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SOLID-PHASE EXTRACTION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE FOR ISOLATION AND ESTIMATION OF PLATELET ACTIVATING FACTOR IN PLASMA

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SUMMARY

A solid-phase extraction technique for the isolation of platelet activating factor [1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC)] from biological matrices was developed. Amberlite XAD-2 was effective in retaining different molecular species of AGEPC from plasma and incubation media. The recovery of the three molcular species (C_{14}-, C_{16}-, and C_{18}-AGEPC) was greater than 95%. XAD-2 also removed large amounts of plasma impurities, giving a cleaner high-performance liquid chromatographic (HPLC) profile. AGEPC in plasma or incubation media was not significantly removed by passage of the sample through a column packed with ODS-silica. A reversed-phase HPLC technique for separation and estimation of different species of AGEPC was developed. Resolution of C_{14}-, C_{16}- and C_{18}-AGEPC was accomplished on a Hamilton PRP-1 resin column using an aqueous acetonitrile gradient containing 1 m*M* methanesulphonic acid. The detection limit was of the order of 50 ng of AGEPC at 210 nm. The AGEPC purified by the technique described retained its biological activity as determined by its ability to release endogenous serotonin from rabbit platelets.

INTRODUCTION

Currently, the procedure used for the extraction of platelet activating factor (PAF) [1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC)] from biological matrices is liquid—liquid extraction [1]. No quantitative results for removal of AGEPC using liquid—liquid extraction are available, the method is time-consuming and the formation of emulsion may result in poor recovery.

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Liquid—solid extractions using solid adsorbents, such as silica, octadecylsilane silica or amberlite resins, have been widely employed to extract lipids, steroids and various other drugs from biological matrices [2—6].

The separation of AGEPC from other phospholipids and its subsequent extraction utilizes either thin-layer chromatography (TLC) or normal-phase high-performance liquid chromatography (HPLC) [7, 8]. However, neither of these chromatographic techniques has proven to be effective in separating the different specis of AGEPC (C_{14}-, C_{16}- and C_{18}-AGEPC). Recently, it has been shown that reversed-phase HPLC using ODS-silica as stationary phase and methanol—acetonitrile—water containing choline chloride as mobile phase is effective for the separation of AGEPC species [9]. Choline chloride has an appreciable absorbance at 210 nm and therefore limits the sensitivity of the procedure. In the technique reported here the separation was accomplished using a more hydrophobic HPLC column and a solvent system of very low UV absorption, which permitted greater sensitivity for AGEPC at 210 nm.

EXPERIMENTAL

HPLC-grade organic solvents were purchased from Aldrich (Milwaukee, WI, U.S.A.) and used as received. [^{3}H]AGEPC (30—60 Ci/mmol; C_{14}, C_{16} and C_{18} species) was purchased from New England Nuclear (La Chine, Canada). [^{3}H]AGEPC (115 Ci/mmol; C_{16} and C_{18} species) was purchased from Amersham International (Oakville, Canada). 1-O-Hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine, 1-O-octadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine and 5-hydroxytryptamine (5-HT; serotonin) were purchased from Sigma (St. Louis, MO, U.S.A.). PAF was obtained from Calbiochem Behring (San Diego, CA, U.S.A.). Octadecylsilane (40 μm) disposable extraction columns (1 g) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Amberlite XAD-2 resin (50—100 μm) was obtained from Serva Feinbiochemica (Canadian supplier: Terochem Labs., Edmonton, Canada) and 1 g (dry weight) was packed into a 10-ml glass syringe. Hamilton PRP-1 resin column (150 mm × 4.1 mm; 10 μm) was purchased from Mandel Sceintific (Rockwood, Canada). Hibar silica column (125 mm × 4 mm; 5 μm) was purchased from E. Merck (Canadian supplier: BDH Chemicals, Toronto, Canada).

