Journal of Chromatography, 416 (1987) 170–175
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3534

Note

Determination of leukotriene B_4 by high-performance liquid chromatography with electrochemical detection

THOMAS HERRMANN*, DIETER STEINHILBER and HERMANN J. ROTH

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

(First received May 6th, 1986; revised manuscript received December 3rd, 1986)

Leukotriene B_4 (LTB_4) is an arachidonic acid metabolite isolated from human polymorphonuclear leukocytes and has several physiological effects at trace level [1–3]. There exists a need to develop a convenient method for the determination of LTB_4, particularly in clinical and experimental medicine. The current approach to LTB_4 separation is high-performance liquid chromatography (HPLC) with quantification by ultraviolet (UV) spectrometry. Since LTB_4 contains four double bonds, including a conjugated triene unit flanked by two hydroxyl groups, the relatively high extinction coefficient of the triene chromophore at ca. 280 nm allows quantification of nanogram amounts of LTB_4. Also, radioimmunoassay [4] and gas chromatography–mass spectrometry have been reported, but HPLC with UV detection has turned out to be the method of choice for routine separation and quantification of LTB_4.

Electrochemical detection (ED), which is widely used for readily oxidized compounds, has proved to be a highly selective method for HPLC at lower oxidative voltages and sensitive even at higher oxidative voltages [5]. Several species, such as phenols, aromatic amines and carbonyl compounds, have been detected at picogram levels. Thus, HPLC–ED has become a popular technique for the determination of trace levels of organic compounds.

This report presents an investigation of the advantages of ED and quantification for LTB_4 and its 20-hydroxy metabolite (20-OH-LTB_4) [6] after reversed-phase HPLC separation.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Waters Assoc. 590 pump, a Waters U6K injector, a Waters 481 variable-wavelength UV detector and a Waters 460 elec-

0378-4347/87/$03.50

trochemical detector with a thin-layer glassy carbon electrode assembly. The detector output was displayed on a Waters M 730 data module.

The surface of the electrode was treated with a solution of dichromate–sulphuric acid for 10 min [7], cleaned with water in an ultrasonic bath, polished mechanically with alumina slurry [8, 9] and finally ultrasonicated to remove the residue remaining on the glassy carbon surface. The electrode was pretreated routinely at the start of each day's work to obtain reproducible and well defined electrochemical conditions. No deterioration in electrode performance was observed with any amount of the sample injected in the 1–30 ng range.

All potentials were measured against a silver/silver chloride reference electrode.

Chemicals

LTB_4 was a generous gift from Dr. C.O. Meese (Dr. Margarete Fischer-Bosch Institute, Stuttgart, F.R.G.), 20-OH-LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE) were purchased from Paesel (Frankfurt, F.R.G.), Ca^{2+} ionophore A 23187 (free acid) from Sigma (St. Louis, MO, U.S.A.), PM 16 buffer from Serva (Heidelberg, F.R.G.), methanol (LiChrosolv®) from Merck (Darmstadt, F.R.G.), water (Ampuwa®) from Fresenius (Bad Homburg, F.R.G.) and alumina powder (0.3 μm) from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

The concentration of the stock solutions of leukotrienes in methanol were established with a Beckman DU-50 spectrophotometer by measuring the UV absorption at 280 nm ($\varepsilon = 40\,000$) [2].

Column and mobile phase

The separations were carried out on a column (250×4.6 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 water–methanol (25:75, v/v), containing an electrolyte (1 g/l lithium perchlorate and 0.05 g/l sulphuric acid). A flow-rate of 1 ml/min was used, and all experiments were carried out at ambient temperature.

Isolation of polymorphonuclear leukocytes and leukotriene extract

Suspensions of human polymorphonuclear leukocytes (PMNLs) were prepared according to the method of Hjorth et al. [10]. The human PMNLs [11] ($1.0–1.4 \cdot 10^7$ cells per ml) were suspended in 1 ml of PM 16 Serva buffer salt solution, supplemented with 1 m*M* Ca^{2+}, 0.1% glucose and 0.1% human albumin. The reaction was initiated with 2.5 μl of Ca^{2+} ionophore solution (1 mg/ml in methanol). After 6 min the reaction was stopped with 1 ml of methanol. The tubes were chilled on ice and acidified with 1 *M* hydrochloric acid. After centrifugation the leukotrienes were extracted from the supernatant liquid as previously described [12]. The extraction of eicosanoids was carried out with a modified Baker 10 extraction system, using Baker C_{18} disposable columns. The samples were applied to the preconditioned columns and washed with water and 25% methanol. Finally the leukotrienes were eluted with 100% methanol. The extract was evaporated to dryness with a stream of nitrogen at 37°C and resuspended in

