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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC EXTRACTION AND QUANTIFICATION PROCEDURE FOR LIPOXYGENASE METABOLITES

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SUMMARY

A number of methods have been used to measure various lipoxygenase metabolites in aqueous samples. These methods, however, suffer from three major limitations: first, they require extensive extraction and isolation from protein-containing media; second, mainly due to the first limitation, they have poor recoveries; and third, these methods usually require a two-step procedure, one for the actual extraction and the other for the quantification of the lipoxygenase metabolites. We have developed a fully automated high-performance liquid chromatographic method which circumvents these limitations. As a result, we are able to obtain high recoveries of various lipoxygenase metabolites from protein-containing samples (i.e. biological samples) while simultaneously quantifying each metabolite. The method employs a column venting technique, whereby the fatty acids are extracted by a pre-column and the proteins are vented to waste. The pre-column eluate is then directed through the analytical column which separates the lipoxygenase metabolites. The described method is reproducible and minimizes both the time and the cost involved in assaying a sample.

INTRODUCTION

The lipoxygenase metabolites of arachidonic acid have become of interest since their initial discovery in platelets and polymorphonuclear leukocytes [1,2]. Recently, other lipoxygenase-derived metabolites from fatty acids, such as linoleic acid, have also been described in a variety of cell types [3–6]. The identification of these metabolites has generated the interest to study what role these compounds play in the biology of various cell interactions. This has resulted in the need to develop an assay system which can quantify these metabolites in various biological preparations. Unfortunately, unlike the metabolites of the cyclo-oxygenase pathway, which are readily detectable by radioimmunoassay, the majority of lipoxygenase metabolites must, at present, be quantified either by high-per-

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formance liquid chromatography (HPLC) or by gas chromatography–mass spectrometry (GC–MS). Presently, the quantification of these metabolites by both normal-phase HPLC and by GC–MS requires that the aqueous biological samples first be extracted and then concentrated in organic solvents prior to their measurement. The use of reversed-phase HPLC offers advantages as the aqueous samples can be analyzed directly, providing that the metabolite levels are sufficiently high and the protein content sufficiently low. However, since most cell suspensions contain low metabolite and high protein levels, sample concentration and/or protein precipitation are still prerequisites for their analysis.

To overcome these technical problems, both lipoxygenase and cyclo-oxygenase metabolites have been extracted from acidified aqueous samples using organic solvents such as diethyl ether, chloroform–methanol mixtures or ethyl acetate. These procedures are both time-consuming and require the evaporation of large volumes of solvent. A variety of solid-phase extraction techniques have also been introduced, including the use of Amberlite XAD resins and octadecyl (C_{18}) extraction columns [7–11]. These techniques result in good to excellent recoveries of various prostaglandins (PGs), monohydroxy fatty acids and leukotrienes (LTs), but they are also time-consuming and require a certain technical expertise in order to obtain satisfactory quantitative results.

Recently, Borgeat et al. [12] described a reversed-phase HPLC method that overcame the problem of cell particulates in the sample. This method allowed for a direct analysis of relatively large sample volumes (2 ml) derived from various cell suspensions. The major limitation of this method is, however, that cell suspensions containing protein [e.g. 0.35% bovine serum albumin (BSA)] cannot be assayed, as the quantitative precipitation of all proteins is not obtained. To overcome this latter limitation, we developed an automated extraction procedure which permits the direct analysis of lipoxygenase metabolites in proteacious media. This automated method provides excellent recoveries of various lipoxygenase metabolites including the peptido-leukotrienes which, in the past, have posed a particular problem due to their positive charge and liability at low pH values.

