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Simultaneous reversed-phase extraction of lipoxygenase and cyclooxygenase metabolites of arachidonic acid in nasal secretions: methodological aspects

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ABSTRACT

An improved analytical method for the simultaneous solid-phase extraction of arachidonic acid metabolites in biological samples is described The major aim of the work was to define the cause of the different recoveries reported in the literature for leukotrienes and hydroxyeicosatetraenoic acids We used nasal lavages to carry out a comparative study of solid-phase extraction parameters of practical importance in securing good recoveries of leukotrienes and hydroxyeicosatetraenoic acids from biological samples We evaluated the influence of the pH of the sample, the pH of the water used to wash the extraction cartridge before elution of the adsorbed analytes, the comparative behaviour of commercially available octadecyl adsorbents and the influence of the concentration–evaporation step on final recoveries Data thus obtained show that there is no significant difference in results when the samples and the water used to wash the cartridge before analyte elution are adjusted to pH values ranging between 4.0 and 7.4. Below pH 4 0, losses may be significant. Furthermore, recoveries can be very much dependent on the type of solid-phase cartridge material and on the eluent evaporation method, especially with regard to aqueous phase removal.

INTRODUCTION

The nose has been used as an *in vivo* model for the study of the mechanisms involved in allergic and non-allergic inflammatory reactions. Since the nose is readily affected by different stimuli, the protection given by a variety of drugs can be easily evaluated Nasal secretions have been studied in rhinitic patients to assess the role of inflammatory mediators, such as kinins [1], prostaglandins [2] and histamine [3]. However, this model can be expanded to evaluate new mediators and additional stimuli. Many research teams are now involved in these stud-

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ies, so standarization of nasal secretion, collection and metabolite extraction procedures is necessary to facilitate comparative studies. Prostaglandins (PGs), leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs), collectively known as eicosanoids because of their 20-carbon-atom backbone, are metabolites of arachidonic acid (AA) that play an important role in the pathophysiology of asthma [4] and allergic rhinitis [5].

Several authors have described the *in vivo* release of PGs and LTs into nasal secretions from healthy volunteers [6] and allergic patients after antigen challenge provocation [7,8]. We have recently reported the first identification of 15-HETE in nasal secretions from healthy individuals [9]. However, simultaneous extraction of the different AA metabolites is difficult owing to the diverse and multifunctional nature of these compounds. Solid-phase C_{18} cartridges have been used, although recoveries reported for the sulphido-peptide LTs are poor [10,11]. A limiting step for the determination of AA metabolites is the required evaporation of the organic eluent fraction containing the eicosanoids. For their determination by high-performance liquid chromatography (HPLC) and/or radioimmunoassay (RIA) [12], there is a need to concentrate these fractions.

To establish the profile of the AA metabolites released by the human nasal mucosa and to assess the role of these compounds in allergic rhinitis, we have exhaustively evaluated the extraction of LTs and HETEs from nasal secretions. In our investigation we have considered some controversial aspects of practical importance, such as the pH of the biological sample, the influence of the pH of the water used to wash the C_{18} solid-phase cartridge and the losses associated with the concentration and evaporation of organic eluates from these cartridges. Finally, the use of different commercially available C_{18} adsorbents was also studied. This kind of information is not sufficiently well covered by the manufacturer's or the scientific literature. There are no data available on the simultaneous extraction of cyclo- and lipoxygenase metabolites of AA.

This paper describes a rapid, reliable and reproducible simultaneous extraction procedure, which is an improvement over other reported methods for either class of these metabolites.

EXPERIMENTAL

Materials

Tritiated LTB_4 (32 Ci/mmol), LTC_4 (38.4 Ci/mmol), 15-HETE (213 Ci/mmol), PGD_2 (185 Ci/mmol), PGE_2 (160 Ci/mmol) and $PGF_{2\alpha}$ (180 Ci/mmol) were purchased from Amersham International (Amersham, U.K.). PGB_2 was obtained from Sigma (St. Louis, MO, U.S.A.). C_{18} reversed-phase extraction cartridges were purchased from three sources: J. T. Baker (Phillipsburg, NJ, U.S.A.), (SPE) Baker, 100 mg adsorbent, mean particle size 40 μm and mean pore size 6 nm; Waters Assoc. (Milford, MA, U.S.A.), Sep-Pak, 400 mg adsorbent, particle size 55–105 μm, pore size 5–30 nm; and Amersham, Amprep, 100 mg adsorbent, mean particle size 40 μm, mean pore size 6 nm.

Nasal lavage samples

An isotonic solution (4 ml, 0.9% NaCl) was instilled into each nostril, while the subjects extended the neck *ca.* 30° from the horizontal and abstained from breathing or swallowing. After 10 s the volunteers expelled the mixture of saline and nasal secretions into polypropylene tubes. A pool of nasal lavages was immediately used for the extraction studies.