Extraction procedure

Fresh human plasma (1 ml) (heparin anticoagulant) was cooled to 4°C and spiked with either a mixture of purified C_{14}-, C_{16}- and C_{18}-AGEPC ($4 \cdot 10^5$ dpm; mass of C_{16}-AGEPC = 50 ng) or with purified AGEPC compounds in 5 μl of ethanol. The samples were diluted with 2 vols. of Tyrod's buffer (phosphate-buffered saline, PBS) and the mixture was acidified to pH 5 with 1 *M* hydrochloric acid. The acidified solution was passed through C_{18} or XAD-2 columns prewashed sequentially with 20 ml of acetonitrile, 10 ml of methanol and 40 ml of water. The columns were allowed to run at a flow-rate of ca. 0.5 ml/min. Plasma eluates were collected for determination of unretained AGEPC. The columns were washed with 3 × 5 ml of water and each individual 5-ml water eluate was collected. The AGEPC was eluted from the column with acetonitrile or with 15 ml of different solvents (see Table II) at a flow-rate of ca. 1.5

ml/min. A 200-μl volume of each fraction was counted in a β-liquid scintillation counter to determine the recovery.

Thin-layer chromatography

Silica gel plate was either prepared as reported [10] or purchased from E. Merck (Canadian supplier: Mandel Scientific). AGEPC was applied to the plates in chloroform—methanol and developed with chloroform—methanol—water (65:35:4) as described [11, 12]. After drying, 0.5-cm bands were scraped off. The compounds were eluted with chloroform—methanol—water (1:2:0.8) for 30 min at room temperature and centrifuged at 250 *g* for 15 min. A fraction of the supernatant was counted to determine the radioactivity.

Normal-phase high-performance liquid chromatography

Chromatography was performed using a Shimadzu Model LC-6A system fitted with a silica column (Hibar) wich was connected to a Shimadzu Model SPD-6AV UV spectrophotometer (210 nm) and a Gilson Model FC-80K fraction collector. AGEPC was dissolved in 20 μl of 2-propanol—hexane (1:1), injected onto the column and analyzed isocratically at 25°C with 2-propanol—hexane (1:1)—water (94:6) at a flow-rate of 1 ml/min. Fractions (1 ml) were collected and 200 μl of each were counted in a β-liquid scintillation counter. The radioactivity per fraction was plotted manually.

Reversed-phase high-performance liquid chromatography

Chromatography was performed using a Shimadzu Model LC-6A dual-pump system fitted with a Hamilton PRP-1 resin column, which was connected to a Model SPD-6AV variable UV—visible detector (210 nm) and a Gilson fraction collector. AGEPC (20 μl) in acetonitrile was injected with a Rheodyne injector fitted with a 50-μl sample loop. Analysis was performed using a linear gradient from 40 to 70% acetonitrile in water over 30 min at flow-rate of 1 ml/min at 30°C. The mobile phase also contained 1 m*M* methanesulphonic acid. Fractions of 1 ml were collected, the amount of radioactivity in each fraction was determined and the radioactivity per fraction was plotted manually.

Serotonin measurement

5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were measured using a Brinkman Metrohm electrochemical detector fitted with a glassy carbon cell and operated at +750 mV vs. Ag/AgCl and 5 nA. Samples were deproteinized with 0.1 *M* perchloric acid and centrifuged at 3000 *g*. The supernatant was injected into a reversed-phase HPLC system fitted with a 125 × 4.6 mm Hypersil-C_{18} (5 μm) column (Mandel Scientific). The solvent system was 18% methanol in 0.1 *M* KH_2PO_4, adjusted to pH 3.78 with phosphoric acid, and the flow-rate was 1 ml/min at 21°C.

Preparation of rabbit platelets

Blood (50 ml) was drawn by cardiac puncture from healthy white rabbits into plastic tubes containing citrate—dextrose anticoagulant (0.1 mol trisodium citrate, 0.2 mol citric acid, 0.1 mol dextrose). The blood was centrifuged at room temperature for 15 min at 100 *g* to obtain platelet-rich plasma (PRP).

