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Note

Rapid, simple and efficient extraction of arachidonic acid metabolites, including the sulphidopeptide leukotrienes LTC_4 and LTD_4, using octadecyl reversed-phase extraction columns

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Leukotrienes (LT) are biologically active metabolites of arachidonic acid formed via an initial oxygenation by a lipoxygenase. LTB_4 is strongly chemotactic for human granulocytes [1]. The sulphidopeptide leukotrienes LTC_4 and LTD_4 possess potent bronchoconstrictive properties in vitro [2] as well as in vivo [3]. A variety of cell types is capable of synthesizing these compounds, e.g. neutrophils [4], eosinophils [5], monocytes [6], macrophages [7] and

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mast cells [8]. Reversed-phase high-performance liquid chromatography (HPLC) is used for the separation and quantitation of the different leukotrienes [9]. Before chromatographic analysis can be carried out, it is necessary to extract the leukotrienes from the aqueous incubation medium. Previous extraction procedures involved solvent—solvent extraction or adsorption to resins like Amberlite XAD-7 or XAD-8. Usually diethyl ether was used for LTB_4 and other less polar arachidonic acid metabolites, and Amberlite XAD-8 or XAD-7 for the sulphidopeptide leukotrienes.

Recently, a rapid and convenient extraction procedure has been introduced using octadecyl reversed-phase extraction columns. Until now, two studies have been published concerning the efficiency of these columns for leukotrienes and other arachidonic acid metabolites [10, 11]. Metz et al. [10] reported that with octadecyl reversed-phase Sep-Pak columns, recoveries for LTB_4, LTC_4, LTD_4 and prostaglandin B_2 (PGB_2) were poor, e.g. being 57 ± 20, 39 ± 18, 44 ± 8 and 87 ± 15%, respectively. Therefore, these authors proposed the use of Sep-Pak silica columns which showed recoveries for LTB_4, LTC_4, LTD_4 and PBG_2 of 76 ± 8, 93 ± 12, 86 ± 8 and 82 ± 7%, respectively, being slightly dependent on the buffering which the extraction sample was applied to the column. Although these recoveries are much better than with the Sep-Pak octadecyl column, they are still not quantitative, particularly for LTB_4, and moreover, when protein is present, a time-consuming protein precipitation step is necessary. Better recoveries for LTB_4 have been reported by Luderer et al. [11], who used Baker octadecyl reversed-phase extraction columns. Although these authors were mainly interested in the recoveries of cyclooxygenase products, they also determined recoveries for some lipoxygenase products. Recoveries for LTB_4, 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE and PGB_2 were 98 ± 2, 77 ± 10, 88 ± 9 and 87 ± 2%, respectively. Unfortunately, recoveries for the sulphidopeptide leukotrienes LTC_4 and LTD_4 were not determined. Therefore, we determined the recoveries for the sulphidopeptide leukotrienes LTC_4 and LTD_4 as well as for LTB_4, 5-HETE and PGB_2, using octadecyl reversed-phase columns from Baker and from Waters (Sep-Pak) and compared the results with the recoveries of these compounds obtained by us using Amberlite XAD-8 or diethyl ether.

EXPERIMENTAL

Materials

Tritiated LTB_4 (180 Ci/mmol), LTC_4 (40.0 Ci/mmol), LTD_4 (42.8 Ci/mmol), 5-HETE (94.8 Ci/mmol) and the LTC_4 radioimmunoassay kit were purchased from New England Nuclear (Boston, MA, U.S.A.). Synthetic LTB_4, LTC_4 and LTD_4 were a kind gift from Dr. J. Rokach (Merck-Frosst Labs., Pointe Claire/Dorval, Canada). Arachidonic acid (purity > 99%) was obtained from Fluka (Buchs, Switzerland). PGB_2, calcium ionophore A23187 and reduced glutathione were purchased from Sigma (St. Louis, MO, U.S.A.). 15(*S*)-HETE was prepared as described previously [12]. All solvents were of HPLC quality and obtained from Baker (Phillipsburg, NJ, U.S.A.) or Merck (Darmstadt, F.R.G.). Octadecyl reversed-phase extraction columns were purchased from Baker (10 SPE, 6 ml, mean particle size 0.04 mm, mean pore size 6 nm) and

from Waters (Sep-Pak, particle size 0.055—0.105 mm, pore size 5—30 nm, Milford, MA, U.S.A.). Servachrome (Amberlite) XAD-8 (particle size 0.3—0.5 mm) was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.). Bovine blood was obtained from the local slaughterhouse.

