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DETERMINATION OF PICOGRAM AMOUNTS OF LIPOXIN A_4 AND LIPOXIN B_4 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

THOMAS HERRMANN*, DIETER STEINHILBER, JOHANNA KNOSPE and HERMANN J. ROTH

Pharmaceutical Institute, University of Tübingen, Auf der Morgenstelle 8, D-7400 Tübingen (F R G)

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SUMMARY

A new method for the determination of lipoxins with electrochemical detection after high-performance liquid chromatography is described. The half-wave potentials of lipoxin A_4 and lipoxin B_4 at a glassy carbon electrode and a mobile phase of methanol–water (65.35, v/v) and 1 m*M* trifluoroacetic acid was found to be +1.14 V versus an Ag/AgCl reference electrode The use of trifluoroacetic acid instead of sulphuric acid and lithium perchlorate led to a background current of 6–8 nA at +1.20 V. The detection limits for both lipoxins, based on a signal-to-noise ratio of 3 1, were found to be 5–10 pg (15–30 fmol). The new method was applied to an extract of human polymorphonuclear granulocytes, preincubated with 15-hydroxyeicosatetraenoic acid and stimulated with Ca^{2+} ionophore A23187.

INTRODUCTION

Lipoxins, which are arachidonic acid metabolites formed by interactions between the 5- and 15-lipoxygenase pathways, have recently been shown to have various biological activities [1,2]. If added to neutrophils, lipoxin A_4 (LXA_4) stimulates superoxide anion generation without provoking aggregation [3]. In addition, LXA_4 and lipoxin B_4 (LXB_4) alter human killer cell activity [4] and activate protein kinase C in vitro [5]. Since only trace amounts of the lipoxin isomers are released by biological sources, there is a need to develop a convenient method for the extraction and determination of picogram amounts of these compounds. Moreover, the method should be capable of separating the lipoxin isomers.

Recently, we reported that the combination of high-performance liquid chromatography (HPLC) and subsequent electrochemical detection (ED) is a highly sensitive method for the determination of readily oxidizable compounds, such as leukotriene B_4 (LTB_4) [6]. Here, we describe a similar, highly sensitive method

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for the determination of lipoxins using HPLC–ED. This method allows the determination and separation of lipoxin isomers after solid-phase extraction of human polymorphonuclear granulocytes using C_{18} disposable columns. Our chromatographic conditions combine low background current with high electrochemical response and a sufficient separation of the lipoxin isomers.

EXPERIMENTAL

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 Water 590 pump and a Waters U6K injector. Both detector outputs were displayed simultaneously on a Waters M 730 data module. The surface of the electrode was treated as previously described [6]. All potentials were measured against an Ag/AgCl reference electrode filled with 3 *M* lithium chloride in 65% methanol.

The conductivities of various mobile phases were measured with a Metrohm E 365B conductor in an EA 645-7 cell. The exact cell constant was determined by using a 0.0200 *M* potassium chloride solution [7].

Chemicals

The lipoxins were generous gifts from J. Rokach (Merck Frosst, Quebec, Canada) and the abbreviations used are: LXA_4, (5*S*,6*R*,15*S*)-5,6,15-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 6S-LXA_4, (5*S*,6*S*,15*S*)-5,6,15-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 11-*trans*-LXA_4, (5*S*,6*R*,15*S*)-5,6,15-trihydroxy-7,9,11,13-*trans*-eicosatetraenoic acid; LXB_4, (5*S*,14*R*,15*S*)-5,14,15-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 14S-LXB_4, (5*S*,14*S*,15*S*)-5,14,15-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 8-*trans*-LXB_4, (5*S*,14*R*,15*S*)-5,14,15-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid [1,2]. 15-HETE (15-hydroxyeicosatetraenoic acid) was purchased from Paesel (Frankfurt, F.R.G.), trifluoroacetic acid (TFA) and Ca^{2+} ionophore A23187 (free acid) from Sigma (St. Louis, MO, U.S.A.), PM 16 buffer from Serva (Heidelberg, F.R.G.), methanol (LiChrosolv®), sulphuric acid (Suprapur®) and lithium chloride from Merck (Darmstadt, F.R.G.), water (Ampuwa®) from Fresenius (Bad Homburg, F.R.G.) and lithium perchlorate from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

Column and mobile phase

The separations were carried out on a column (250 mm × 4.6 mm I.D.) packed with Nucleosil® C_{18} (particle size 5 μm), which was obtained from Macherey-Nagel (Düren, F.R.G.). The solvent system was methanol–water (65:35, v/v) containing an electrolyte. A flow-rate of 1.0 ml/min was used. All experiments were carried out at ambient temperature.