EXPERIMENTAL

HPLC-grade acetonitrile was obtained from BDH (Mississauga, Canada). Hank's balanced salt solution (HBSS) was purchased from Gibco (Burlington, Canada). All other solvents were purchased from Fisher Scientific (Mississauga, Canada). Unlabelled fatty acids, prostaglandin B_2 (PGB_2) and essentially fatty acid-free BSA were obtained from Sigma (St. Louis, MO, U.S.A.). The BSA was further purified by precipitating 5 g of the BSA in 100 ml of acetonitrile–water (90:10, v/v) and lyophilized prior to use. ACS scintillation fluid, ^{3}H-labelled leukotriene C_4 (LTC_4), 12-hydroxyeicosatetraenoic acid (12-HETE) and 5-HETE were obtained from Amersham (Oakville, Canada). These ^{3}H-labelled metabolites were purified by HPLC prior to use. Synthetic LTC_4, LTD_4 and 8,15-dihydroxyeicosatetraenoic acids (diHETEs) were kind gifts from Drs. G. Letts and J. Rokach (Merck Frosst Canada, Dorval, Canada). Unlabelled 13-hydroxyoctadecadienoic acid (13-HODE), 12-HETE, 15-HETE, 15-hydroxyeicosatrienoic

acid (15-HETE:3) and 15-hydroxyeicosapentaenoic acid (15-HEPE) were prepared as described previously [13,14]. HPLC-grade water was prepared using a Milli-Q water purifier obtained from Millipore (Mississauga, Canada). The data were analysed by two-way analysis of variance and linear regression analysis using minitab software, version 5.1.

Reversed-phase high-performance liquid chromatography

All reversed-phase HPLC equipment and columns were purchased from Waters Scientific. The instrumentation was composed of two pumps, an RCM 100 cartridge module, a Wisp™ automatic injector, an M490 programmable detector, an M720/730 system controller/integrator and a WAVS column/solvent selector. A Nova-Pak cartridge (3 μm, 100 mm$\times$5.0 mm I.D.) and a μBondapak C_{18} pre-column were used in system 1 for the separation of monohydroxy fatty acid, PGB_2 and the diHETEs. A resolve-C_{18} cartridge (5 μm, 100 mm$\times$8.0 mm I.D.) and a resolve C_{18} pre-column were used in system 2 for the detection of the LTs, PGB_2 and the diHETEs.

Three solvents were used: solvent A was composed of acetonitrile–water–orthophosphoric acid (15:85:0.01, v/v/v); solvent B was composed of acetonitrile–water–orthophosphoric acid (80:20:0.01); and solvent C was composed of acetonitrile–water–orthophosphoric acid (40:60:0.03), pH adjusted to 6.0 with 6 *M* sodium hydroxide. The solvent gradients used are described under *Automated extraction procedure.*

All metabolites were detected and quantified by UV spectrophotometry at the wavelength of their maximum absorbance, i.e. 234 nm for the monohydroxy fatty acids, 270 nm for PGB_2 and the diHETEs, and 280 nm for the LTs.

Automated extraction procedure

All samples to be analyzed were brought to a final acetonitrile concentration equal to that of the initial HPLC solvent used for that analysis (i.e. solvent A or another loading solvent) prior to their injection. The WAVS automatically controlled the solvent pathway from the pre-column and directed the solvent flow either through the analytical column or to waste. Automated HPLC extractions were performed using the pre-column method. The extractions were performed by injecting the sample into the solvent pathway and eluting it onto the pre-column. All unretained material which eluted through the pre-column during its loading and subsequent washing was diverted to waste (Fig. 1a). After 3 min, the flow of solvent from the pre-column was diverted from waste to the analytical column and all retained metabolites were eluted from the pre-column using an acetonitrile gradient (Fig. 1b).