Preparation of a mixture of unlabelled leukotrienes

Bovine granulocytes were isolated as described previously [13]. $1.8 \cdot 10^9$ Cells were incubated in 45 ml of Dulbecco's salt solution with arachidonic acid (80 μM), calcium ionophore A23187 (20 μM), Ca^{2+} (final concentration 2 mM) and reduced glutathione (5 mM) at pH 7.4 and 37°C for 10 min. After termination of the reaction by adding ice to the solution, the cells were removed by centrifugation at 46 000 *g* for 10 min. The clear supernatant was used as described hereafter.

Extraction procedure

Tritiated LTB_4 (0.25 pmol), LTC_4 (1.14 pmol), LTD_4 (1.06 pmol) or 5-HETE (0.48 pmol) were each dissolved in 1 ml of water or in 1 ml of the supernatant of the bovine granulocytes incubation mixture, containing approximately 0.7 nmol of LTB_4, 1.1 nmol of LTC_4, 1.5 nmol of 12-*epi*-6-*trans*-8-*cis*-LTB_4 (sometimes referred to as 5(*S*),12(*S*)-diHETE), 0.5 nmol of 12-*epi*-6-*trans*-LTB_4 + 6-*trans*-LTB_4 and 3.2 nmol of 12-HETE. Unlabelled PGB_2 (8.93 nmol) was dissolved in 1 ml of water. After extraction of the eicosanoids using the hereafter described procedures, recoveries of tritiated LTB_4, LTC_4, LTD_4 and 5-HETE were obtained by determination of the radioactivity of the appropriate fractions by liquid scintillation counting. Recoveries of PGB_2 were obtained by measuring the UV absorption at 278 nm (ϵ = 28 650 M^{-1} cm^{-1}) of the appropriate fractions.

Extraction with octadecyl reversed-phase columns. The columns (Baker and Sep-Pak) were successively washed with ca. 10 ml of methanol, 5 ml of water and 5 ml of 0.1% EDTA solution. Washing with the EDTA solution was found essential for a quantitative recovery of the sulphidopeptide leukotrienes LTC_4 and LTD_4. The aqueous samples (pH 7.4) were applied to the columns without prior acidification, which was found not to be necessary. After washing the column twice with 5 ml of water, the adsorbed lipids were eluted with 3 ml of methanol. It should be noted that the sorbent is not allowed to dry, in particular during the conditioning of the columns prior to adsorption of the lipids. The volumes of the wash solvents are not critical. The washing of the Baker columns can be speeded up by applying a slight (nitrogen) overpressure. However, this should be avoided during the adsorption stage, because then it results in a reduced recovery. Radioactivity was determined in the methanol fraction (3 ml) and in the combined aqueous fractions (6 ml). In the case of PGB_2, the distribution of this prostaglandin over the methanolic and aqueous fractions was determined by measuring the UV absorption at 278 nm.

Extraction with Amberlite XAD-8. Before use, Amberlite XAD-8 was thoroughly cleaned according to the procedure described by Mathews et al. [14] for Amberlite XAD-7. XAD-8 resin (100 g) was added to 500 ml of ethanol and refluxed for 4 h. After removal of the turbid ethanol, another 500 ml of ethanol were added and again refluxed for 4 h.

The XAD-8 was next washed three times with 500 ml of chloroform for 0.5 h each under vacuum and then twice with 500 ml of methanol for 1 h. The methanol was removed by washing four times with 500 ml of water for 2 h under vacuum. The XAD-8 was stored as a suspension in water (500 ml) at 4°C. As much suspension was transferred to a glass column as necessary for a bed volume of ca. 1.5 ml. The aqueous extraction sample (1 ml) was then applied to the column. After adsorption of the sample, the column was washed with 1.5 ml of a 0.19 m*M* ammonium chloride solution and the adsorbed lipids were then eluted with 2.25 ml of ethanol—water (4:1). The ethanol—water fraction as well as the collected aqueous fractions (2.5 ml) were then counted for tritium. In the case of PGB_2, the amounts in both fractions were determined by measuring the UV absorption at 278 nm.

