phosphorus from the neutral fraction and 17 nmol from the acidic fraction was applied per single analysis

The same lipids were also quantified by preparative TLC For this analysis,

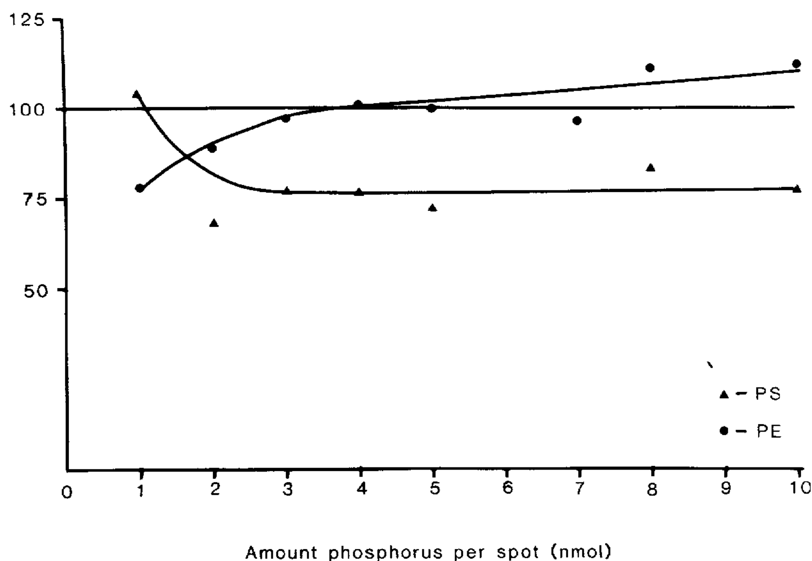

Fig 7 Comparison of molar absorbances of PC, PE and PS The absorbance values of PE and PS were related to PC as in Fig 5

TABLE I

COMPARISON OF RESULTS AND PRECISION FROM ANALYSIS OF RAT BRAIN EXTRACTS PERFORMED WITH THE MOLYBDENUM BLUE DENSITOMETRIC METHOD AND THE PREPARATIVE TLC METHOD

Phospholipid	Molybdenum blue densitometry			Preparative TLC		
	Concentration (nmol per 100 µl extract)	Coefficient of variation (n = 10) (%)		Concentration (nmol per 100 µl extract)	Coefficient of variation (n = 10) (%)	
		Within-series	Between-series		Within-series	Between-series
PC	14 5	2 9	9 5	11 9	3 5	2 7
PE	12 9	3 7	8 8	11 2	3 1	2 9
PC/PE	1 12	2 4	3 5	1 07	3 1	2 3
PI	9 9	5 7	7 9	9 9	10 1	7 8
PS	12 0	4 3	9 0	12 6	9 7	10 3
PS/PI	1 22	3 0	10 9	1 29	13 9	15 6

five to ten times the amount used in the densitometric method was required The results are presented in Table I The within-series variations for all the lipids analysed by the densitometric method were between 2 9 and 5 7% The preparative TLC method had a similar within-series variation for PC but a larger C V. for PI and PS The between-series variation for densitometric determinations was ca 9% With the preparative TLC method, the between-series variations for PC and PE were lower but for PI and PS they were similar to those for densitometry.

The concentration values in the lipid extract obtained for PC and PE were higher with the densitometric method than with preparative TLC Calculation of the PC/PE ratio, however, gave similar values for both methods.

The concentration values obtained for PI and PS were almost the same with the two methods

Comparison with the copper acetate reagent method

The visualization of lipids with copper acetate reagent is dependent on the degree of saturation of the fatty acids in the phospholipids This is illustrated on the HPTLC plate in Fig 1. Dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidic acid of lipid mixture A and palmitoyllysophosphatidylcholine of mixture B could not be detected with this reagent, compared to the molybdenum blue reagent.

The neutral fraction of rat brain extract was analysed by densitometry after staining with copper acetate The PC/PE ratio was determined as 0.36 with this reagent, compared to 1.12 and 1 07 with molybdenum blue densitometry and preparative TLC, respectively (Table I)

DISCUSSION

For accurate quantifications of phospholipids from biological sources, it is important to have a method that is independent of factors such as length and degree of saturation of the fatty acids or polar head groups. This problem has been discussed by several authors [11, 22, 23]. An assay involving detection with a phosphate-specific reagent would overcome these difficulties. The possibility of analysing phospholipids on a molar basis is also preferable, since the differences in molecular weight due to fatty acid variations are difficult to calculate Furthermore a phosphate-specific reagent makes it possible to use densitometry after one-dimensional HPTLC separation of complex lipid mixtures, without interference from lipids other than phospholipids

This report presents a densitometric method that involves detection with the phosphate-specific molybdenum blue reagent according to principles previously reported by others [16, 17]. The introduced technique has made it possible to put these theories into practice and particularly the problems with coloured background on the HPTLC plates and instability in colour intensity have been solved

When densitometric analysis of phospholipids is performed with the molybdenum blue reagent, it is important to use a standardized procedure as described in this report, e.g. the time for the colour reaction to proceed and then the time until the plates are scanned The suitable range for applied amount of each phospholipid to be analysed is between 3 and 10 nmol. The standard curves allow measurements down to at least 1 nmol, but below 3 nmol the absorbances per nmol of the different phospholipids deviate from each other, thereby requiring one standard for each lipid

Most of the analysed phospholipids had a similar densitometric detector response per nmol with the presented method. No significant differences were observed among phosphatidylcholines with totally different fatty acid composition. Sphingomyelin, lysophosphatidylcholine and phosphatidylserine had a lower absorbance than the other phospholipids It is important to choose an appropriate standard to meet the different purposes of the experiments The simplest way is to use one lipid standard for determinations of several phospho-

lipids, simultaneously However, accurate measurement of phospholipids with different detector responses requires the use of a mixture of different phospholipids as standard

In comparison with the commonly used method including phosphate determination after preparative TLC, this molybdenum blue densitometric assay offers several advantages. It is more convenient, since it is considerably more rapid and simple It is also more suitable when small sample sizes are available, since the method requires five to ten times less material However, in some cases, when unlimited amounts of material are available, the preparative TLC method may be preferable due to its higher accuracy

Of the previously used densitometric assays, the copper acetate and phosphomolybdic acid methods are reported to be the most suitable [10, 11, 24] However, these reagents stain all lipid classes indiscriminately and the colour intensity is dependent on fatty acid saturation [11, 25] For example, phospholipids with two palmitic acids are not detectable with the reagents. This is also pointed out in this study. The value for the PC/PE ratio measured by the copper acetate method in rat brain lipid extracts deviated to a great extent from the values obtained by preparative TLC and molybdenum blue densitometry (0.36 compared to 1 07 and 1 12) This discrepancy may be explained by the higher degree of unsaturation of fatty acids in PE than in PC in biological tissues, thereby causing a more pronounced colour development per nmol of PE

In conclusion, this is a rapid and simple method for the quantification of individual phospholipids This densitometric assay is practicable in studies of biological phospholipids, since it is based on a specific reaction of phosphorus-containing compounds and requires small amounts of material

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