In order to calculate the recovery of the lipoxygenase metabolite, a method had to be used which gave complete recovery of all metabolites injected. The method developed (100% recovery method) was identical to the pre-column method except that the flow of solvent from the pre-column was always directed through the analytical column. As the solvent continually flowed through the analytical column using the 100% recovery method, only protein-free media could be injected. Confirmation that this method gave complete recovery of all metabolites

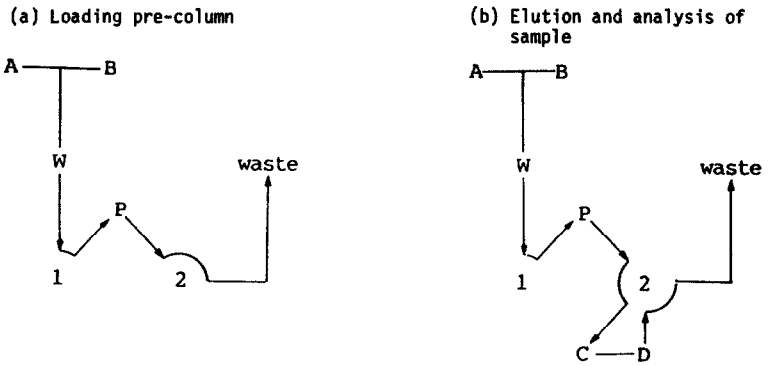

Fig. 1. Solvent pathway during the extraction and analysis of a sample. (a) Solvent flow for first 3 min; (b) solvent flow after 3 min. A = Pump A; B = pump B; C = column; D = detector; P = precolumn; W = Wisp injector; 1 = event 1 of WAVS; 2 = event 2 of WAVS.

TABLE I

SOLVENT GRADIENT 1, USED FOR THE ANALYSIS OF MONOHYDROXY FATTY ACIDS AND PGB_2

Analysis time (min)	Flow-rate (ml/min)	Solvent		Rate of change
		%A	%B	
0.0- 2.0	1.0	100	0	Isocratic
2.0- 2.5	1.5	50	50	Linear
2.5-12.0	1.5	35	65	Linear
12.0-18.0	1.5	35	65	Isocratic
18.0-20.0	1.5	0	100	Linear
20.0-30.0	2.5	0	100	Convex
30.0-30.5	1.5	100	0	Linear
30.5-35.0	1.0	100	0	Delayed
40	Injection of the next sample			

was performed by injections of various radiolabelled metabolites and determining the amount of radioactivity which eluted at the appropriate retention time.

The analytical column and the pre-column used for the quantification of the monohydroxy fatty acids were a Nova-Pak cartridge and a μBondapak pre-column (system 1), respectively. Solvent gradient 1, which was the gradient used in system 1, is given in Table I. It should be noted that this solvent gradient will not separate all of the diHETEs, which require a methanol–acetontrile mixture and a more gradual gradient between 2.5 and 12 min [7,15]. However, this solvent gradient was adequate for calculating recovery yields of PGB_2.

A modification of the solvent gradient of Borgeat et al. [12] was used in system 2 for the analysis of the LTs and is given in Table II (gradient 2). The WAVS commands were the same as those shown in Fig. 1, but a resolve C_{18} cartridge and pre-column replaced the Nova-Pak cartridge and the μBondapak pre-column that were used in system 1.

TABLE II

SOLVENT GRADIENT 2, USED FOR THE ANALYSIS OF PGB_2, THE diHETEs AND THE LTs

Analysis time (min)	Flow-rate (ml/min)	Solvent			Rate of change
		%A	%B	%C	
0.0- 2.5	1.0	100	0	0	Isocratic
2.5- 3.0	2.0	50	50	0	Linear
3.0-15.0	2.0	50	50	0	Isocratic
15.0-15.5	4.0	0	100	0	Linear
15.5-20.0	4.0	0	100	0	Isocratic
20.0-20.5	2.0	100	0	0	Linear
20.5-24.0	2.0	0	0	100	Delayed
24.0-40.0	2.0	100	0	0	Delayed
40.0-45.0	1.0	100	0	0	Delayed
45.0	Injection of next sample				

RESULTS AND DISCUSSION

In a preliminary study using a C_{18} extraction column (Sep-Pak, Waters Scientific), we found that the addition of an organic solvent, such as acetonitrile or methanol, to plasma increased the recovery of lipoxygenase metabolites by at least 10% (data not shown). In light of this observation, this study investigated the effects of acetonitrile concentrations, sample volume and protein content on the recovery of lipoxygenase metabolites, using an automated HPLC extraction method.