Isolation of polymorphonuclear leukocytes and lipoxin extract

Suspensions of human polymorphonuclear leukocytes (PMNLs) were prepared according to the method of Hjorth et al. [8]. The cells were suspended in 10 ml of PM 16 Serva buffer salt solution [1,2] ($2 \cdot 10^7$ cells/ml), supplemented with 1 m*M* Ca^{2+}, 0.1% glucose and 0.1% human albumin. They were preincubated for 2 min with 15-HETE (10 μM), and the reaction was initiated with 25 μl of Ca^{2+} ionophore solution (1 mg/ml in methanol). After 15 min the reaction was stopped by addition of 10 ml of methanol. The tubes were chilled on ice and acidified with 1 *M* hydrochloric acid. After centrifugation, the lipoxins were extracted from the supernatant liquid as previously described [6].

RESULTS AND DISCUSSION

Several electrode materials, such as gold, platinum and glassy carbon, were considered. These were tested in the same mobile phase (methanol–water, 65:35, v/v, with 1 m*M* TFA) for background current. At +0.6 V oxidation potential, the current rose to 100 nA if platinum was used. The same effect was observed with the gold electrode at +1.0 V. At these voltages either lipoxins were still not detectable or only negligible signals appeared at concentrations of 5 ng. Since the glassy carbon electrode showed a background current of only 6–8 nA at +1.2 V, this electrode was used for further experiments.

The concentration of methanol had a profound influence on the retention times of the lipoxins. In order to obtain retention times between 6 min ($k' = 1.8$) for LXB_4 and 9 min ($k' = 3.1$) for 6S-LXA_4, a solvent mixture containing 65% methanol was found to produce the desired results. A pH decrease from 5 to 3, variation

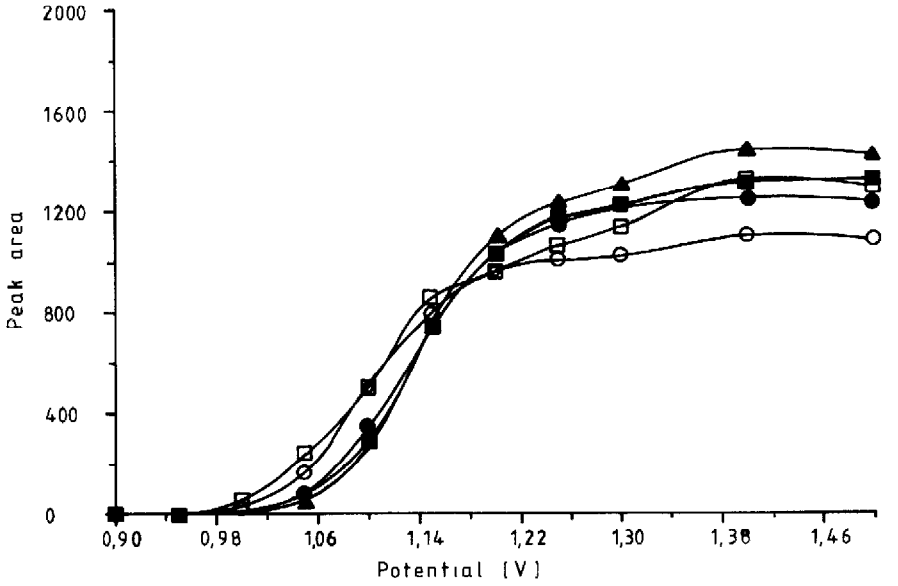

Fig. 1. Dependence of the electrochemical response on the applied potential (vs. Ag/AgCl) for LXA_4 (●), 11-*trans*-LXA_4 (○), LXB_4 (■), 8-*trans*-LXB_4 (□) and 14S-LXB_4 (▲) (10 ng each).

