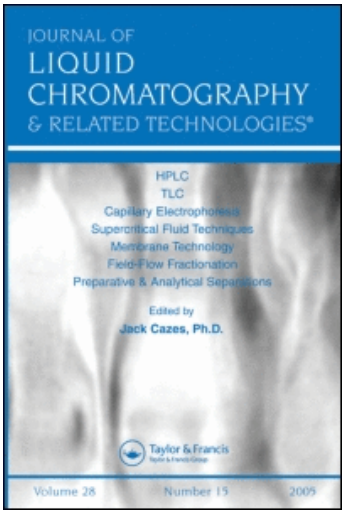

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CONCURRENT C18 SOLID PHASE EXTRACTION OF PLATELET ACTIVATING FACTOR (PAF) AND ARACHIDONIC ACID METABOLITES

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ABSTRACT

Concurrent solid phase extraction of arachidonic acid metabolites and platelet activating factor in human vein umbilical endotelial incubates has been achieved on commercial C18 reversed phase cartridges. Control of sample pH, elution solvents and C18 adsorbent material are important for selective recoveries. Samples were acidified at pH=4.0 and processed through the C18 cartridges which were washed with 10 ml of water/methanol 9/1, 10 ml of water at pH=4.0 and 20 ml of petroleum ether. Arachidonic acid metabolites and platelet activating factor adsorbed on the solid phase material were sequentially separated by elution with 4.5 ml of methyl formate and 4.5 ml of methanol, respectively. Recoveries thus obtained were 65.0 ± 2.5%, 89.1 ± 4.6%, 91.1 ± 7.5%, 94.8 ± 1.6 % for PAF, 6-keto $PGF_{1\alpha}$, 15-HETE and PGE_2 respectively. The method was also compared with the classical Bligh & Dyer liquid-liquid extraction procedure.

INTRODUCTION

Platelet activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and arachidonic acid (AA) metabolites are biologically active substances concomitantly synthesized following cell activation in hypersensitivity and inflammatory reactions (1,2,3). The simultaneous extraction of these lipid mediators in a biological matrix has not yet been established. Classical liquid-liquid extraction with a mixture of methanol/chloroform (2/1, v/v), as described by Bligh & Dyer (4) is generally employed for the extraction of platelet activating factor (PAF) in biological samples (5). However, liquid-liquid extraction methods can be highly time consuming since multi-step procedures may be necessary for efficient extraction. Recently, both normal and reversed solid phase extraction (SPE) on silica (6) and C18 (2) cartridges, respectively, have been applied to PAF enrichment and analysis. On the other hand, although liquid-liquid extraction methods were initially used for the AA metabolites (7,8), presently the more specific C18 solid phase extraction methods are preferred for these metabolites (9,10,11). In this case methanol or methyl formate are used as solvent eluents.

Although PAF and AA metabolites are individually extracted using liquid-liquid (5) or solid phase extraction (9,10), respectively, there are no studies on the development of useful methods for the concurrent extraction of these lipid mediators from biological samples.

The aim of this work has been to study the applications of commercial C18 solid phase cartridges for the extraction of PAF in biological samples, as well as to develop a concurrent extraction method for PAF and AA acid metabolites suitable to be combined to high performance liquid chromatography (HPLC) (12,13), gas chromatography-mass spectrometry (GC-MS) (14), radioimmunological assay (RIA) (15,16) or bioassay (17)

techniques. For this purpose we have also evaluated the incidence on PAF recoveries of different factors such as a) initial pH of the sample, b) different solvent elution mixtures and c) different commercial solid phase cartridges.

EXPERIMENTAL

Chemicals

Tritiated PAF (80 Ci/mmol), 15-HETE (213 Ci/mmol), 6-keto $PGF_{1\alpha}$ (183 Ci/mmol) and PGE_2 (186 Ci/mmol) were purchased from Amersham International (Amersham, Buckinghamshire, U.K.). Chloroform, methanol and petroleum ether were from Merck (Darmstadt, Germany). Hexane, isopropanol and acetonitrile were HPLC grade from Scharlau/Ferosa (Barcelona, Spain). C18 reversed cartridges were obtained from Amersham (Amprep, 100 mg adsorbent, mean particle size 40 µm, mean pore size 6 nm) and Waters Associates (Mildford, Mass. USA (Sep Pak, 400 mg adsorbent, particle size 55-105 µm, pore size 5-30 nm)).