The effects of varying the acetonitrile concentrations in both the sample and the loading solvent on the recoveries of 13-HODE, 15-HETE and PGB_2 from HBSS are shown in Table III. Gradient No. 1 was used when the loading acetonitrile concentration was 15%. For the loading solvents other than 15% acetonitrile, the composition of solvent A and the steepness of the gradient were changed accordingly to allow for the proper separation of all metabolites studied. When 40% acetonitrile was used as the loading solvent, there was a complete loss of PGB_2, indicating that no diHETEs were recovered. Lowering the acetonitrile concentration to 20 and 15%, increased the recovery of PGB_2 to 96 and 100%, respectively. There was a 100% recovery of the monohydroxy fatty acids at both concentrations. A further lowering of the acetonitrile concentration to 5% resulted in poor qualitative recoveries, as there was considerable variation. Therefore, since there was poor recovery of PGB_2 at high acetonitrile concentrations and considerable variation at low concentrations, all further recoveries were studied at an acetonitrile concentration of 15% (solvent A).

The extraction of various monohydroxy fatty acids was then evaluated over a metabolite range (5–200 ng). The recoveries of each metabolite at the different concentrations were similar and, therefore, the average overall recoveries are shown in Table IV. The recoveries for all metabolites exceeded 99% at all concentrations tested, when a concentration of 15% acetonitrile was used as the load-

TABLE III

EFFECT OF ACETONITRILE CONCENTRATION ON THE RECOVERY OF MONOHYDROXY FATTY ACIDS AND PGB_2

The metabolites were dissolved in HBSS and were brought to a final acetonitrile concentration equal to that used in the loading HPLC solvent. With the flow of solvent eluting from the pre-column directed to waste, 500 μl of each sample were injected onto the pre-column. The pre-column was then washed for 3 min using the loading acetonitrile concentration. The solvent pathway from the pre-column was then directed through the analytical column and a gradient was then used to elute the metabolites through the analytical column. The amount of metabolites extracted were quantified by UV absorption.

Loading acetonitrile concentration (%)	n	Recovery (mean ± S.D.) (%)		
		13-HODE	15-HETE	PGB_2
40	6	96.5 ± 1.0	99.6 ± 1.3	< 0.1
20	3	100.4 ± 0.8	99.9 ± 0.6	95.9 ± 0.3
15	3	100.2 ± 0.2	99.5 ± 0.8	100.3 ± 0.1
5	3	104.1 ± 8.9	91.9 ± 5.2	109.8 ± 6.3

TABLE IV

AVERAGE RECOVERY OF MONOHYDROXY FATTY ACIDS FROM HBSS OVER A METABOLITE CONCENTRATION RANGE

Volumes of 500 μl of HBSS containing different concentrations of each metabolite (15–200 ng) were injected using the pre-column method and the 100% recovery method. There were no differences in the recoveries of each metabolite at the various concentrations, and therefore the data are expressed as the mean percentage recovery ± S.D. of the six concentrations tested, with $n = 3$ at each concentration.

Metabolite	Recovery (%)
13-HOTE	100.1 ± 0.4
13-HODE	99.5 ± 0.6
12-HETE	100.7 ± 1.5
15-HEPE	100.6 ± 1.2
15-HETE	99.5 ± 1.8
15-HETE:3	99.6 ± 1.0

