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Note

# Rapid isocratic high-performance liquid chromatographic purification of platelet activating factor (PAF) and *lyso*-PAF from human skin

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Platelet activating factor (PAF) is a mixture of at least two 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholines in which the length of the alkyl substituent can vary. It is reported that the natural product is a mixture of straight chain $C_{16}$ and $C_{18}$ homologues in 9:1 mol/mol proportions [1]. Although PAF was first recognised by virtue of its potent effect on the aggregation and degranulation of platelets [2], more recent research has demonstrated a wider spectrum of biological activity including induction of leukocyte aggregation, chemotaxis and chemokinesis, generation of superoxide and lysosomal enzyme release [3]. A variety of cell types synthesise and release PAF following stimulation [4].

Psoriasis is an inflammatory skin disorder characterised by an epidermal neutrophil infiltrate. Biologically active concentrations of arachidonic acid metabolites have been shown to be present in the involved skin of psoriatics [5] and may, by virtue of their chemotactic properties, be relevant to the pathogenesis of the neutrophil infiltrate. As PAF is also chemotactic and chemokinetic for human neutrophils [6], we were interested in measuring PAF in the skin of psoriatic patients and have developed a rapid, isocratic high-performance liquid chromatographic (HPLC) procedure for the recovery of substantial amounts of PAF-like biologically active material from psoriatic plaque.

PAF itself does not absorb in the UV above 200 nm and, in order to permit the use of phosphorylcholines as markers for the elution of PAF, solvents which permit the eluent to be monitored at 210 nm or lower must be employed. To be able to process a reasonably large number of samples an isocratic HPLC method is preferred, and it is essential that the column provides

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reproducible retention volumes for the analyte. In addition, the method should ensure the separation of PAF from other lipids which have similar biological activity, and from the metabolite, *lyso*-PAF, which is biologically inactive [7].

The HPLC separation of phospholipids on a variety of stationary phases has been reviewed [8]. The majority of systems described have employed silica gel phases eluted with hexane—alcohol mixtures containing varying proportions of water often with the admixture of acid or base [9]. In 1981 Briand et al. [10] described gradient elution HPLC using a DIOL-bonded LiChrosorb packing material, eluted with acetonitrile—water solvent mixtures, for the separation of phosphorylcholines and reported the improved peak shapes obtained using this material. Several HPLC separation methods have been reported for PAF itself [11—13], but none of these has, to date, satisfied all of the criteria mentioned above.

## MATERIALS AND METHODS

Psoriatic plaque was obtained from volunteer patients by gentle scraping of the lesional areas, a technique yielding 250—1000 mg scale. Scale from separate individuals was used, none of whom had received any treatment for at least two weeks.

Lipids were extracted by disintegrating (Polytron, Kinematica, Luzern, Switzerland) in chloroform—methanol (2:1, v/v) (100 ml/g scale). After centrifugation (2000 *g*, 10 min) and decanting, 20% of its volume of water was added to the supernatant, the mixture was shaken and allowed to separate. The lower layer was collected and evaporated to dryness. The extract was then applied, in solution in chloroform—methanol (2:1), as a streak to a 20 × 20 cm thin-layer chromatographic (TLC) plate (Silica gel G, 500 μm; Anachem, Luton, U.K.) and developed in chloroform—methanol—acetic acid—water (50:25:10:4, v/v), in a filter-paper-lined, pre-saturated glass tank at room temperature. A second TLC plate was developed simultaneously, with two marker spots of lecithin and *lyso*-lecithin, which were visualised using iodine vapour. PAF runs on this system between the two markers (typical $R_F$ 0.26) and this region of the plates was scraped off and extracted with chloroform—methanol—water (1:2:0.8, v/v). After centrifugation (2000 *g*, 10 min), 25% by volume each of water and chloroform were added to the supernatant and the mixture was shaken. The lower layer was recovered and evaporated to dryness. This procedure obviates the carry-over of silica gel into the organic extract [14].