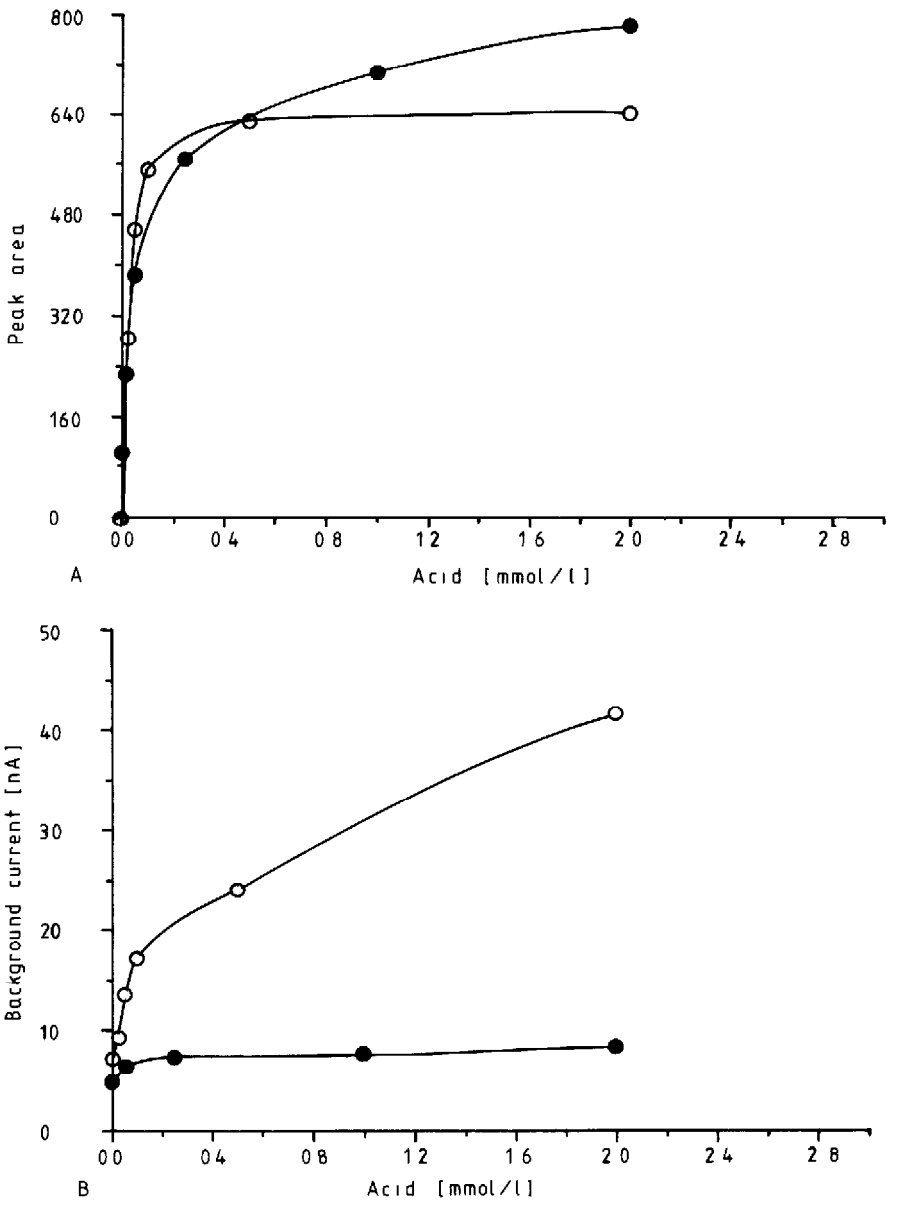

Fig. 2. (A) Effect of acid concentration in the mobile phase (methanol–water, 65.35, v/v) on the peak area of LXA_4. (○) Sulphuric acid; (●) TFA; detector potential +1.2 V (vs. Ag/AgCl). (B) Effect of acid concentration on the background current. Mobile phase (methanol–water, 65:35, v/v) containing sulphuric acid (○) or TFA (●); detector potential +1.2 V (vs. Ag/AgCl).

of the concentration of lithium perchlorate (0–100 m*M*) and the presence of sulphuric acid and/or TFA did not affect the retention time.