ing solvent. This indicated that the percentage recovered by this extraction method was not affected by the metabolite level. In order to see if there was any difference between the two methods (100% recovery versus pre-column), the lines of best fit of the data for 13-hydroxyoctadecatrienoic acid (13-HOTE) extraction from HBSS were calculated. The regression lines of the form $y = (a \pm s)x + (b \pm s)$ where y = area under the peak, x = amount of 13-HOTE injected in ng and s = standard deviation were as follows: for the 100% recovery method, $y = (28.63 \pm 0.04)x - (28.82 \pm 4.79)$; and for the pre-column method, $y = (28.57 \pm 0.04)x - (23.50 \pm 4.53)$. Statistical analysis of the data indicated that both regression lines were linear over the metabolite range, and that the equa-

tions for each line were similar ($\alpha = 0.10$) and highly correlated, $r \geq 0.998$ intra- and inter-method. Similar results were found for all the other monohydroxy fatty acids tested (data not shown). This regression analysis indicates the efficiency of the extraction methodology, as the pre-column method and the 100% recovery method were almost identical.

We then wanted to determine whether protein content or sample volume influenced the recoveries of the lipoxygenase metabolites since we had only demonstrated excellent recoveries in protein-free media. System 1 was used to measure the recoveries of the monohydroxy fatty acids and PGB_2, and system 2 to measure the recoveries of PGB_2, the diHETEs and the LTs. A resolve C_{18} pre-column and analytical cartridge were used for the extraction of the LTs as they contain free silanol groups which would likely aid in the retention of positively charged metabolites [12,16]. A μBondpak pre-column and a Nova-Pak analytical cartridge were used for the detection of monohydroxy and dihydroxy metabolites as they are endcapped (no free silanol groups), which minimized the effects of band broadening and/or tailing and the retention of unwanted material. The recoveries of all metabolites exceeded 90% in HBSS both in the absence and the presence of 0.35% albumin (Table V). Thus, there was virtually complete recovery of all metabolites. The data in Table V were obtained for injection volumes of 2000 μl. Similar results were obtained with injection volumes of 500 or 1000 μl (data not shown). These data indicate the flexibility of this method as excellent recoveries of all lipoxygenase metabolites were obtained regardless of the presence of protein in the sample or the volume of sample injected.

Additional studies were performed to determine the effect of acidification on the recoveries and stabilities of PGB_2, the diHETEs and the LTs over time at 20°C. There was less than 1% degradation of PGB_2 and the diHETEs in protein-

TABLE V

RECOVERIES OF VARIOUS LIPOXYGENASE METABOLITES FROM 2000 μl HBSS OR HBSS CONTAINING 0.35% ALBUMIN

A 100-ng amount of each metabolite dissolved in 2000 μl of HBSS or HBSS containing 0.35% albumin were injected using the pre-column method. Recoveries of 100% were calculated from the amount of each metabolite found using the 100% recovery method with an injection volume of 500 μl HBSS.

Metabolite	Recovery (mean ± S.D., $n=3$) (%)	
	HBSS	HBBS + 0.35% albumin
13-HOTE*	99.4 ± 1.1	91.2 ± 1.6
13-HODE*	102.2 ± 1.5	95.6 ± 2.3
12-HETE*	97.8 ± 1.3	93.4 ± 1.4
PGB_2*	99.8 ± 1.1	89.2 ± 1.3
LTC_4**	99.4 ± 2.5	95.6 ± 1.1
LTD_4**	98.9 ± 3.1	100.2 ± 1.3
PGB_2**	99.8 ± 2.8	93.2 ± 0.7
diHETE**	99.1 ± 1.2	91.9 ± 0.8

* Gradient No. 1 used.

** Gradient No. 2 used.

free HBSS at pH 7.35 over a period of 48 h, and there was $\simeq 1\%$ degradation of LTD_4 over 8 h. When the medium was acidified to pH 4.0, the stability and recovery of PGB_2 and diHETEs were unaffected; however, there was increased degradation of LTD_4 to 5%. LTC_4 was labile at both pH values, degrading at least 5% at pH 7.35 and at least 20% at pH 4.0 over 8 h. In contrast to the detrimental effect of acidification on the stability of LTC_4 and LTD_4, acidification of the media when injection volumes of 2000 μl were used, resulted in the beneficial effects of increased peak sharpness and a longer retention time (Fig. 2). These latter beneficial effects were likely due to the rapid absorption of the LTs positive amino groups onto the negative silanol groups of the pre-column. However, simply reducing the injection volume from 2000 to 500 μl resulted in an increased peak sharpness, making acidification redundant.