Extraction and purification

Tritiated standards (14 pg/ml 15-HETE, 15 pg/ml LTB_4, 111 pg/ml LTC_4, 18 pg/ml $PGF_{2\alpha}$, 20 pg/ml PGE_2 and 18 pg/ml PGD_2) were added to supernatants of centrifuged (10 000 *g*, 15 min, 4°C) nasal lavages at different pH values (4.0 and 5.8). Samples were processed through the three different commercial C_{18} cartridges (Baker, Sep-Pak and Amprep). After sample addition the cartridges were washed with 10 ml of water at different pH values (4.0, 5.8 or 7.4). Finally, LTs and HETEs were eluted with 5 ml of methanol–water (90:10, v/v) [13]. PG recoveries (only at pH 4) were also evaluated.

C_{18} cartridge recoveries were measured by scintillation counting of the eluates directly collected into scintillation vials, after evaporation of the organic phases, without water elimination. Complete process recoveries were estimated after vacuum evaporation of eluates to dryness in a Savant concentrator–evaporator (Savant Instruments, Hicksville, NY, U.S.A.). Dried residues were redissolved in acetonitrile and aliquots were also withdrawn for scintillation counting using an LKB rackbeta counter (Turku, Finland). The recoveries of LTs and HETEs were evaluated using Powell's generally applied method for prostanoid extraction [14].

To evaluate losses upon evaporation to dryness of cartridge eluates, recoveries of [^{3}H]15-HETE were obtained after subjecting these eluates to forced evaporation in three different ways: under a helium stream at 20°C; under vacuum to complete dryness; and under vacuum just to removal of methanol in the eluent followed by lyophilization to complete dryness.

The mobile phase used to separate lipoxygenase metabolites and PGB_2 was methanol–water–trifluoroacetic acid–triethylamine (80:20:0.5:0.01, v/v). Separation of cyclooxygenase metabolites was carried out with 40 m*M* formic acid (pH 3.15) with triethylamine–acetonitrile (68:32, v/v) as a mobile phase. All separations were carried out on C_{18} reversed-phase HPLC columns.

For evaluation of HPLC recoveries, aliquots of tritiated samples were injected into the chromatograph, and fractions corresponding to retention times of tritiated compounds were individually collected. The mobile phase of each fraction was evaporated, and the remaining water was lyophilized. The resulting residues were redissolved in acetonitrile and counted by liquid scintillation. Samples containing non-tritiated PGB_2 (25 ng/ml was added to supernatants of nasal lavages) were monitored at 280 nm, and quantification was made by comparison with peak areas obtained from a PGB_2 standard.

RESULTS AND DISCUSSION

The effect of sample pH on eicosanoid extraction recoveries is shown in Table I. The good recoveries obtained for LTC_4, LTB_4 and both HETEs would not justify the acidification of samples, as proposed by several authors [11,13], when extracting lipoxygenase metabolites from biological samples. This is in line with previous studies of Verhagen *et al.* [15], who reported good recoveries without acidification. Also, similar results were obtained in a study of the influence of the pH of the cartridge water-wash on lipoxygenase product recoveries (Table II). In all cases, the final elution of the AA metabolites was carried out with methanol–water (90:10, v/v) which was found to improve LTC_4 recovery relative to the use of absolute methanol [13].

Results shown in Tables I and II demonstrate that prior acidification to pH 4.0, both of the nasal lavage samples and of the water used to wash the cartridges before analyte elution, is not necessary. Omission of the acidification of nasal lavages, which are usually at at pH of *ca.* 5.8 when collected, would help to prevent LTC_4 degradation, which is reported to occur in acid media [16]. Such an effect may be noticeable when recoveries are obtained after acidification of the samples to pH 3.15 prior to their loading onto the cartridges as described by Powell [14] (Table III). Powell [14] eluted the samples subsequently with water (pH 3.1), petroleum ether and methyl formate. These results suggest that this extraction procedure may not be suitable for the sulphido-peptide LTC_4, although it can be considered adequate for the simultaneous extraction of PGs, HETEs and LTB_4 from biological extracts.

Evaporation to dryness of eluates from C_{18} minicolumns is an obligatory step for subsequent quantitative analyses by HPLC and/or RIA [12]. Since solvent removal is in general a critical step leading to significant sample losses, we have

TABLE I

COMPARISON OF PERCENTAGE RECOVERIES OF TRITIATED LTs AND HETEs FROM NASAL LAVAGES AT TWO SAMPLE pH VALUES

C_{18} Baker minicolumns; $n = 6$, mean ± S.D.