Fig. 1 shows the peak areas of LXA_4, LXB_4, 14S-LXB_4, 11-*trans*-LXA_4 and 8-*trans*-LXB_4 as a function of the applied oxidation potential. For the *cis*-lipoxins the half-wave potential was +1.14 V, and for the *trans*-lipoxins it was only 0.06 V lower. In subsequent experiments a potential of +1.20 V was chosen.

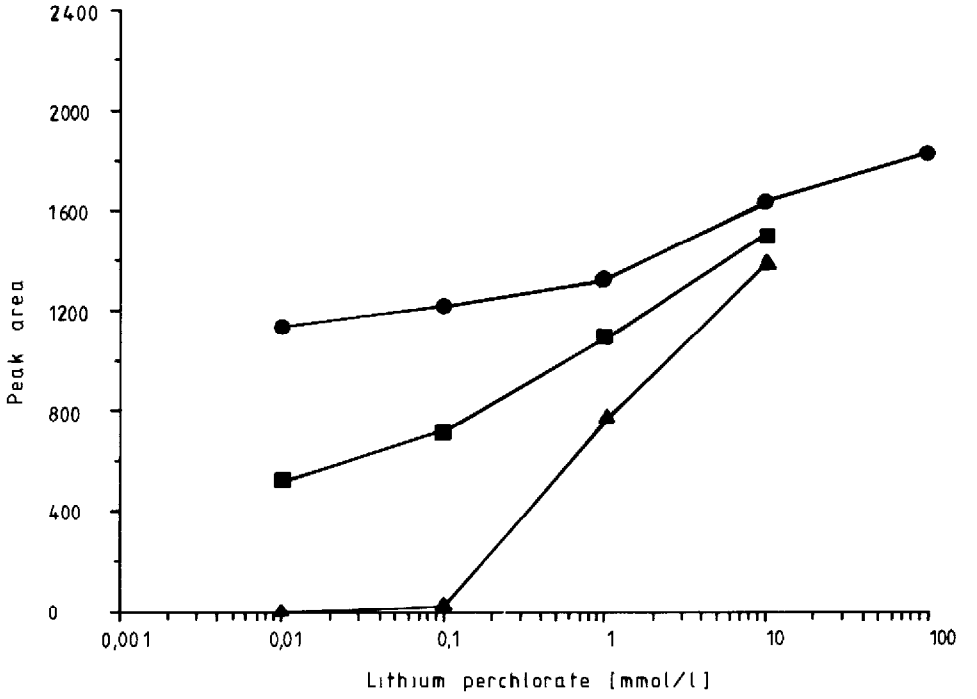

Fig. 3. Graph of peak area (10 ng of LXA_4) versus lithium perchlorate concentration at three different pH values: pH 5 (▲), pH 4 (■), pH 3 (●). Eluent, methanol–water (65:35, v/v) with TFA; detector potential, +1.2 V (vs. Ag/AgCl).

