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Detection of lyso-platelet-activating factor by high-performance liquid chromatography after derivatisation with fluorescent fatty acids

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SUMMARY

Lyso-platelet-activating factor (lyso-PAF) was derivatised with 9-anthracenepropionic acid in the presence of dicyclohexylcarbodiimide, *p*-toluenesulfonic acid and 4-dimethylaminopyridine. The reaction yield exceeded 90% when the fatty acid was present in double molar amounts versus lyso-PAF. The procedure was equally effective in the derivatisation of other lysophospholipids. The derivatized phospholipids are detected by ultraviolet absorption ($\lambda = 253$ nm) or fluorescence detection (using excitation at 254 nm and emission at 450 nm). The technique was applied successfully to the detection of lyso-PAF in complement activated rabbit plasma.

INTRODUCTION

In recent years, it has been speculated that platelet-activating factor (PAF, Fig. 1) is involved in the pathogenesis of several inflammatory diseases, particularly asthma, and septic shock [1–5]. However, direct evidence to support the above hypothesis remained weak due to the inability to detect the presence of PAF in the plasma or body fluids of patients with the above diseases. The major obstacle to the detection of PAF in body fluids is its rapid metabolic

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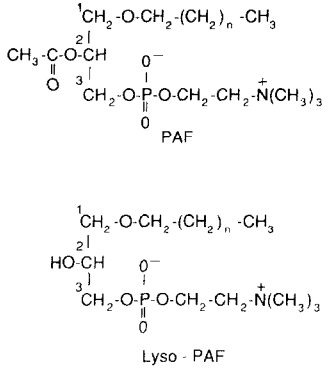

Fig. 1. Structures of platelet-activating factor (PAF) and lyso-PAF.

transformation to lyso-PAF (Fig. 1) [6,7]. Although sub-nanomolar levels of PAF can be measured by bioassay techniques [8,9], these methods do not detect lyso-PAF because it is biologically inactive [10,11]. The most common procedures for detection of lyso-PAF employ thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) with radioactive labels or mass spectrometric (MS) techniques [12,13]. However, in clinical investigations PAF radiolabelling is not possible because of the inability to introduce the label in vivo. MS techniques can be used for detection of lyso-PAF in body fluids. In most cases MS requires isolation of the compound from the biological fluid. In addition, MS instrumentation is very costly thus precluding widespread use.

In view of the above difficulties, it is desirable to have access to a selective HPLC technique which permits detection of lyso-PAF routinely. Thus, derivatisation of lyso-PAF for routine HPLC analysis would hold promise for a successful methodology of detection in biological samples.

The most suitable position for derivatisation of the lyso-PAF molecule is the hydroxyl group at the 2-position in the glycerol backbone (Fig. 1). A number of phospholipid analogues have already been prepared by substitution at this position using dimethylaminopyridine (DMAP) [14]. Gupta et al. [14] synthesized several phospholipid analogues having a photoactive group at the 2-position. Although the yields were quite good (>80%), the separate preparation of fatty acid anhydrides using N,N-dicyclohexylcarbodiimide (DCC) requires strict anhydrous conditions which are difficult to achieve in small-scale synthesis. Orlando et al. [15] synthesized various phospholipid analogues from lyso-phosphatidylcholine (lyso-PC) in a 'one-pot' system. These investigators added lyso-PC, fatty acids, DCC and DMAP all in one step. Reaction at 20°C required 20 h and provided yields of 20–30%. Although this technique is more convenient, the yield is low, particularly for detection of low levels of biological compounds. In addition the presence of DCC could provide N-acylurea as a

by-product [16]. This not only decreases ester formation but it also produces significant contaminating peaks in the HPLC pattern. One way to reduce N-acylurea formation is by using an acid catalyst such as *p*-toluenesulfonic acid (pTSA). Holmberg and Hansen [17] obtained esters of primary and secondary alcohols as well as phenols using DCC and fatty acids in the presence of pTSA in pyridine almost quantitatively with minimal N-acylurea formation.

In this study we obtained quantitative esterification of various lyso-phospholipids by using DCC, DMAP and pTSA in a one pot procedure.