Eicosanoid	Recovery (%)	
	pH 4.0	pH 5.8[a]
LTC_4	89.8 ± 1.9	85.4 ± 2.7
LTB_4	103.3 ± 2.0	100.1 ± 1.4
15-HETE	97.0 ± 1.8	92.8 ± 2.7
5-HETE	103.2 ± 1.0	96.7 ± 2.2

[a] Non-adjusted natural sample pH.

TABLE II

COMPARISON OF PERCENTAGE RECOVERIES OF TRITIATED LTs AND HETEs FROM NASAL LAVAGES AT NATURAL SAMPLE pH AND AT DIFFERENT pH VALUES OF THE WASHING WATER

C_{18} Baker minicolumns, $n = 6$, mean ± S.D.

Eicosanoid	Recovery (%)		
	pH 4 0	pH 5.8	pH 7 4
LTC_4	84.3 ± 1.7	83 9 ± 2.6	86 3 ± 1.8
LTB_4	99.7 ± 2.1	100 1 ± 1.4	100 8 ± 0 8
15-HETE	90 1 ± 1.3	95 3 ± 1.6	96.9 ± 1.8
5-HETE	95 9 ± 1.6	96 7 ± 2 2	94.9 ± 1 8

evaluated the overall recoveries of PGs, LTs and HETEs along the complete analytical process of sample extraction and concentration by solvent evaporation prior to HPLC and/or RIA determinations.

Table IV shows the solid-phase extraction recoveries for the sulphido-peptide leukotriene C_4, as well as for LTB_4 and 15-HETE, on three different commercial C_{18} adsorbents (Sep-Pak, J. T. Baker and Amprep). Results indicate that the extraction process is equally efficient for LTB_4 and 15-HETE on each of the three cartridge types. However, for LTC_4, significantly lower recoveries were obtained with the Baker cartridges.The latter was observed with six different samples. This result cannot be related to the known physicochemical characteristics of the three commercial C_{18} materials or to their carbon coverages determined by elemental organic analyses, which were 12.5, 16.9 and 19% for Sep-Pak, Baker and Amprep, respectively.

These results were obtained by direct scintillation counting of the 5-ml metha-

TABLE III

PERCENTAGE RECOVERIES OF PGs, LTs AND 15-HETE FROM NASAL LAVAGES AT pH 3 15, AS IN POWELL'S METHOD

C_{18} Baker minicolumns; $n = 6$; mean ± S.D

Eicosanoid	Recovery (%)
LTC_4	14 1 ± 1.5
LTB_4	94 1 ± 1.5
15-HETE	58 5 ± 3.1
PGE_2	90 1 ± 2.7
PGD_2	90.2 ± 3.4
$PGF_{2\alpha}$	91 9 ± 2.0

nol–water (90:10) eluates before their evaporation to dryness. Therefore possible additional losses upon evaporation were not taken into account. On the other hand, losses were important for LTC_4 and 15-HETE if recoveries were determined after the complete evaporation of the methanol–water phase (see Table IV under C_{18} + evaporation) In order to establish the origin of these losses we evaluated the role of the evaporation procedure. As illustrated in Table V, large variations in recoveries for 15-HETE can be observed at this stage, depending both on the physical characteristics of the type of solid-phase material used and on whether samples were evaporated (a) under a gentle helium stream with the vial inserted in a heated aluminium block at 20°C, thus forcing the preferential removal of the methanol phase over the aqueous phase, (b) under vacuum followed by lyophilization of any remaining water, or (c) under vacuum to complete dryness. The results shown in Table V confirm the data shown in Table IV for 15-HETE and prove that vacuum evaporation to dryness of the 5-ml methanol–water (90:10) eluate in a Savant concentrator–evaporator system could be critical, both for 15-HETE and LTC_4.

The differences observed between cartridge systems could be related to the higher retention of water in the Sep-Pak cartridge during sample loading and subsequent water-wash stages. The fraction of immobilized water retained in the cartridge seems to be eluted with the final methanol–water mixture and thus extends the evaporation time. Experimentally, it has been observed that the final eluate volume from the Sep-Pak columns is always on the average 6% higher than the volume collected from the Baker or Amprep columns.

This observation would be consistent with the lower carbon coverage of the Sep-Pak material. On the other hand, the effect of the larger amount of adsorbent

TABLE IV

COMPARATIVE RECOVERIES FOR LIPOXYGENASE AA METABOLITES FROM NASAL LAVAGES ON THREE C_{18} ADSORBENTS BEFORE (C_{18}) AND AFTER EVAPORATION TO DRYNESS (C_{18} + EVAPORATION)

n = 6, mean ± S.D.