C18 solid phase extraction

A tritiated standard of PAF activity (55 pg/ml) was added to supernatants obtained from human umbilical vein endothelial cell (HUVEC) cultures. Samples adjusted at pH=4.0 or pH=7.4 were processed through C18 solid phase Amprep cartridges. Cartridges previously activated with 10 ml of methanol and 10 ml of water were washed with 10 ml of water/methanol 9/1, 10 ml of water (pH=4.0 or pH=7.4) and 20 ml of petroleum ether. Recoveries of PAF were evaluated using either one of the following eluents: 4.5 ml of methanol/chloroform (2/1), 4.5 ml of methanol/chloroform with 2% of acetic acid (2/1) or 4.5 ml of methanol. Moreover, C18 Sep-Pak cartridges were also evaluated using methanol only as eluent. For concurrent extraction of PAF and AA metabolites tritiated

standards (55 pg PAF/ml, 53 pg 15-HETE/ml, 51 pg 6-keto $PGF_{1\alpha}$/ml, 52 pg PGE_2/ml and 46 pg $PGF_{2\alpha}$/ml) were added to HUVEC supernatants. Samples were adjusted to pH 4 and were processed through C18 Amprep cartridges, which were activated and washed as described above for PAF. Finally, cartridges were eluted in two sequentally steps with 4.5 ml of methyl formate (AA metabolite elution) and 4.5 ml of methanol (PAF elution). Eluates were vacum evaporated-concentrated (Savant, Hinksvill, NY, USA) to dryness for direct scintillation counting (LKB Rackbeta counter, Turku, Finland).

Bligh & Dyer extraction

1 ml of chloroform and 2 ml of methanol (acidified or not with 2% of acetic acid) were added to 1 ml of HUVEC supernatants, previously adjusted to pH=4.0. The sample was vortexed and an additional 1 ml of chloroform and 1 ml of water were added up to achieve a final composition of chloroform/methanol/water 2/2/2 (v/v/v). After centrifugation (250 g, 10 min, 4°C), the lower chloroform phases were collected and evaporated to dryness for scintillation counting.

RESULTS

Table 1 shows the recoveries of PAF from Amprep and Sep-Pak C18 cartridges for samples loaded at pH 7.4 and at pH 4.0 and eluted under different conditions. Significant differences were observed for PAF recoveries between pH 7.4 and pH 4.0 in all conditions assayed. PAF recoveries using a mixture of methanol/chloroform 2/1 or methanol alone as solvent eluents were not statistically significant. The presence of 2% of acetic acid in the methanol/chloroform (2/1,v/v) mixture slightly improved PAF recovery. On the other hand, the recovery of PAF using Sep-Pak cartridges eluted with methanol was significantly lower at both pH values than that obtained with the Amprep cartridges.

Table 1. Comparative ^{3}H-PAF recoveries (%) from HUVEC supernatants in C18-Amprep and C18-Sep Pak solid pase extraction cartridges. Values are mean ± SD, n = 8. *: $p < 0.05$ vs pH 4.0, a: $p < 0.05$ vs Sep-Pak (Student t-test).

	C18 Amprep			C18 Sep pak
pH	methanol/ chloroform (2/1)	methanol/ chloroform (2/1) 2% acetic acid	methanol	methanol
7.4	77.6 ± 3.7 *	80.1 ± 5.6 *	76.7 ± 2.1 a *	69.2 ± 5.1 *
4.0	62.2 ± 2.8	69.0 ± 5.8	65.7 ± 4.7 a	44.0 ± 6.0

Table 2 gives the recoveries on C18 Amprep for PAF, 6-keto $PGF_{1\alpha}$, 15-HETE, and PGE_2 corresponding to HUVEC supernatants processed at pH 4.0 and sequentially eluted with 4.5 ml of methyl formate and 4.5 ml of methanol. The acidic pH is required for the concurrent extraction of cyclooxygenase (6-keto $PGF_{1\alpha}$, PGE_2) and lipoxygenase (15-HETE) AA metabolites, as previously reported (11).

