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Determination of picomole amounts of lipoxins C_4, D_4 and E_4 by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A method for the sensitive determination of the sulphopeptide lipoxins (LXs) C_4, D_4 and E_4 by high-performance liquid chromatography with subsequent electrochemical detection is described. The best results were obtained when the analysis was carried out with the solvent system methanol–water–trifluoroacetic acid (66:34:0.008, v/v/v). The acquired half-wave potentials were different for all investigated compounds: +1.18 V for LXC_4 +1.3 V for LXD_4 and +1.25 V for LXE_4. The detection limits of LXC_4, LXD_4 and LXE_4, based on a signal-to-noise ratio of 3:1, were found to be 200–700 fmol. Although sulphopeptide lipoxins possess a high molar absorptivity, electrochemical detection still is three times more sensitive than ultraviolet detection.

INTRODUCTION

Lipoxins (LXs), which are arachidonic acid metabolites formed by the interaction of the 5- and 15-lipoxygenase pathways, have been shown to exhibit various biological activities. LXA_4 leads to superoxide anion generation and the degranulation of human neutrophils, promotes chemotaxis, contracts lung parenchymal stripes and activates protein kinase C *in vitro*. LXA_4 and LXB_4 inhibit natural killer cell activity [1].

(15*S*)-Hydroxy-5,6-epoxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid has been suggested to be a common intermediate in the biosynthesis of LXA_4 and LXB_4 in human granulocytes [2]. LXA_4 has been found in extracts from human eosinophils stimulated with the ionophore A23187 and arachidonic acid [3]. Moreover, human eosinophils contain considerable amounts of glutathione-S-transferase [4]. This enzyme catalyses the conjugation of glutathione to another well known allylic epoxide, LTA_4, leading to the sulphopeptide leukotrienes (LTs) LTC_4, LTD_4 and LTE_4. Combining the 5- and 15-lipoxygenase with the glutathione-S-transferase pathway, it has been shown that human eosinophils can release amino acid containing lipoxins. Owing to the similar biosynthetic route of these compounds to LXA_4 and LXB_4, and in accordance with the nomenclature of leukotrienes, the trivial names LXC_4, LXD_4 and LXE_4 were suggested [5] (structures given in Fig. 1).

The method of choice for the qualitative determination of lipoxins is separation by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [6]. The conjugated tetraene system shows a typical absorbance maximum at 301 nm and a molar absorptivity of 50 000 [2]. The new lipoxins C_4, D_4 and E_4 present a bathochromic shift to 307 nm. A method has been reported [7] for the highly sensitive determination of oxidizable compounds, such as lipoxins, using HPLC and subsequent electrochemical detection (ED). In this paper the application of this technique to the new lipoxins is described, including a study of the electrochemical behaviour of these compounds under certain chromatographic conditions.

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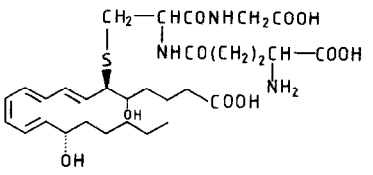
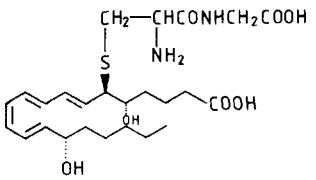
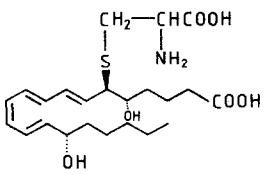


Lipoxin C_4



Lipoxin D_4



Lipoxin E_4

Fig. 1. Structures of the sulphopeptide lipoxins.

EXPERIMENTAL

Materials

LTC_4, LTD_4 and LTE_4 were obtained from Paesel (Frankfurt, Germany). Soybean lipoxygenase, trifluoroacetic acid (TFA) and Ca^{2+} ionophore A23187 (free acid) were purchased from Sigma (St. Louis, MO, USA), phosphate-buffered saline (PBS) buffer PM 16 without Ca^{2+}, Mg^{2+} and phenol red from Serva (Heidelberg, Germany), methanol (LiChrosolv) and lithium chloride from Merck (Darmstadt, Germany) and alumina powder (0.3 μm), lithium perchlorate and sodium borohydride from Fluka (Buchs, Switzerland). Water used for the mobile phase was freshly distilled. All other chemicals used were of analytical-reagent grade.

The concentration of the stock solutions of lipoxins in methanol were established with a Beckman DU-50 spectrophotometer by measuring the UV absorption at 307 nm. A molar absorptivity of 50 000 was used for the calculations.