EXPERIMENTAL

Materials

Lyso-PAF, lyso-PC-decanoic acid, lyso-phosphatidylethanolamine (lyso-PE) and lyso-phosphatidylinositol (lyso-PI) were purchased from Sigma (St. Louis, MO, U.S.A.). 10-(9-Anthroyloxy)stearic acid, 11-(9-anthroyloxy)undecanoic acid, 9-anthracenepropionic acid, 1-pyrenedocosanoic acid, 1-pyrenebutanoic acid and 1-pyrenenonanoic acid were purchased from Molecular Probes (Eugene, OR, U.S.A.). Analytical-grade *p*-nitrobenzylalcohol, gold-labelled DMAP, DCC and pTSA were purchased from Aldrich (Milwaukee, WI, U.S.A.). ^{3}H-Labelled lyso-PAF (3.0–4.4 TBq/mmol) was purchased from Amersham International (Arlington Heights, IL, U.S.A.). A silica gel column (150 mm × 4.6 mm I.D., 5 μm particle size), a C_{18} column (150 mm × 4.6 mm, 5 μm particle size) and the silica TLC plates were purchased from Alltech (Deerfield, IL, U.S.A.). HPLC-grade solvents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Derivatization

Lyso-PAF (100 μg) + 9-anthracenepropionic acid (400 μg) were dissolved in dry pyridine (200 μl). pTSA (40 μg in 100 μl pyridine) and DMAP (100 μg in 100 μl pyridine) were added to the above solution, mixed and stirred at 20°C for 15 min. DCC (200 μg) in dry pyridine (100 μl) was added and stirring was continued for 6 h at 20°C in sealed glass reaction vials. Water (1 ml) was added and the mixture was kept overnight at −20°C. Methanol (4 ml), chloroform (4 ml) and deionized water (3 ml) were added, and the mixture was extracted according to the method of Bligh and Dyer [18]. A similar procedure was used for lyso-PAF derivatization with the other acids listed under *Materials*.

When low levels of lyso-PAF (e.g. biological samples) were derivatized, the amounts of fatty acid, DMAP, pTSA and DCC were reduced by a factor of 10 in a total pyridine volume of 250 μl, and the reaction time was extended to 24 h at 20°C.

Thin-layer chromatography

TLC was performed on silica gel plates of 20 cm×5 cm or 20 cm×20 cm, using a mixture of chloroform–methanol–water (65:35:6, v/v). Prior to use, the TLC tank was saturated with the above solvent's vapor for 1 h. Derivatized PAF was identified with aid of a fluorescent lamp (Spectronics, New York, NY, U.S.A.). Lyso-PAF was detected after spraying the plates with cupric sulfate in orthophosphoric acid [10% (w/v) $CuSO_4$ in 8% (v/v) H_3PO_4] followed by heating at 170°C for 15 min. Charred spots appeared for lyso-PAF.

Normal-phase high-performance liquid chromatography

Normal-phase HPLC was performed on a silica column using a mobile phase of chloroform–methanol–water (60:40:2, v/v) at a flow-rate of 1 ml min^{-1}. Samples were injected (50 μl) and detected with a fluorescent detector (RF 530, Shimadzu, Kyoto, Japan), using excitation at 254 nm and emission at 450 nm.

Reversed-phase high-performance liquid chromatography

Reversed-phase HPLC was performed on a C_{18} column using a mobile phase of acetonitrile–methanol–85% H_3PO_4 (8:90:2, v/v) containing 10 m*M* choline chloride. Samples were injected in 50 μl of methanol–chloroform (1:9, v/v) and analyzed at a flow-rate of 1 ml min^{-1}. Elution was monitored with a fluorescent detector as above or with a UV detector at 254 nm.

Mass spectrometric analysis

Fast atom bombardment (FAB) spectra (positive ions) were obtained on an AEI MS9 mass spectrometer equipped with a home-built FAB ion source and gun. The latter was operated with xenon at 7.5–8 kV and 1 mA, the ion source potential was maintained at 6 kV. Samples were dissolved in methanol, and 1 μl of solution was applied to the FAB probe copper tip which had been previously wetted with *p*-nitrobenzylalcohol. Spectra were recorded at a scan rate of 10 s per decade and recorded on UV-sensitive paper.