Eicosanoid	Recovery (%)			
	Sep-Pak	Baker	Amprep	
LTC_4	95.5 ± 2.0[a]	85.4 ± 2.7	94.5 ± 2.6[a]	C_{18}
	46.3 ± 16.5	39.2 ± 9.4	58.8 ± 8.5	C_{18} + evaporation
LTB_4	95.2 ± 1.1	96.3 ± 1.3	96.6 ± 1.4	C_{18}
	89.7 ± 6.9	83.2 ± 6.6	89.1 ± 1.1	C_{18} + evaporation
15-HETE	97.5 ± 1.2	100.4 ± 1.9	100.0 ± 1.7	C_{18}
	49.9 ± 15.4	92.9 ± 6.1	98.2 ± 1.1	C_{18} + evaporation

[a] $p < 0.001$ *versus* Baker (Student's *t*-test).

TABLE V

COMPARATIVE RECOVERIES OF 15-HETE ON THREE C_{18} ADSORBENTS WITH DIFFERENT SYSTEMS OF EVAPORATION OF ELUENT

$n = 6$; mean ± S D

Method of evaporation	Recovery (%)		
	Sep-Pak	Baker	Amprep
Helium stream at 20°C, uncomplete evaporation	100 1 ± 2.1	96 9 ± 4 9	97 3 ± 1.5
Vacuum evaporation and lyophilization to complete dryness	82.7 ± 0 9	88.9 ± 1.5	90 7 ± 2.5
Vacuum evaporation to complete dryness	44.6 ± 4.4	91.3 ± 2 5	93.5 ± 0 7

in Sep-Pak cartridges (400 *versus* 100 mg in Amprep and Baker cartridges) cannot be excluded. Furthermore, the progressive enrichment of the aqueous component in the methanol–water mixture during the evaporation would result in increasing boiling points of the mixture and thus longer total evaporation times. Both of these factors would adversely affect the stability of labile compounds. Thus, these results show that total evaporation of the eluent liquid phase is best carried out either under vacuum or a helium stream, and then completed by lyophilization.

Results shown in Tables II and III indicate that lipoxygenase metabolites from nasal lavages can be extracted by C_{18} minicolumns without acidification. However, this would not be applicable in principle to the extraction of PGs, which requires the acidification of the sample prior to its loading into the C_{18} cartridges. On the other hand, as indicated before, acid conditions could promote LTC_4 degradation [16].

Taking the results shown in Tables I and II into account, we have evaluated the individual C_{18} solid-phase extraction and HPLC purification recoveries, as well as the complete process recoveries for PGE_2, PGD_2 and $PGF_{2\alpha}$ at pH 4.0 in C_{18} Amprep columns by comparing them with those obtained for the lipoxygenase metabolites under the same conditions. As shown in Table VI, recoveries obtained for HETEs, LTs and PGs would facilitate the simultaneous extraction of these AA metabolites from nasal lavages, even though in this case PGs were not extracted at pH 3.1, as in Powell's method [14]. Although PGB_2 has been included because of its widespread use as an internal standard in many eicosanoid determinations, its relatively higher recoveries are most likely due to the fact that its recoveries had to be evaluated at the nanogram level (no tritiated standards of PGB_2 are available) owing to insufficient sensitivity of the UV detector.

TABLE VI

OVERALL RECOVERIES OF EICOSANOIDS AFTER C_{18} AMPREP EXTRACTION, HPLC PURIFICATION AND COMPLETE EVAPORATION UNDER VACUUM

Sample adjusted to pH 4; $n = 6$; mean ± S D

Eicosanoid	Recovery (%)		
	C_{18} Amprep	HPLC	Complete process
LTC_4	58 8 ± 8 5	72.1 ± 5.7	45 9 ± 4.8
LTB_4	95.4 ± 0 4	80.6 ± 6 5	76.9 ± 6.1
15-HETE	97 1 ± 2.1	69.0 ± 3 7	66 7 ± 4.0
PGD_2	82 9 ± 2.4	68.7 ± 4.2	61 7 ± 7 1
PGE_2	86 4 ± 3.8	74 0 ± 2 9	65 1 ± 4 5
PGF_2	83.1 ± 5.5	69 3 ± 2 9	62.3 ± 4.3
PGB_2			85.2 ± 2 5

CONCLUSIONS

We have evaluated in detail the simultaneous extraction of the cyclooxygenase and lipoxygenase AA metabolites from nasal secretions. Our results show that (1) nasal secretions do not need to be acidified for the extraction of lipoxygenase metabolites, (2) the prior acidification of samples to pH 4.0 allows simultaneous extraction of cyclooxygenase and lipoxygenase metabolites, and (3) the evaporation–concentration process in C_{18} cartridge eluates is critical for LTC_4 and 15-HETE and depends on the technique selected for solvent removal.

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