As shown on this table, for all practical assay purposes the AA metabolites and PAF were efficiently extracted and separated when the C18 cartridges were firstly washed with 4.5 ml of methyl formate and then with 4.5 ml of methanol. The methyl formate fraction is enriched with the metabolites of AA whereas the methanol preferentially carries the PAF

Alternatively, we have also studied Bligh & Dyer recoveries for PAF, 15-HETE, 6-keto $PGF_{1\alpha}$ and PGE_2 in samples previously adjusted to pH=4.0

Table 2. C18 Amprep solid phase extraction recoveries (%) of PAF, 6-keto $PGF_{1\alpha}$, 15-HETE and PGE_2 in HUVEC supernatants adjusted to pH=4.0 and sequentally eluted into separate fractions, first with 4.5 ml of methyl formate and second with 4.5 ml of methanol. Values are mean ± SD. n = 8.

	Methyl Formate	Methanol
PAF	7.5 ± 1.5	65.0 ± 2.5
6-keto $PGF_{1\alpha}$	89.1 ± 4.6	2.7 ± 0.6
15-HETE	91.1 ± 7.5	3.1 ± 3.0
PGE_2	94.8 ± 1.6	0.72 ± 0.1

Table 3. Bligh & Dyer recoveries (%) of PAF, 15-HETE, 6-keto $PGF_{1\alpha}$ and PGE_2, with sample pH adjusted at 4.0 and with methanol containing 2% of acetic acid. Values are mean ± SD. n=6.

Lipid mediator	Recovery (%)
PAF	89.3 ± 2.9
15-HETE	73.3 ± 6.1
6-keto $PGF_{1\alpha}$	78.4 ± 1.2
PGE_2	81.7 ± 2.6

(see Table 3). Under these conditions PAF and AA metabolites were also efficiently extracted and PAF recoveries were similar (89.3 ± 2.9 %, n = 6) to those obtained for the same samples at neutral pH (90.8 ± 4.7 %, n=6).

DISCUSSION

The higher PAF recovery values obtained at pH 7.4 vs those found at pH 4.0 indicated that acidification of the sample would not be required when only PAF must be extracted from a sample.However, these conditions may be unacceptable in terms of good recoveries if it should become necessary to co-extract acidic compounds, such as the AA metabolites or eicosanoids. At pH 7.4 the concurrent extraction of some of these other compounds such as the prostaglandins is not possible because sample acidification is required to prevent the protonation of the carboxylic and hydroxylic groups of eicosanoids (7,9) and enhance the overall sample hydrophobicity with the consequent retention on the reversed phase adsorbent. In contrast, no differential recoveries were observed between the three elution solvents assayed (see Table 1). Nevertheless, the use of a mixture of methanol/chloroform (2/1) for the exclusive PAF extraction would be recommended in order to facilitate the evaporation of the organic solvent. In all cases, Amprep cartridges gave PAF recoveries significantly higher than Sep-Paks (see Table 1).

The recoveries of PAF, 15-HETE, 6-keto $PGF_{1\alpha}$ and PGE_2 obtained at pH 4.0, confirm the utility of C18 solid-phase extraction procedures for the concurrent extraction of PAF and arachidonic acid metabolites at this acidic sample pH value (see table 2). This agrees with data previously reported for arachidonate metabolites at pH 4.0, which showed that this pH is the most suitable for the concurrent extraction of prostaglandins, HETEs and LTs from nasal washes (11). As reported in table 2, the sequential elution using methyl formate and methanol is very suitable for accomplishing the practical

separation of AA metabolites from PAF using C18 cartridges. This provides a rapid and useful method for the concurrent extraction of PAF and AA metabolites and subsequent individual fraction separation. The method is susceptible to be combined with HPLC and bioassay or RIA techniques. Furthermore, this method of extraction allows for a more selective clean up of the sample than the Bligh & Dyer method and also offers the possibility to recover PAF and AA metabolites in separate fractions.

On the other hand, the classical Bligh & Dyer extraction method can only be applied for the concurrent extraction of PAF, 6-keto $PGF_{1\alpha}$, 15-HETE, and PGE_2 when the sample has previously been acidified to pH 4.0. It is known that liquid-liquid extraction is more time consuming and unspecific than C18 solid phase extraction methods. This is specially important when subsequent elaborated HPLC-RIA (AA metabolites) or HPLC-bioassay (PAF) determinations must be carried out.

In summary, the acidification of the sample to pH 4.0 is the limiting step for the concurrent extraction of PAF and AA metabolites in biological samples which can be carried out using both the classical Bligh & Dyer and the C18 SPE. Nevertheless, the C18 SPE methodology affords a more selective sample clean up and allows the recovery of PAF and AA metabolites in two separate fractions being suitable for combination with further HPLC, GC-MS, RIA or bioassay techniques.

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