RESULTS AND DISCUSSION

The results from acylation of the hydroxyl group in lyso-PAF with six different fluorescing fatty acids are shown in Fig. 2. As can be seen from the relative spot sizes the smaller-chain fatty acids usually provided a higher yield of ester formation. The most reactive fatty acids were found to be 9-anthracenepropionic acid and pyrenebutanoic acid. The long-chain fatty acids such as anthroyloxyundecanoic acid (Fig. 2, lane 1) or pyrenedocosanoic acid (Fig. 2, lane 4) did not provide quantitative derivatisation. The reaction of 9-anthracenepropionic acid with lyso-PAF was strongly dependent on the presence of both catalysts, i.e. DMAP and pTSA. Removal of either catalyst resulted in

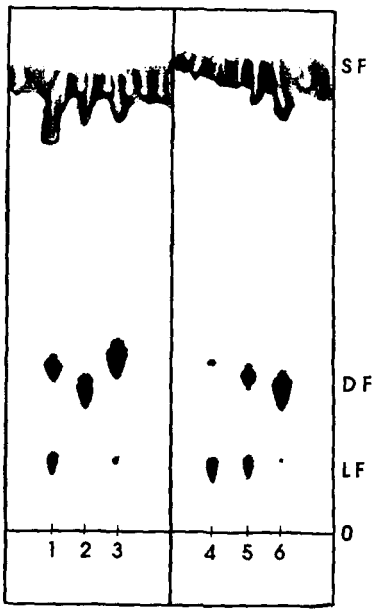

Fig. 2. TLC of derivatised lyso-PAF with anthroyloxyundecanoic acid (1), anthracenepropionic acid (2), anthracenestearic acid (3), pyrenedocosanoic acid (4), pyrenenonanoic acid (5) and pyrenebutanoic acid (6). O = samples origin; LF = non-derivatized lyso-PAF; DF = derivatised lyso-PAF; SF = solvent front.

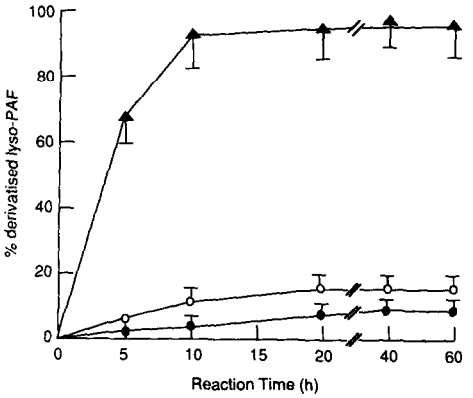

Fig. 3. Rate of formation of 9-anthracenepropionyl-PAF in the presence of DMAP+pTSA (▲), DMAP (○) and pTSA (●). DCC was present in all three cases. Reactions were performed at 20°C with vigorous stirring. The reaction progress was monitored by TLC. Mean ± S.D., $n = 8$.

a significant reduction in derivatisation yield (Fig. 3). The reaction of lyso-PAF with 9-anthracenepropionic acid was found to be dependent on the molar ratio of the reagents (Fig. 4). At a molar ratio of 1:1 (lyso-PAF/9-anthra-

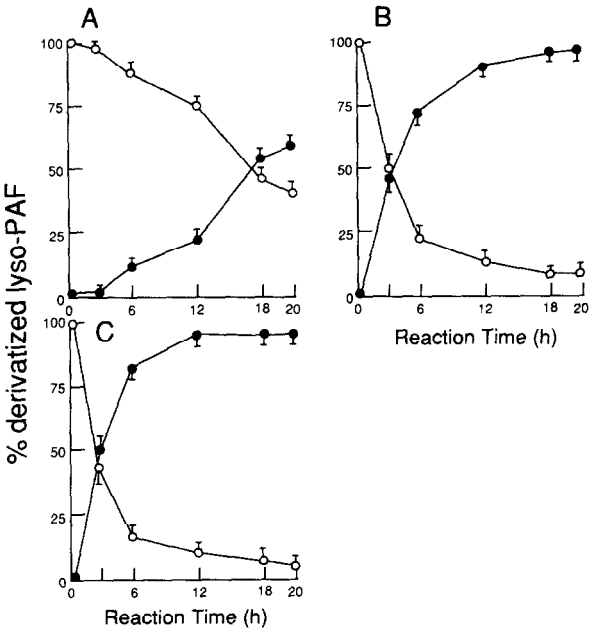

Fig. 4. Rate of lyso-PAF acylation at C_2-OH with 9-anthracenepropionic acid at molar ratios of 1:1 (A), 1:2 (B) and 1:10 (C). (○) Lyso-PAF; (●) 9-anthracenepropionyl-PAF. The reaction progress is monitored by TLC. Mean ± S.D., $n = 8$.