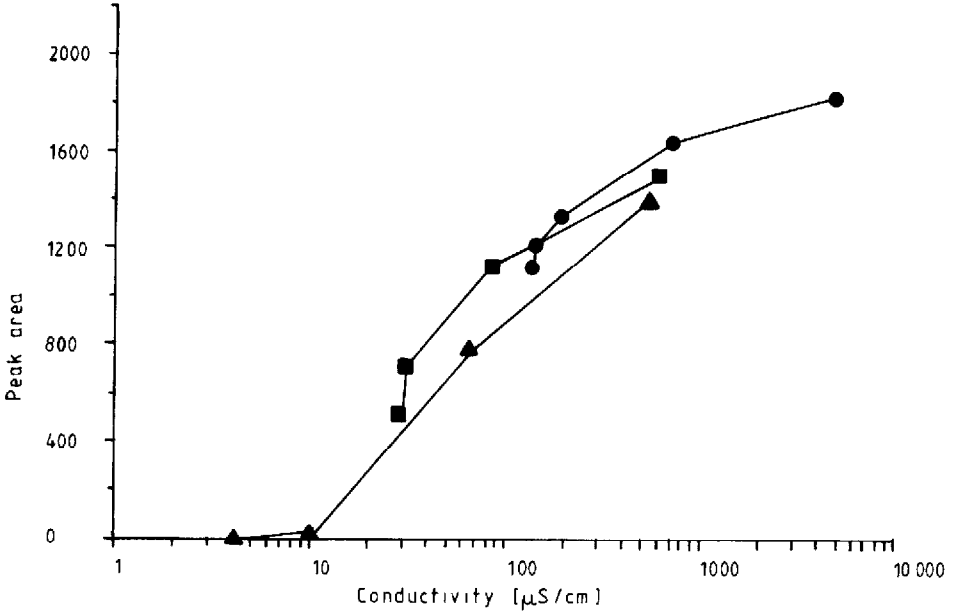

Fig. 4. Graph of peak area (10 ng of LXA_4) versus conductivity of the mobile phase, methanol–water (65·35, v/v) containing lithium perchlorate (0.01–100 m*M*) and TFA, at three different pH values: pH 5 (▲), pH 4 (■), pH 3 (●). Detector potential, +1.2 V (vs. Ag/AgCl).

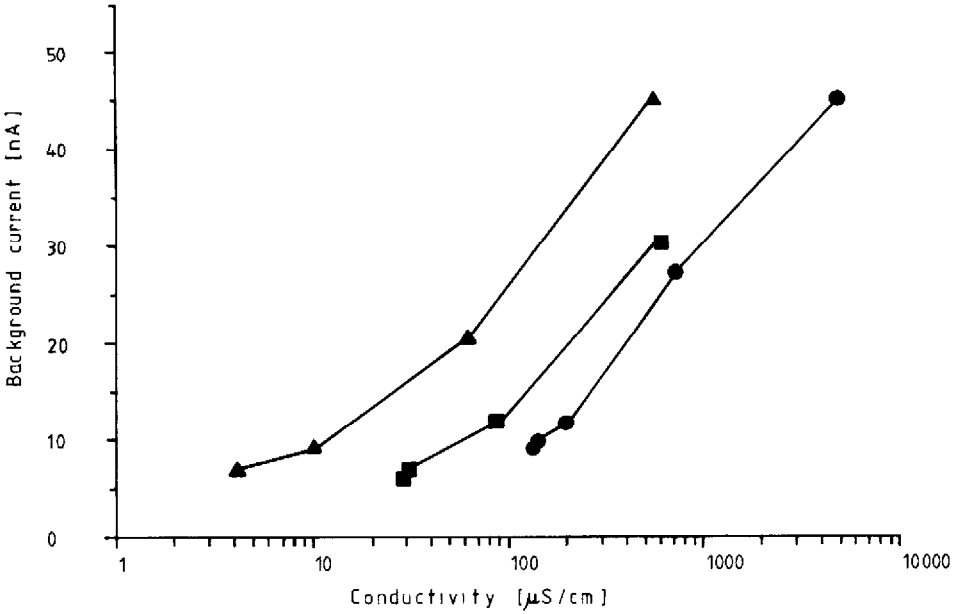

Fig. 5. Graph of background current versus conductivity at three different pH values: pH 5 (▲), pH 4 (■), pH 3 (●). Detector potential and mobile phase as in Fig. 4.

TABLE I

EQUATIONS FOR CALIBRATION GRAPHS AND CORRELATION COEFFICIENTS (r) FOR LIPOXINS

The equation is defined as $y=ax+b$, where y is the peak area and x is the amount of sample up to 5000 pg.