The PRP was removed and ethylene glycol bis(β-aminoethyl ether) N,N'-tetra-acetic acid (EGTA) was added (0.1 m*M* final concentration). The platelets were sedimented at room temperature at 1500 *g* for 15 min and washed with PBS without Ca^{2+}. The platelets were resuspended in PBS without Ca^{2+} at a dilution of $2 \cdot 10^9$ per ml. The platelets were counted using a bacterial counting cytometer.

Preparation of human polymorphonuclear leukocytes (PMNLs)

PMNLs were prepared from healthy subjects as described [13]. Red cells were lysed with ammonium chloride [14] and PMNLs were suspended in Tyrod's buffer at $1 \cdot 10^7$ per ml. The cell viability was determined by trypan blue exclusion dye and 1-ml incubation were performed.

Fast atom bombardment mass spectrometry (FAB-MS)

Ionization was performed on a Kratos MS902 mass spectrometer equipped with a fast atom bombardment source and probe. The FAB gun was of the saddle-field type. Gun voltage was 7–8 kV at 1 mA, xenon was used as the bombarding gas. Analyses were performed at ambient temperature and 6 kV accelerating voltage. The C_{16}-AGEPC sample after reversed-phase HPLC was rechromatographed by normal-phase HPLC and extracted with dichloro-methane—water. For control analysis, a sample of commercially available PAF was subjected to FAB-MS. Samples were dissolved in methanol and subjected to FAB-MS ionisation using glycerol and thioglycerol as the matrices.

RESULTS

Table I shows the recovery of AGEPC from plasma using XAD-2 and C_{18} materials. The XAD-2 column retained 99% of the AGEPC and the C_{18} column 28%. Following the first 5-ml water wash, another 20% of AGEPC was removed from C_{18}, whereas none was removed from XAD-2. The washing of columns was carried out until radioactivity was no longer detected. When the columns were eluted with acetonitrile, almost the entire AGEPC was recovered from XAD-2, whereas C_{18} contained only 3% AGEPC.

TABLE I

RECOVERY OF AGEPC (C_{14}, C_{16} AND C_{18}) FROM 1 ml PLASMA USING XAD-2 AND C_{18} COLUMNS

Step	Eluent	Elution volume (ml)	Recovery (mean ± S.D.; n = 5) (%)	
			XAD-2	C_{18}
1	Plasma	3	0.5 ± 0.3	72 ± 4.5
2	Water	5	0	20 ± 2
3	Water	5	0	1 ± 0.5
4	Water	5	0	0
5	Acetonitrile	5	76.0 ± 2.5	3 ± 0.7
6	Acetonitrile	5	21.0 ± 3	0
7	Acetonitrile	5	1.5 ± 1	0
8	Acetonitrile	5	0	0

TABLE II

RECOVERY OF AGEPC (C_{14}, C_{16} AND C_{18}) FROM 1 ml PLASMA USING XAD-2 AND 15 ml ELUENT

Eluent	Recovery (mean ± S.D.; n = 5) (%)
Ethanol	98 ± 3.5
Methanol	96 ± 2.5
Acetonitrile	99 ± 3
Propan-2-ol	77 ± 4.3
Pyridine	100 ± 2
Tetrahydrofuran	66 ± 3.7
Acetone	56 ± 5
Ethyl acetate	53 ± 2.3
Dichloromethane	5.5 ± 1
Chloroform	4 ± 0.5
Hexane	4.5 ± 1
Toluene	1 ± 0.5

A comparison of the elution of AGEPC from XAD-2 with various solvents is shown in Table II. The higher polarity solvents were more efficient in removing the AGEPC from XAD-2. The results in Table I and II were obtained using a mixture of C_{14}-, C_{16}- and C_{18}-AGEPC; similar results were also obtained when each individual species was studied.