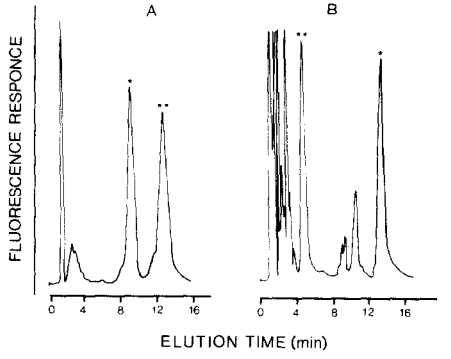

Fig. 5. (A) Normal-phase HPLC and (B) reversed-phase HPLC analysis of crude mixture containing 10 ng anthracenepropionyl PAF (⋆) and 10 ng anthracenepropionyl PC (⋆⋆) using fluorescence detection; emission at 450 nm and excitation at 254 nm.

cenepropionic acid), the yield of reaction was found to be 50% (Fig. 4A). Whereas the yield increased to >90% when the molar ratio increased to 1:2 or 1:10 (Fig. 4B and C, respectively). Increasing the molar ratios to 1:10 did not proportionally increase the yield of reaction over the 1:2 ratio, but de-

creased the derivatisation time (faster reaction). The yield from esterification did not improve much upon elevation of temperature (e.g. 65°C).

In addition to TLC the derivatized phospholipids can be separated by both normal-phase or reversed-phase HPLC.

A crude mixture of the 9-anthracenepropionic acid derivatives of lyso-PAF (⋆) and 1-decyl-lyso-PC (⋆⋆) was separated by both of these HPLC methods, and, as seen from Fig. 5, isocratic runs lead to good separation in both cases. However, reversed-phase HPLC provided better peak profiles and superior separation (Fig. 5B).

Application

In order to investigate the applicability of the present technique for isolation and estimation of lyso-PAF from body fluids, lyso-PAF (25 or 1 ng) containing [^{3}H]lyso-PAF (0.02 TBq) was injected into 1 ml of rabbit plasma. After deproteinisation of plasma with methanol, the residue was dried and derivatized with 9-anthracenepropionic acid. The resultant product was analyzed by normal-phase HPLC as indicated above. The chromatogram (Fig. 6) revealed a peak (⋆) with retention time identical to the authentic anthracenepropionate ester of PAF (Fig. 6A). However, approximately 30% of lyso-PAF was lost during the various manipulating steps. Fig. 6B shows the chromatogram of 1 ng lyso-PAF injected into 1 ml blood and then extracted and derivatised with 9-anthracenepropionic acid. Fig. 6C shows the derivatised lyso-PAF released into the blood (1 ml plasma) of rabbit injected with cobra venom. The indicated peak (⋆) had a retention time identical to authentic anthracene propionate PAF.

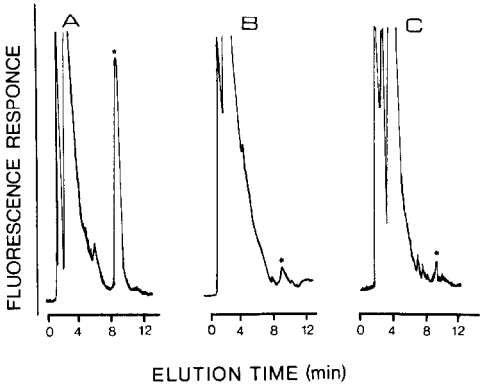

Fig. 6. Normal-phase HPLC analysis of (A) 25 ng and (B) 1 ng lyso-PAF added to 1 ml plasma followed by derivatization with 9-anthracenepropionic acid. (C) Normal-phase HPLC of biologically derived lyso-PAF in 1 ml plasma of rabbit injected with cobra venom after derivatisation with 9-anthracenepropionic acid. Anthracenepropionyl PAF (⋆) was detected by fluorescent detection; emission at 450 nm and excitation at 254 nm.