Extraction with diethyl ether. After acidification of the extraction sample (1 ml) with hydrochloric acid to pH 3, the lipids were extracted by three successive shakings with 2 ml of diethyl ether. The combined ether fractions (6 ml) were washed three times with 0.3 ml of water, evaporated to dryness and then brought up in methanol. Both the aqueous fraction and the methanol fraction were counted for tritium. In the case of PGB_2, the amounts in both phases were determined by measuring the UV absorption at 278 nm.

Reversed-phase HPLC analysis

Leukotrienes were separated and quantified as described previously [9] using a CP Spher 8 C_{18} column (250 × 4.6 mm I.D., Chrompack, Middelburg, The Netherlands) attached to a Perkin-Elmer Series 1 pump and an LC 85 detector. The solvent system was tetrahydrofuran–methanol–water—acetic acid (25:30:45:0.1) which had been brought to pH 5.5 with ammonium hydroxide. The aqueous phase contained 0.1% EDTA to prevent binding of cations to the column [10]. A flow-rate of 0.9 ml/min was maintained and the effluent was monitored at 280 nm (leukotrienes: ϵ = 40 000 M^{-1} cm^{-1}, PGB_2: ϵ = 28 650 M^{-1} cm^{-1}).

Radioimmunoassay of LTC_4

A commercially available LTC_4 radioimmunoassay (New England Nuclear) was used in accordance with the manufacturer's instructions.

RESULTS AND DISCUSSION

In Table I (columns A), recoveries are summarized of pmol quantities of tritiated LTB_4, LTC_4, LTD_4 and 5-HETE and of nmol quantities of unlabelled PGB_2 after extraction of these compounds from aqueous solutions using Baker octadecyl extraction columns, Amberlite XAD-8 or diethyl ether. Recoveries were excellent with the Baker extraction columns. However, with Amberlite XAD-8 low recoveries of 5-HETE were obtained, whereas with diethyl ether recoveries of both leukotrienes and 5-HETE were very poor. These low recoveries might be explained by the well known phenomenon that some lipids are easily adsorbed to glass walls. Contrary to the octadecyl extraction method, glass materials are normally used in Amberlite XAD-8 and diethyl ether extractions. The amount of adsorbed lipid is negligible when the total

TABLE I

RECOVERIES OF SOME TRITIATED EICOSANOIDS IN THE ABSENCE (A) AS WELL AS IN THE PRESENCE (B) OF A LARGE EXCESS OF UNLABELLED EICOSANOIDS, USING VARIOUS EXTRACTION PROCEDURES

The tritiated eicosanoids were each dissolved, in the indicated amount, in 1 ml of water (A) or 1 ml of a solution containing approximately 7 nmol of unlabelled eicosanoids (B) as described under Experimental. Unlabelled PGB_2 was only dissolved in 1 ml of water (A). After the extraction procedures had been performed, recoveries were obtained by determination of the radioactivity by liquid scintillation counting or by measuring the UV absorption at 278 nm (PGB_2). Recoveries are given as mean percentages ± standard error of the mean of five extractions. N.D. = Not determined.

Compound	Amount (pmol)	Recovery (%)							
		C_{18} Baker		C_{18} Sep-Pak		XAD-8		Diethyl ether	
		A	B	A	B	A	B	A	B
LTB_4	0.25	98 ± 4	97 ± 4	N.D.	96 ± 1	98 ± 1	98 ± 1	78 ± 1	94 ± 1
LTC_4	1.14	98 ± 3	98 ± 3	N.D.	97 ± 2	98 ± 1	97 ± 1	14 ± 1	16 ± 3
LTD_4	1.06	94 ± 3	94 ± 3	N.D.	95 ± 1	94 ± 1	94 ± 1	18 ± 1	25 ± 4
5-HETE	0.48	94 ± 3	94 ± 3	N.D.	93 ± 1	55 ± 5	94 ± 1	44 ± 1	84 ± 3
PGB_2 (unlabelled)	8930	99 ± 1	—	97 ± 4	—	99 ± 5	-	98 ± 4	-