When a mixture of C_{14}-, C_{16}- and C_{18}-AGEPC was analyzed by normal-phase HPLC, two radioactive peaks were obtained (Fig. 1A). The first peak (I) was attributed to degradation products of [^{3}H]AGEPC, since there was negligible mass and no biological activity. The second peak (II) was determined to be a mixture of C_{14}-, C_{16}- and C_{18}-AGEPC, indicating that normal-phase HPLC failed to separate the different species of AGEPC. A similar observation was also made when AGEPC was analyzed by TLC (data not shown). When the second peak (II) of the normal-phase HPLC profile was analyzed by reversed-phase HPLC three major peaks were observed (Fig. 1B), indicating that reversed-phase HPLC was able to separate these three compounds. The order of elution was determined to be C_{14}, C_{16} and C_{18}, since, when a mixture of C_{16}-

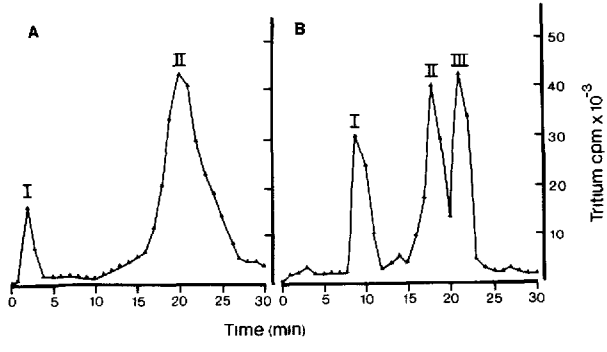


Fig. 1. (A) Normal-phase HPLC profile of a mixture of ^{3}H-labelled C_{14}-, C_{16}- and C_{18}-AGEPC. Points (▲) represent the distribution of radioactivity in 1-min (1 ml) fractions. (I) Unretained material; (II) mixture of C_{14}-, C_{16}- and C_{18}-AGEPC. (B) Reversed-phase HPLC profile of peak II from A. (I) C_{14}-AGEPC; (II) C_{16}-AGEPC; (III) C_{18}-AGEPC.

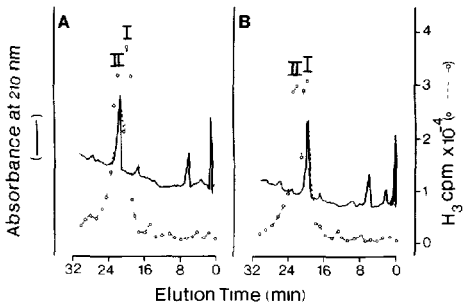
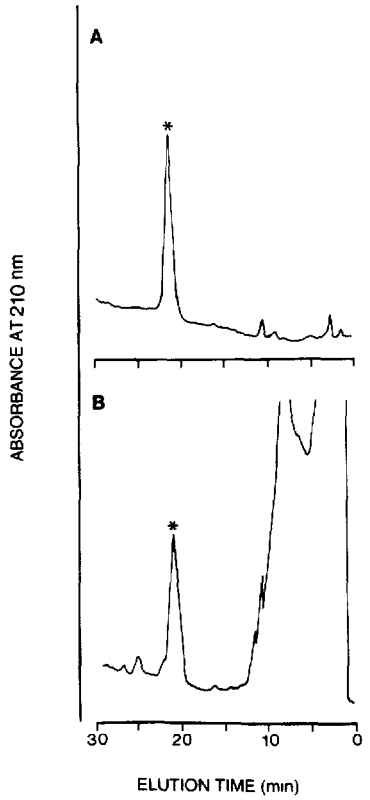


Fig. 2. (A) Reversed-phase HPLC analysis of 50 ng of C_{18}-AGEPC and a mixture of C_{16}-[^{3}H]AGEPC and C_{18}-[^{3}H]AGEPC. Peak II corresponds to C_{18}-AGEPC. (B) Reversed-phase HPLC analysis of 50 ng of C_{16}-AGEPC and a mixture of C_{16}-[^{3}H]AGEPC and C_{18}-[^{3}H]-AGEPC. Peak I corresponds to C_{16}-AGEPC. Sensitivity 0.02 a.u.f.s at 10 mV.