The degradation of LTC_4 and LTD_4 at both pH values was accelerated when 0.35% albumin was added to the media but had no effect on the stability of PGB_2 or the diHETEs at pH 7.35. The stabilities and recoveries of PGB_2 and the diHETEs in the acidified, albumin-containing media, however, could not be assessed as acidification led to the retention and subsequent elution of unwanted materials that completely masked the diHETE region of the chromatogram (Fig. 2b). These observations indicate that acidification should be avoided as it leads to decreased sensitivity and increased degradation of the lipoxygenase metabolites.

Unacidified albumin-containing media also lead to the retention of some unwanted material. However, this only occurred when a total of 10–14 ml of media had been injected. Even so, the diHETE region of the chromatogram was not totally obstructed as was seen after only one injection (500 μl) of acidified medium. We are presently performing additional experiments to determine what conditions can be used to increase the amount of sample that can be injected before there is excess build-up of unwanted material.

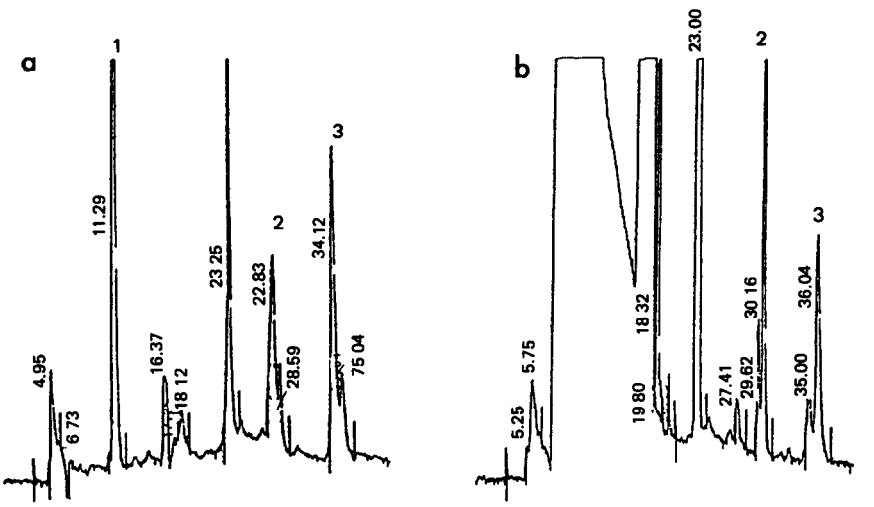

Fig. 2. Extraction of PGB_2, LTC_4 and LTD_4 from HBSS containing 0.35% albumin using gradient No. 2. (a) Unacidified HBSS; (b) acidified HBSS. Peaks: 1 = PGB_2; 2 = LTC_4; 3 = LTD_4.

In conclusion, we have demonstrated that, with the described method, we can achieve excellent recoveries for a variety of lipoxygenase metabolites both in protein-free and protein-containing media when the acetonitrile concentrations in both the sample and the loading solvent are 15%. The quantitative and qualitative results obtained with this automated system appear to be markedly improved as compared to previous methods over the ranges of sample volume, media and metabolite levels tested. Sample acidification and protein precipitation are not required. This significantly reduces the analysis time and cost. In addition, avoiding the acidification step reduces the probability of sample degradation, particularly in the case of the LTs. For these reasons, we recommend this automated reversed-phase extraction method as a relatively simple but reliable and reproducible method for the quantitative analysis of lipoxygenase metabolites in aqueous and protein-containing media.

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