Lipoxin	Equation	r
LXA_4	$y=0.100x+1.28$	0.99989
6S-LXA_4	$y=0.109x+0.126$	0.99999
LXB_4	$y=0.0967x+0.91$	0.99995
14S-LXB_4	$y=0.107x+1.13$	0.99998

With the aim of finding a mobile phase with a low background current and a high sensitivity, various concentrations of sulphuric acid and TFA were tried. Fig. 2A illustrates the peak area of LXA_4 and Fig. 2B the background current, at different concentrations of the acids. In the range examined, the sensitivity to detection was similar for both acids. However, the background current proved to be quite different: with 0.5 m*M* sulphuric acid it rose to 20 nA, whereas with 0.5 m*M* TFA the background current rose to less than 10 nA. Thus, it was advantageous to use TFA for higher sensitivity to detection at lower background currents.

In addition, the influence of various concentrations of lithium perchlorate and TFA on the peak area of LXA_4 and the background current was investigated. Fig. 3 shows the dependence of the peak area on the added amount of lithium per-

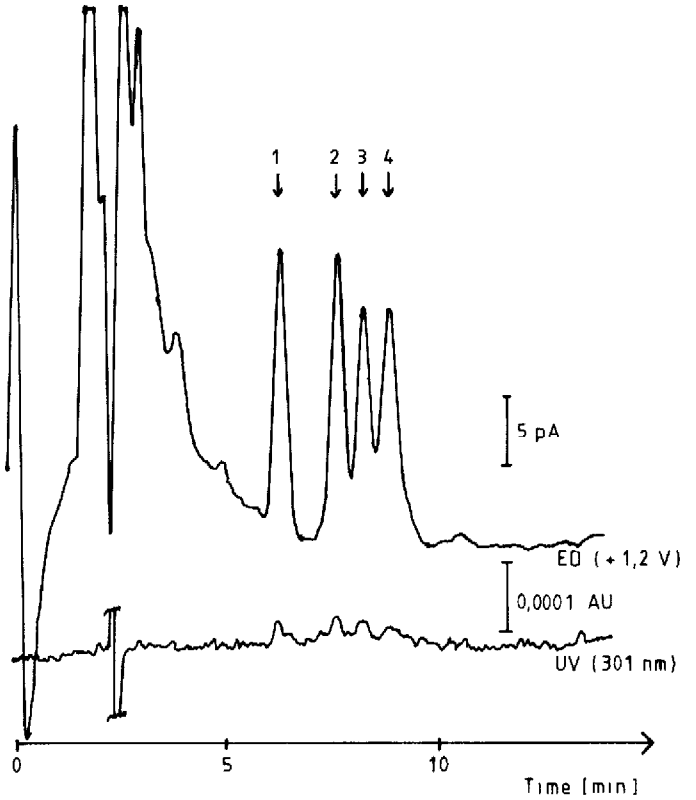

Fig. 6. HPLC–ED and HPLC–UV profiles of four lipoxins Eluent, methanol–water (65·35, v/v) containing 1 m*M* TFA; flow-rate, 1.0 ml/min; electrochemical detector, +1.2 V (vs. Ag/AgCl), response time, 5 s; filter, 2; background current, 3.1 nA; UV detector set at $\lambda = 301$ nm, with response time 5 s. Peaks: 1 = LXB_4 (118 pg); 2 = LXA_4 (131 pg); 3 = 14S-LXB (113 pg); 4 = 6S-LXA_4 (114 pg). Injected volume, 1 μl.

chlorate at three different pH values (3.0, 4.0 and 5.0). The figure clearly demonstrates that the detection at pH 5.0 is improved quite significantly by lithium perchlorate whereas at pH 3.0 there is hardly any increase in the intensity of the signal. It can, therefore, be concluded that the specific conductivity, as a measure of the ionic strength of the mobile phase, has to reach a minimum value for good detection of lipoxins. Fig. 4 illustrates the dependence of the peak area of the specific conductivity of the mobile phase at different concentrations of lithium perchlorate and pH values. In Fig. 5 the background current has been plotted against the specific conductivity. When the pH was held constant, the current rose with an increase in the concentration of lithium perchlorate. Therefore, a sensitive method of detection of lipoxins with a lower background current was established at a specific conductivity above 100 μS/cm using only TFA and no lithium perchlorate.