[^{3}H]AGEPC and C_{18}-[^{3}H]AGEPC was co-chromatographed with 50 ng each of C_{16}-AGEPC or C_{18}-AGEPC, C_{18} was found to be eluted with the last radioactive peak (Fig. 2A) and C_{16} was eluted with the first radioactive peak (Fig. 2B).

In order to determine whether extraction of AGEPC from biological



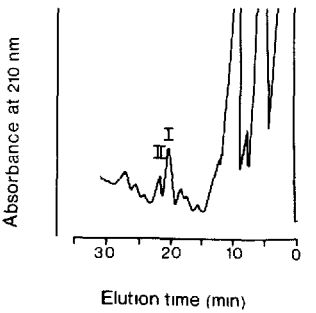
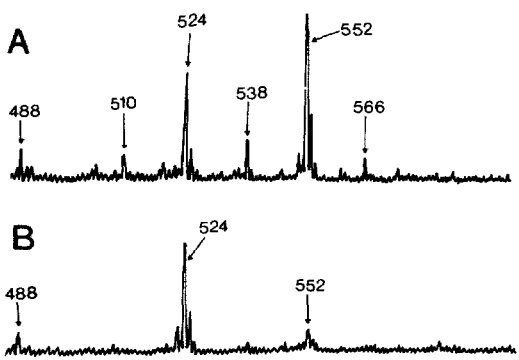


Fig. 3. Reversed-phase HPLC analysis of 100 ng of C_{16}-AGEPC (*) in PBS (A) and in 1 ml of human plasma (B) following extraction with XAD-2.

Fig. 4. Reversed-phase HPLC analysis of AGEPC released by human PMNLs after stimulation by ionophore A23187 Sensitivity at 210 nm: 0.02 a.u.f.s. Peak I corresponds to C_{16}-AGEPC and peak II corresponds to C_{18}-AGEPC.

matrices using XAD-2 provides a clean chromatogram at 210 nm, 100 ng of AGEPC were added either to 1 ml of plasma or 1 ml of buffer containing 0.3% human serum albumin (HSA). Fig. 3A shows a chromatogram of AGEPC extracted from buffer (*) and from plasma (Fig. 3B). Thus, as Fig. 3B shows, the plasma impurities did not significantly interfere with AGEPC absorption at 210 nm. After establishment of the extraction and HPLC techniques for analysis and estimation of synthetic AGEPC, similar procedures were used to extract naturally formed AGEPC from biological samples. Therefore, 10^7 PMNLs per ml were stimulated with 1 μg/ml ionophore A23187 for 2 min. After extraction of supernatants, the residue was analyzed by reversed-phase HPLC and AGEPC was detected at 210 nm. The results of stimulation of PMNLs by ionophore A23187 are shown in Fig. 4. Although the fraction corresponding to C_{14}-AGEPC was contaminated with tissue materials, two peaks were observed corresponding to C_{16}-AGEPC and C_{18}-AGEPC, with C_{16} being the major peak (Fig. 4, peak I) and the second peak (Fig. 4, peak II) corresponding to C_{18}-AGEPC. A quantitative study was not performed; however, in relation to the peak areas of synthetic AGEPC (25, 50, 100 and 200 ng) the amount of C_{16}-AGEPC released by PMNLs was less than 50 ng. To further confirm the identity of the eluted peak as C_{16}-AGEPC, those materials from at least 10^9 PMNLs were pooled, further purified (see Experimental) and subjected to FAB-MS ionisation analysis. As Fig. 5A shows, commercially available PAF gave at least five major ions corresponding to C_{15}- (*m/e* 510), C_{16}- (*m/e* 524), C_{17}- (*m/e* 538), C_{18}- (*m/e* 552) and C_{19}-AGEPC (*m/e* 566). However, the spectrum from HPLC-eluted materials derived from PMNLs gave a major ion at *m/e* 524 corresponding to C_{16}-AGEPC as expected [15] (Fig. 5B) and a minor ion at *m/e* 552 corresponding to C_{18}-AGEPC (derived