HPLC was performed on a 10-μm LiChrosorb DIOL (Technicol, Stockport, U.K.) 25 cm × 4.6 mm column using a solvent mixture of *tert.*-butylmethyl ether—methanol—water—ammonia (200:100:10:0.02, v/v). The relative amounts of the first two solvents may be varied over a wide range in order to achieve convenient retention volumes for specific compounds. Using the solvent mixture described above, the UV monitor could be operated down to a limit of 207 nm. The chromatography was performed on a Waters Model 6000 pump with a Rheodyne valve injector and a Pye Unicam PU 4020 variable-wavelength detector. Solvent flow-rates between 1 and 2 ml/min were used

throughout. Radioactivity eluting from the column was monitored, either on a Reeve Analytical (Glasgow, U.K.) radioactivity monitor, or by fraction collection and scintillation counting.

The chemokinetic activity of the fractions collected from the HPLC system was determined by an agarose micro-droplet assay [15].

[$^{14}$C]PAF, [$^{14}$C]*lyso*-PAF and [$^{3}$H]PAF were obtained from Amersham International (Amersham, U.K.) and all phospholipids were supplied by Sigma (Poole, U.K.). The chromatographic solvents were of HPLC grade (Fisons, Loughborough, U.K.). Synthetic PAF and *lyso*-PAF were obtained from Cambridge Research Biochemicals (Cambridge, U.K.). A 9:1 w/w mixture of $C_{18}$ and $C_{16}$ PAF has been used to represent "authentic" PAF.

## RESULTS AND DISCUSSION

The majority of published methods for the HPLC of phosphorylcholines which have used solvents with sufficient UV transparency to permit monitoring at 210 nm or lower, employed silicic acid columns with aqueous organic solvents. In our hands these methods failed to give reproducible retention times for the two phosphorylcholines, lecithin and lysolecithin, the major sources of difficulty lying in the varying activity of the column and in the near saturating quantities of water required to provide reasonable retention volumes for these very polar substances.

One publication has reported [10] the use of DIOL LiChrosorb packing material in this field, and its bonded nature might be expected to provide a packing material with more controlled activity than silicic acid itself. Polar solvents are required to elute the cholines from this column and we have used a mixture of *tert.*-butylmethyl ether, methanol and water with the addition of a trace of ammonia. The latter improves the peak shape considerably over the use of a neutral solvent mixture. This solvent can be used down to 207 nm and is sufficiently volatile to be removed easily by blowing dry with nitrogen at 45° C.

Fig. 1 shows a chromatogram obtained from the injection of a mixture of phospholipids onto this system. The profile of recovered radioactivity from coinjected [$^{14}$C]PAF and [$^{14}$C]*lyso*-PAF is also shown. PAF is separated from lecithin, *lyso*-lecithin and from *lyso*-PAF. Sphingomyelin, with which PAF comigrates on TLC, is also separated from PAF.

We have applied this system to the extraction of PAF from human psoriatic scale. The final analysis for PAF depends upon chemokinetic activity and it has been important to employ a procedure which would separate PAF from other biologically active lipids, such as arachidonic acid metabolites [5] and from complement-derived peptides [16]. The lesional psoriatic scale obtained from volunteers was first extracted using the method of Folch et al. [17]. The total lipid fraction produced was then separated on a TLC system in which PAF ran at a low $R_F$ between lecithin and *lyso*-lecithin. All the arachidonic acid metabolites were eluted to the top of the plate. The region of the TLC plate containing the PAF was then extracted and the material thus obtained applied to the HPLC system described above. One-minute fractions (1.5 ml) of the eluting material were collected, evaporated to dryness and assayed for