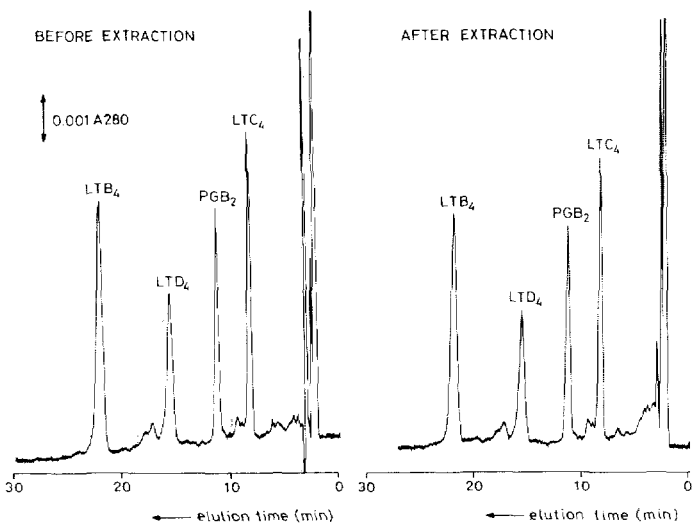

Fig. 1. Reversed-phase HPLC profiles of a mixture of LTB_4 (0.19 nmol), LTC_4 (0.13 nmol), LTD_4 (0.09 nmol) and PGB_2 (0.14 nmol), before and after extraction using octadecyl extraction columns (Baker). For experimental details, see Experimental.

amount of lipid is at the nmol level, but it can represent a considerable part of the total amount of lipid when the latter is present at the pmol level. Therefore, extractions of tritiated eicosanoids were also carried out in the presence of a large excess of unlabelled eicosanoids (7 nmol) as described under Experimental. In this case, octadecyl extraction was also performed with Sep-Pak octadecyl extraction columns (Waters). From Table I it is clear that

recoveries with diethyl ether, although much improved for LTB_4 and 5-HETE, are still very poor for LTC_4 and LTD_4. Therefore, diethyl ether extraction is unsuitable as a general extraction method for leukotrienes. In contrast, octadecyl extraction and Amberlite XAD-8 extraction show excellent recoveries. Octadecyl extraction is strongly preferred because it is simple and rapid, disposable extraction columns are commercially available, and moreover, the sorbent, unlike Amberlite XAD-8, does not require very time-consuming cleaning.

From the reversed-phase HPLC profiles shown in Fig. 1, it is obvious that extraction using octadecyl extraction columns (Baker) does not result in any quantitative or qualitative changes of the eicosanoid mixture. It has been reported that the sulphidopeptide leukotrienes decompose rapidly at low pH [15]. In fact, we observed a rapid destruction of LTC_4 and in particular LTD_4 on the extraction columns at low pH. Therefore, acidification should be omitted. Quantitative recoveries for the sulphidopeptide leukotrienes were obtained only when the column was prewashed with a 0.1% EDTA solution. Because Metz et al. [10] found poor recoveries of leukotrienes with Sep-Pak octadecyl reversed-phase columns, they advised the use of silica Sep-Pak columns. Although with these columns much better results were obtained, recoveries were still not quantitative, particularly for LTB_4. Moreover, proteins should be removed first by a denaturation—centrifugation step. In contrast, octadecyl reversed-phase extraction of eicosanoids from aqueous samples using Baker columns, can be performed in the presence of a large excess of protein. For example, 0.04 mg of 15-HETE was found to be quantitatively recovered in the presence of 100 mg of albumin. LTC_4 extracted from an aqueous sample with the Baker column, was quantitated with reversed-phase HPLC as well as with an LTC_4 radioimmunoassay. As both assays showed identical results, it can be concluded that no immunoreactive material is eluted from the column that is able to interfere in the LTC_4 radioimmunoassay. In summary, extraction of LTB_4, the sulphidopeptide leukotrienes LTC_4 and LTD_4, 5-HETE, 15-HETE and the internal standard PGB_2 can be performed quantitatively, even at the (sub)pmol level, using octadecyl reversed-phase extraction columns. The procedure is rapid and simple, requiring no extensive and time-consuming cleaning of the sorbent, no acidification of the extraction sample and no prior precipitation of proteins. Therefore, octadecyl reversed-phase extraction is, at present, the most suitable method for the quantitative extraction of oxidative metabolites of arachidonic acid from aqueous media.

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