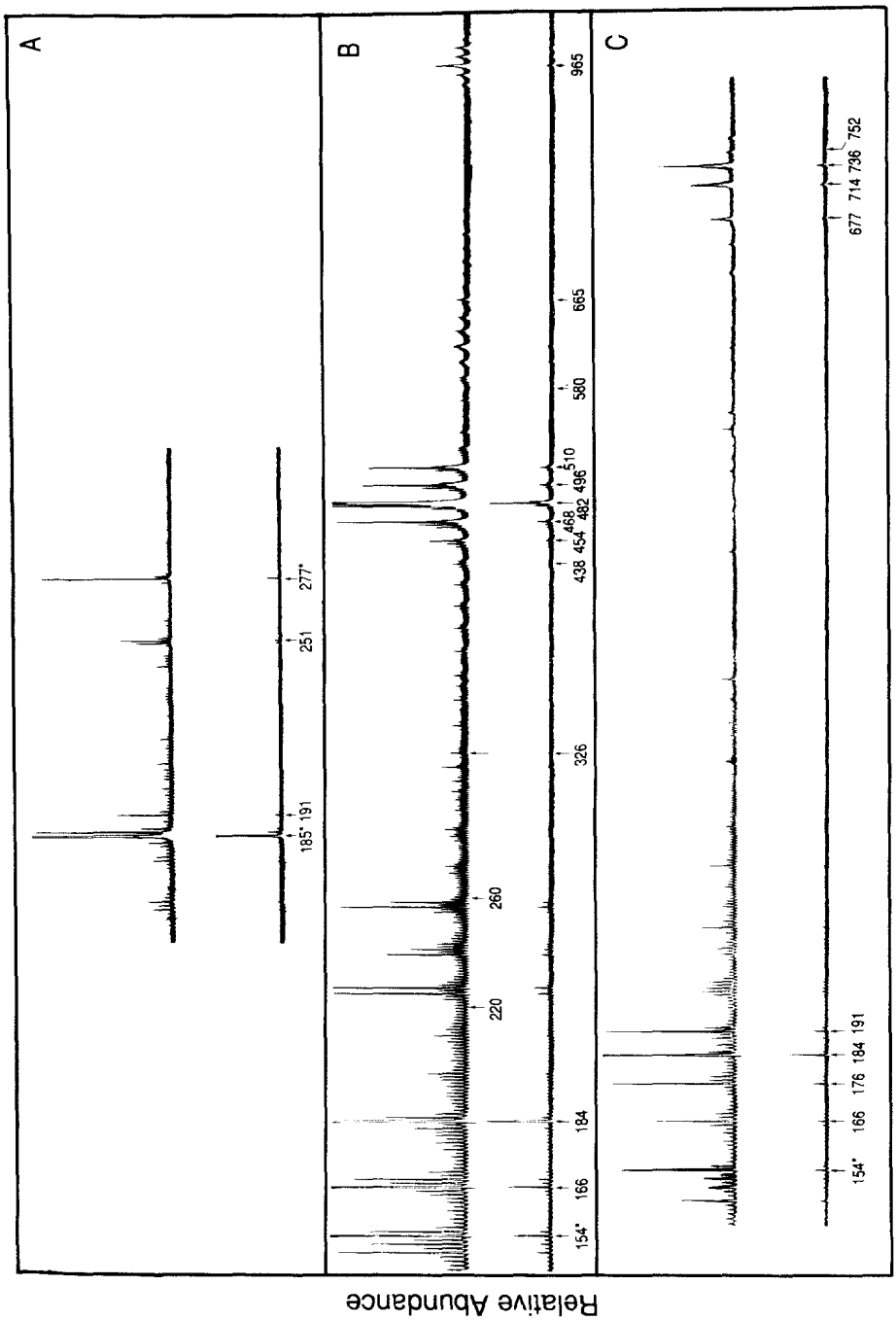

Fig. 7. FAB mass spectra of (A) 9-anthracenepropionic acid, (B) lyso-PAF and (C) anthracenepropionyl-PAF. ★ = Ions due to matrix.

In addition to derivatisation of lyso-PAF and lyso-PC, the present technique was found to be equally effective in derivatisation of other lyso-phospholipids such as lyso-PI and lyso-PE. Table I shows R_F values obtained after TLC of lyso-PC, lyso-PI and lyso-PE before and after derivatisation with 9-anthracenepropionic acid.

In order to confirm the nature of ester formation at the 2-position, MS analysis was performed. The mass spectrum of 9-anthracenepropionic acid (Fig. 7A) showed the protonated molecular ion at *m/z* 251 and a major fragment ion (M−59) at *m/z* 191. Other ions are due to the FAB matrix. The spectrum of lyso-PAF (Fig. 7B) exhibited a major ion at *m/z* 482 ($[M+H]^+$, $n=14$) and smaller ions at 454 ($n=12$), 468 ($n=13$), 496 ($n=15$) and 510 ($n=16$).

Ions of approximately 30% intensity (with respect to the saturated moiety) were also detected at *m/z* 452, 466, 480, 494 and 508, indicating the presence of one unsaturation in the alkyl chain.