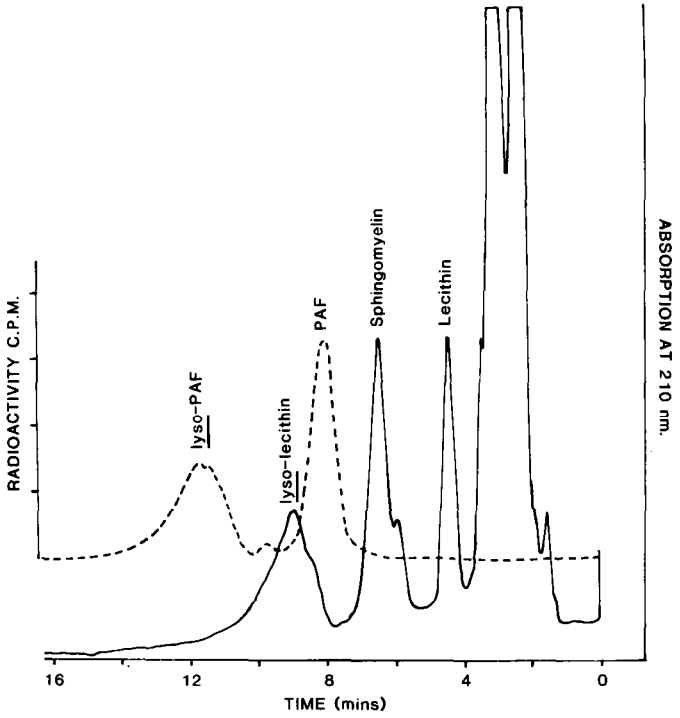


Fig. 1. HPLC traces for the separation of phosphorylcholines at 210 nm (solid line) and of [$^{14}$C]PAF and [$^{14}$C]*lyso*-PAF using an on-line radioactivity monitor (dashed line).

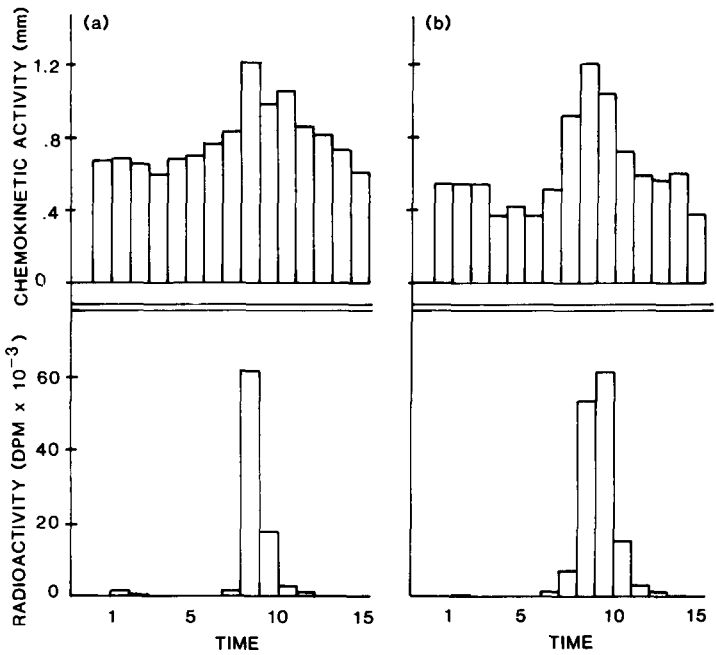


Fig. 2. Profiles of chemokinetic activity (upper part) and radioactivity (lower part) obtained from successive 1-min fractions collected from an HPLC column are shown for 100 ng authentic PAF (a) and for extracted psoriatic scale (b).

leukocyte chemokinetic activity. Samples of authentic PAF and [$^3$H]PAF were also run on the same system and the fractions were collected as before. The profiles of the biological activity and radioactivity so obtained are shown in Fig. 2. Here the excellent coincidence of the maxima of biological activity and radioactivity for a sample of authentic PAF (Fig. 2a) and a sample of extracted psoriatic scale (Fig. 2b), can be seen. This result has been confirmed in three patients and it seems clear that PAF-like material can be extracted from this source. Comparison of the levels of biological activity obtained from psoriatic scale with a dose response curve for authentic PAF indicates that some 20—100 ng of PAF-like material are extractable from 1 g of scale.

Whether the PAF-like material is released from polymorphonuclear leukocytes (PMNs) already present in psoriatic lesions, or is necessary for the initial migration of PMNs into the affected site, remains to be established, as does the structural identity of the PAF-like material we have isolated. We are now preparing mass spectrometric identification and assay procedures firstly to confirm these findings and, secondly, to enable more precise quantitation. The chromatographic methods described here should also prove to be of use in the separation of other classes of phospholipids.

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