Preparation of lipoxin standards

The lipoxin standards were prepared according to the method of Örning and Hammarström [8].

LTD_4 and LTE_4 (2.5 μg) were incubated with 20 μg of soybean lipoxygenase (2500 U) in 1 ml of PBS buffer (9.55 g/l, see under Materials), pH 7.4, at room temperature with vigorous shaking. A 100-μg mass of soybean lipoxygenase was used to transform LTC_4. The UV spectrum was scanned (from 250 to 350 nm every 10 min. The reaction was finished when no further increase in absorption at 307 nm was observed. To stop the enzyme activity, 500 μl of pure methanol were added; 10 mg of sodium borohydride were then dissolved in the incubation mixture to reduce the hydroperoxides formed. This preparation was kept at room temperature for 15 min. The addition of 1 ml of PBS buffer (9.55 g/l) was necessary to reduce the methanol content to less than 30% to obtain better recoveries during the solid-phase extraction. Finally the solution was acidified to pH 6 with 1 *M* hydrochloric acid, extracted and analysed as described in the following section.

Extraction procedure

Using the method reported by Steinhilber and Roth [5], Baker C_{18} disposable columns were conditioned with 2 ml of 100% methanol, 2 ml of water, 2 ml of a 0.1% aqueous EDTA solution and again with 2 ml of water. The samples were then applied to the columns and washed with 3 ml of water and 3 ml of 25% methanol. Finally, the lipoxins were eluted with 100% methanol. The extract was evaporated to dryness under a stream of nitrogen and resuspended in a small volume of methanol. Aliquots of this solution were injected into the HPLC apparatus.

Apparatus

The HPLC equipment consisted of a Waters Assoc. 460 electrochemical detector with a thin-layer glassy carbon electrode assembly, a Waters 481 variable-wavelength UV detector, a Waters 590 pump and a Waters U6K injector. Both detector outputs were connected to an IEEE interface of a Maxima 820 workstation. All data were acquired and processed with the Maxima full control software.

To restore sensitivity to the glassy carbon elec-

trode, its surface was pretreated with a dichromate–sulphuric acid and alumina slurry, as previously reported [9]. All potentials applied were referred to a Ag/AgCl electrode filled with 3 *M* lithium chloride in 65% aqueous methanol.

Column and mobile phase

The separations were carried out using a Waters Radial-Pak cartridge (100 × 5 mm I.D.) packed with 4-μm Novapak C_{18} material obtained from Waters (Eschborn, Germany). The solvent system was methanol–water–TFA (66:34:0.008, v/v/v). A flow-rate of 1.2 ml was used. All experiments were carried out at ambient temperature.

RESULTS AND DISCUSSION

The mobile phase most commonly used to separate lipoxygenase products by reversed-phase HPLC is either acetonitrile–water with TFA or methanol–water containing TFA. Although an excellent separation of the lipoxins can be achieved with the former mobile phase, this solvent system was not suitable for ED owing to a high background current. These findings are in agreement with previously published data [10]. To prevent interaction between the cations and the amino acid containing eicosanoids, a few workers have proposed the addition of EDTA to the mobile phase [11]. This substance was not applicable to ED because it leads to an increase in the background current. For the best electrochemical results the analysis was carried out with a solvent system using only methanol–water–TFA as reported previously [7]. To obtain a low background current and retention times for optimal separation (α = 1.14) of the lipoxins, a solvent mixture containing 66% aqueous methanol and 1 m*M* TFA was applied. Fig. 2 shows a characteristic chromatogram of LXC_4, LXD_4 and LXE_4 standards, obtained as described. Owing to the preparation method, bulk impurities were eluted in front of the lipoxins but they did not influence the determination.

Hydrodynamic voltammograms of LXC_4, LXD_4 and LXE_4 are presented in Fig. 3. At +1.45 V a maximum response was obtained for all lipoxins. The half-wave potentials were different for all compounds investigated: +1.18 V for LXC_4, +1.3 V for LXD_4 and +1.25 V for LXE_4. In contrast,