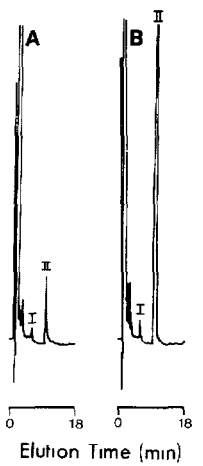


Fig. 5. Fast atom bombardment mass spectrum of (A) PAF obtained from Calbiochem Behring and (B) reversed-phase HPLC-eluted material corresponding to C_{16}-AGEPC derived from stimulation of human PMNLs with ionophore A23187.

Fig. 6. Reversed-phase HPLC analysis of 5-HIAA (I) and 5-HT (II) with electrochemical detection. (A) Unstimulated (control) rabbit platelets; (B) platelets stimulated with C_{16}-AGEPC derived from PMNLs.

from slight contamination of reversed-phase (HPLC peaks). The ion at *m/e* 488 is the ion from the thioglycerol used as the liquid matrix in FAB-MS.

The chromatograms shown in Fig. 6 demonstrate that the AGEPC purified by XAD-2 column and reversed-phase HPLC retained its biological activity. As shown in Fig. 6A, the control incubation (platelets were not stimulated with C_{16}-AGEPC) contained approximately < 1 ng of 5-HIAA and approximately 10 ng of 5-HT per $2 \cdot 10^8$ platelets. Although a quantitative experiment was not performed, these amounts were estimated in comparison to the known amount of synthetic 5-HT or 5-HIAA used as standards. When platelets were incubated with C_{16}-AGEPC derived from 10^7 PMNLs (100 or 50% of the total released) a large amount of 5-HT (Fig. 6B, peak II) was found to be released but there was not a significant increase of 5-HIAA released (Fig. 6B, peak I).

DISCUSSION

Most previous methods for the extraction and estimation of PAF from biological matrices either lack the required sensitivity, specificity, or require tedious and time-consuming analytical procedures. The first aim of the present study was to simplify the extraction procedure for AGEPC from biological matrices as well as to provide an excellent recovery of AGEPC. The second aim was to obtain a complete separation of AGEPC compounds (C_{14}-, C_{16}- and C_{18}-AGEPC) and decrease the detection limits. XAD-2 proved to be an excellent solid-adsorbent material for the extraction of AGEPC from biological matrices and the polar organic solvents were effective for their removal. However, in this study acetonitrile was routinely used since reversed-phase HPLC was performed using aqueous acetonitrile and therefore it was unncessary to evaporate the eluate to dryness. In addition, HPLC-grade acetonitrile was found to contain less UV-absorbing impurities which interfered with chromatography at 210 nm. In this study a comparison was not made to evaluate the AGEPC recovery by a liquid—liquid extraction technique. However, in another study, the AGEPC residue from chloroform- methanol—water (liquid--liquid extraction) was found to contain more plasma impurities which made chromatography with UV absorption at 210 nm at a sensitivity of 0.02—0.1 a.u.f.s. impossible. Extraction with XAD-2 provided a cleaner residue, thus permitting the quantification of AGEPC by reversed-phase HPLC using ultraviolet detection at 210 nm. However, for this purpose it would be advantageous to use an internal standard with similar physicochemical behaviour to AGEPC, such as analogues or derivatives of AGEPC, which permits direct quantification of the compounds without the requirement of bioassay. Nevertheless, a standard curve obtained from synthetic AGEPC could also be used for quantification of unknown AGEPC in biological matrices. The effectiveness of XAD-4 (a resin chemically similar to XAD-2 but with a smaller pore size) to adsorb AGEPC from plasma was also studied and similar results to XAD-2 were obtained (data not shown). However, with XAD-4 a longer elution time was required and it was often necessary to resort to vacuum or pressure in order to increase the elution flow-rate. For the above reason it was found that a solvent mixture of acetonitrile—dimethylformamide (90:10) was more effective than acetonitrile or other polar organic solvents.