For trace determinations a mobile phase containing 1 m*M* TFA and an oxidation potential of +1.20 V were chosen. Table I lists the equations for calibration graphs and correlation coefficients (r) for some lipoxin isomers under these con-

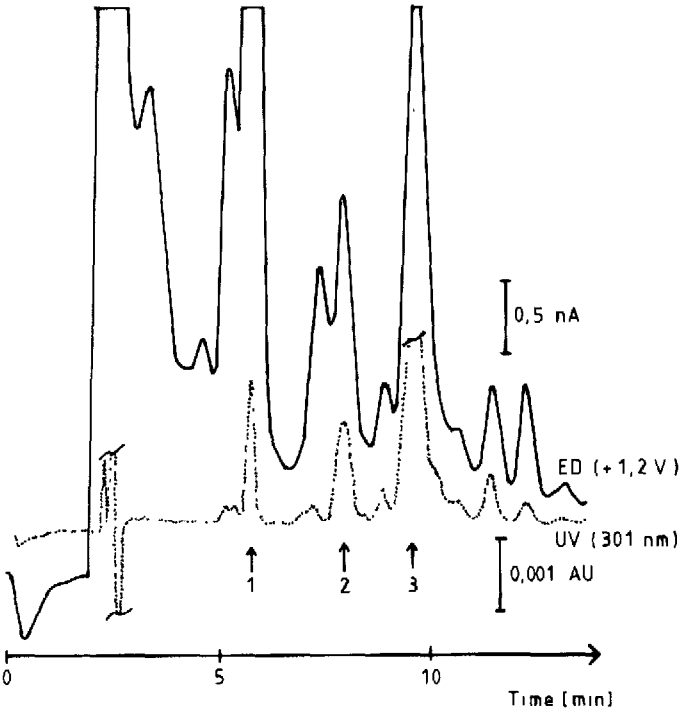

Fig. 7. Chromatograms of PMNLs extract using ED (full line) and UV detection (dotted line). Eluent, methanol–water (63·37, v/v) containing 1 m*M* TFA; detector potential, +1.2 V (vs. Ag/AgCl); UV detection, $\lambda = 301$ nm. Peaks: 1 = 20-OH LTB_4; 2 = LXB_4; 3 = LXA_4.

ditions. The standard deviation of the peak area was ± 0.55 ($n = 8$) for 100 pg of LXA_4 and ± 0.75 ($n = 6$) for the same amount of LXB_4. Because of the same pretreatment of the electrode at the start of every day's work, the inter-assay variability for 3.5 ng of LXA_4 was low (relative standard deviation $\pm 6\%$, $n = 4$).

Using a specially designed mobile phase, the background current could be reduced to 3–4 nA. Fig. 6 represents a chromatogram of different lipoxin isomers using such a mobile phase. The detection limit, based on signal height-to-noise ratio of 3.1, was found to be 5–10 pg (15–30 fmol) for both LXA_4 and LXB_4. For 1 ng of LXA_4 at $k' = 2.5$ the current response was 0.16 nA, and for 18 pmol of LXA_4 a charge of $2 \cdot 10^{-8}$ C was recorded. If the lipoxin undergoes a one-electron oxidation at the electrode, a conversion of 1.2% should have taken place. This is a low conversion rate compared with those reported by Kissinger [9].

An extract of PMNLs was chosen to demonstrate the detectability of lipoxins by this new method. Thus, Fig. 7 shows an HPLC–ED profile of such an extract obtained as described in Experimental. The numbers indicate the retention times of the standards. A UV detector ($\lambda = 301$ nm) also displayed corresponding peaks.

In summary, we report here a new method for the detection for potent biological mediators represented by LXA_4 and LXB_4. The HPLC–ED approach provides a rapid, sensitive and selective method of estimation, without a need for derivatization. For sensitive determination, a low background current (6–8 nA at

+1.20 V) is necessary, which is obtained by the use of TFA instead of sulphuric acid and avoiding the use of lithium perchlorate. Since the electrochemical detector is ten to twenty times more sensitive than the UV detector, it could serve as a powerful tool in the analysis of the biological properties of lipoxins. Since 14*S*-8-*cis*-LXB_4 is well separated from the other lipoxins, and was not found in a PMNLs extract by Serhan et al. [2], it might fulfil the requirements necessary to be used as internal standard.

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