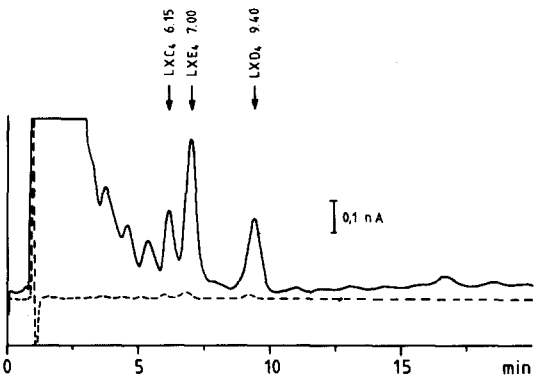


Fig. 2. HPLC–ED (solid line) and HPLC–UV (broken line) profiles of sulphopeptide lipoxins. Eluent, methanol–water (66:34, v/v) containing 1 m*M* TFA; flow-rate, 1.2 ml/min; applied potential, +1.35 V (*versus* Ag/AgCl); UV detection, 308 nm; injection volume, 15 μl containing 5 ng of each lipoxin.

LXC_4, LXD_4 and LXE_4 showed an identical electrochemical response (89 ± 2.25 nA s) per 100 p*M* injected. In subsequent experiments a potential of +1.35 V was used.

With the aim of finding a mobile phase with a low background current and a high sensitivity, various concentrations of TFA (0.1–1 m*M*) and lithium perchlorate (0–3 m*M*) were used. Both compounds strongly affected the elution of every lipoxin (data not shown). Generally, increasing concentrations of both additives decreased the retention times. In a few instances LXC_4 and LXE_4 were co-eluted. Only

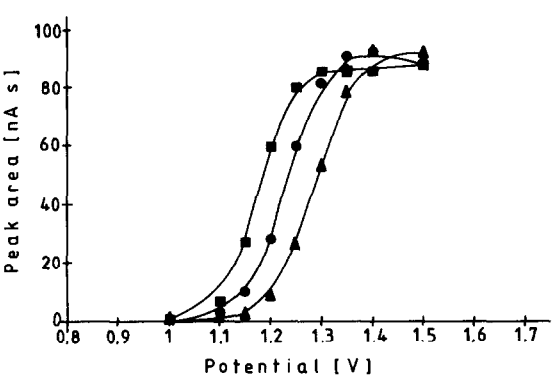


Fig. 3. Hydrodynamic voltammograms of LXC_4 (■), LXD_4 (▲) and LXE_4 (●), each corresponding to 100 pmol of lipoxin.

TABLE I

MEANS OF PEAK AREAS

Injected (ng)	Peak area (mean ±S.D., $n = 3$) (nA s)		
	LXC_4	LXD_4	LXE_4
1	1.74 ± 0.29	2.84 ± 0.47	4.03 ± 0.62
10	11.47 ± 0.17	23.32 ± 1.52	28.12 ± 1.63
20	21.95 ± 0.74	41.02 ± 0.55	51.00 ± 0.82
50	54.37 ± 2.50	106.17 ± 1.43	139.17 ± 1.65

a slight rise in electrochemical response was observed when the background current increased to 19 nA. The best results with respect to chromatographic resolution and electrochemical response were achieved with a mobile phase containing 66% aqueous methanol and 1 m*M* TFA. This solvent mixture established a stable baseline and a typical background current of 5–8 nA, thus allowing measurements at a sensitivity of 100 pA full scale.

Data obtained under these conditions are giving as means ±S.D. of the areas (in nA s), corresponding to the amount of each lipoxin injected (Table I). Table II presents the equations of these graphs and the correlation coefficients for the peptido-lipoxins,

TABLE II

EQUATIONS CORRESPONDING TO THE CALIBRATION GRAPHS AND CORRELATION COEFFICIENT FOR THE LIPOXINS

Equation is defined as $y = ax + b$, where y is the peak area and x is the amount (ng) of sample; r is the correlation coefficient.

Lipoxin	t_R (min)	Equation	r
LXC_4	6.15	$y = 1.074x + 0.641$	1.0000
LXD_4	9.40	$y = 2.098x + 0.857$	0.9995
LXE_4	7.00	$y = 2.776x - 0.503$	0.9990

which were obtained by calculating the results shown in Table I by the least-squares method. The detection limits of LXC_4, LXD_4 and LXE_4, based on a signal-to-noise ratio of 3:1, were 100–500 pg (200–700 fmol).

This method shows that HPLC with ED allows sensitive detection even at high potentials. In spite of the fact that sulphopeptide lipoxins are very sensitive to variations in the mobile phase, relatively small amounts of LXC_4, LXD_4 and LXE_4 can be detected. Although peptido-lipoxins possess a high molar absorptivity, ED still is three times more sensitive than UV detection.

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