Although the most common analytical technique for estimation of AGEPC is normal-phase HPLC, this technique was found to have two major disadvantages. (1) In all eluting solvents examined, it was impossible to resolve different types of AGEPC. (II) Most normal-phase solvents have UV cut-offs at 210 nm or greater. The mobile phase selected, aqueous acetonitrile, has a UV cut-off of ca. 190 nm, thus permitting AGEPC detection at 210 nm. The present reversed-phase HPLC technique provides the separation of AGEPC compounds; the limit of detection of this procedure is between 20 and 50 ng depending on the nature of biological samples (incubation media or plasma). Using a gradient to elute AGEPC and detection at 210 nm caused increasing of the baseline, as the concentration of acetonitrile increased. However, this problem could be eliminated by using a small amount of sodium azide in the first mobile phase (approximately 100 μl of a 1% solution for a litre). This also depends on the purity of water and acetonitrile; thus, the above concentration of sodium azide could be changed to obtain the desired chromatography. The use of 1 m*M* methanesulphonic acid is not absolutely essential: perchloric acid or phosphoric acid could also be used. The advantage of using methanesulphonic acid over phosphoric or perchloric acid was that methanesulphonic acid gives a better separation of C_{16}- and C_{18}-AGEPC and slightly improves peak resolution. The mechanism by which methanesulphonic acid produces those changes is unknown. Another important property of the present reversed-phase HPLC technique was that AGEPC retained its biological activity which would be helpful in assessing individual species activity in pharmacological studies.

It is not yet clear, whether the extraction procedure and HPLC technique reported in this study would be effective in isolation and estimation of other glycerophospholipids. However, further investigations are in progress to determine the recovery of various glycerophospholipids from biological fluids and their subsequent quantification using HPLC.

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REFERENCES

1 E.G. Bligh and W.J. Dyer, Can. J. Biochem. Physiol., 37 (1959) 911.
2 S.A. Metz, M.E. Hall, T.W. Harper and R.C. Murphy, J. Chromatogr., 233 (1982) 193.
3 F.J. Zijlstra and J.E. Vincent, J. Chromatogr., 311 (1984) 39.
4 R. Dixon, R. Evans and T. Crews, J. Liq. Chromatogr., 7 (1984) 177.
5 H.L. Bradlow, Steroids, 11 (1968) 265.
6 K. Green, M. Hamberg, B. Samuelsson and J.C. Frolich, in J.C. Frolich (Editor), Advances in Prostaglandins and Thromboxane Research, Vol. 5, Raven Press, New York, 1978, p. 15.
7 R.N. Pinckard, R.S. Farr and D.J. Hanahan, J. Immunol., 123 (1979) 1847.
8 M.L. Blank and F. Snyder, J. Chromatogr., 273 (1983) 415.
9 K.L. Clay, R.C. Murphy, J.L. Andres, J. Lynch and P. Henson, Biochem. Biophys. Res. Commun., 121 (1984) 815.

10 M. Sanchez-Crespo, P. Inarrea, V. Alvarez, F. Alonso, J. Egido and L. Hernando, Am. J. Physiol., 244 (1983) F706.
11 R.N. Pinckard, R.S. Farr and D.J. Hanahan, Fed. Proc., Fed. Am. Soc. Exp. Biol., 37 (1978) 1667.
12 J.O. Shaw, M.P. Printz, K. Hirabayashi and P.M. Henson, J. Immunol. 121 (1978) 1939.
13 A. Boyum, Scand. J. Immunol., S5 (1976) 9.
14 W. Boyle, Transplantation, 6 (1968) 761.
15 K.L. Clay, D.O. Stene and R.C. Murphy, Biochem. Mass Spectrom., 11 (1984) 47.