Ions in the 580–665 region are due to $[M+matrix]^+$ formations while the ion cluster about mass 965 is due to formation of $[M_2+H]^+$ ions. Ions in the range from *m/z* 326 to 438 are due to fragmentation of the aliphatic chain. Major ions between masses 220 and 260 (224, 226, 238, 240, 256 and 258) are due to fragmentations outlined in Fig. 8, Table II. In some cases the fragmentation is accompanied by a hydrogen migration.

The spectrum of the chromatographically purified anthracene-propionate ester of PAF (Fig. 7C) shows relatively little fragmentation. Ions only due to the $n=14$ lyso-PAF are observed.

Intense ions of *m/z* 714 ($[M+H]^+$), 736 ($[M+Na]^+$) and 752 ($[M+K]^+$) are present. An ion at *m/z* 677 is attributed to the loss of $N(CH_3)_3$ from the $[M+Na]^+$ ion.

An ion at *m/z* 191 was observed previously in the spectrum of 9-anthracenepropionic acid and the ion at 166 is characteristic of the choline phosphate moiety (Table II, ion m) indicating that this unit has not been altered during the derivatisation process. The formation of an ion of *m/z* 176 is attributed to

TABLE I

R_F VALUES OF LYSO-PC, -PI AND -PE BEFORE AND AFTER DERIVATISATION WITH 9-ANTHRACENEPROPIONIC ACID

Compound	R_F value
Lyso-PC	0.18
Anthracenepropionic-PC	0.36
Lyso-PI	0.26
Anthracenepropionic-PI	0.82
Lyso-PE	0.47
Anthracenepropionic-PE	0.84

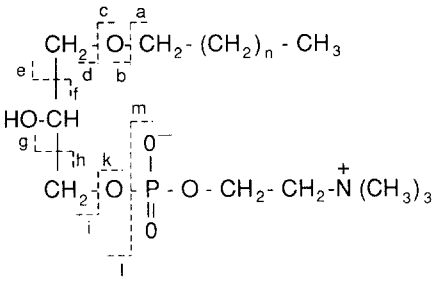

Fig. 8. Proposed fragmentation of lyso-PAF in positive-ion mode FAB-MS. See Table II for masses of fragment ions.

TABLE II

IONS OBSERVED FROM POSSIBLE FRAGMENTATION PROCESSES IN THE FAB ANALYSIS OF LYSO-PAF

Fragment ion	Ion mass				
	$n=12$	$n=13$	$n=14$	$n=15$	$n=16$
a	197	211	225	239	253
b	256	256	256	256	$256-H_2O \rightarrow 238$
c	213	227	241	255	269
d	240	240	240	240	240
e	227	241	255	269	283
f	226	226	226	226	226
g	257	271	285	299	313
h	196	196	196	196	196
i	217	285	299	313	327
k	182	182	182	182	182
l	287	301	315	329	343
m	166	166	166	166	166

loss of the anthracene portion accompanied by hydrogen migration back to the PAF skeleton. These data demonstrate the presence of one anthracenepropionate molecule on the PAF skeleton being located at the 2-position.

The stereochemistry of PAF remained unchanged during the derivatisation process as was determined by treatment of anthracenepropionyl-PAF with phospholipase A_2 [19]. Phospholipase A_2 caused loss of the anthracenepropionyl unit from the C_2 glycerol backbone (2-position) confirming the L *(sn)* orientation of this position.

CONCLUSION

The method described here has several advantages over the previous techniques for detection and estimation of lyso-PAF in biological fluids. Most im-

portant of all, the new technique is sensitive (≥ 1 ng) due to its fluorescent chromophore. With the combination of pTSA and DMAP it became possible to obtain almost quantitative yields of the derivatives. Previous efforts to derivatise lyso-PAF through the cleavage of phosphocholine moiety with phospholipase C [20] has not been much utilized due to the acyl group migration between 2- and 3-positions. Thus, the present technique is advantageous, as only the C-2 derivatives are obtained (as shown by MS). Furthermore, due to the presence of pTSA, the formation of N-acylurea is negligible, thus providing a high yield of the ester formation. The present technique was found to be applicable in detection of lyso-PAF present in rabbit blood; thus, it might be useful for detection of lyso-PAF in other body fluids associated with a variety of diseases. We did not find significant deterioration of the anthracene moiety during long-term (up to 30 days, $-80°C$) storage of the reaction mixture; therefore, several samples can be derivatised and kept for a few weeks before analysis.

In conclusion, the technique described here should be useful in detecting lyso-PAF in body fluids of patients with various pathological states, where the involvement of PAF has been a major question.

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