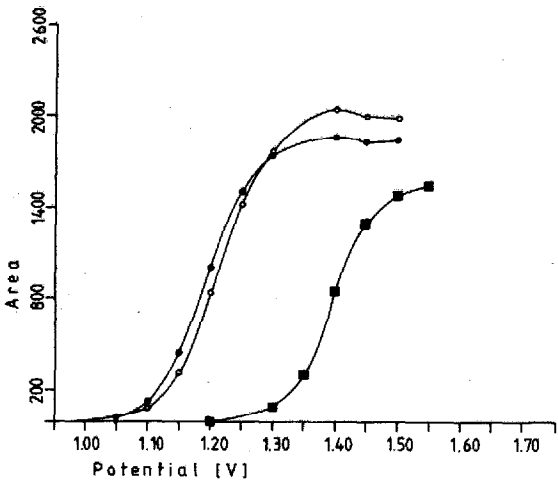

Fig. 1. Dependence of the electrochemical response on the applied potential (V, vs. Ag/AgCl) for LTB_4 (●), 20-OH-LTB_4 (○) and 5-HETE (■) (10 ng, 10 ng and 5 ng, respectively).

1 ml of methanol. This solution was used for the HPLC separation.

RESULTS AND DISCUSSION

The chromatographic approach selected for use in HPLC was reversed-phase chromatography with an octadecylsilane column, since the separation and recovery of the arachidonic acid metabolites have been reported to be generally very high [13].

The most commonly used solvent for separation of lipoxygenase products of arachidonic acid, such as LTB_4, is aqueous methanol with trace amounts of an acid. The addition of a small amount of sulphuric acid and of 1 g/l lithium perchlorate as supporting electrolytes led to no significant change in the retention time of LTB_4, but a high response of current was obtained during ED. Since the UV absorption of LTB_4 and of the 20-hydroxy derivative at 280 nm was not affected by these supporting electrolytes, a series configuration of the UV and electrochemical detectors was possible. This also allowed confirmation and comparison between the two detection modes.

Fig. 1 shows the peak areas of LTB_4, its 20-hydroxy metabolite and 5-HETE as a function of the applied oxidation potential. For LTB_4 the half-wave potential, defined as 50% of the maximum detectable area, was +1.20 V. It was only 0.02 V higher for 20-OH-LTB_4. For 5-HETE the half-wave potential was found to be +1.40 V. It can therefore be concluded that 20-OH group of the LTB_4 metabolite had not affected the electrochemical behaviour in a significant manner. For LTB_4 ca. 90% of the maximum response was detectable at an oxidation potential of +1.28 V. Total response was obtained at +1.34 V. In order to obtain corresponding responses for the 20-hydroxy derivative, ca. 40–50 mV were additionally necessary. Since there was an increase in the background current and in the noise level at higher voltages, an oxidation potential of +1.30 V could be considered as

TABLE I

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

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

Leukotriene	Linear range (pg)	Equation	r
LTB_4	100- 2000	$y = 0.1988x + 0.0119$	0.9997
	1000-30000	$y = 0.1971x + 19.82$	0.9996
20-OH-LTB_4	80- 8000	$y = 0.2101x - 12.67$	0.9992

a reasonable compromise for trace determinations of LTB_4 and the 20-hydroxy metabolite. At this voltage, either 5-HETE is still not detectable or only a small signal appears at high concentrations.

At +1.35 V the current response of 1 ng of LTB_4 ($k' = 3.0$) was 0.4 nA. For 1 ng of 20-OH-LTB_4 ($k' = 0.6$) it was 0.7 nA.

To construct calibration graphs for quantification, decreasing amounts of leukotrienes were analysed as described above. Table I lists the equations and correlation coefficients in the range from 100 pg to 30 ng for LTB_4. A linear relationship was also obtained for the 20-hydroxy metabolite in the range from 80 pg to 8 ng. The standard error was ± 1.5 ($n = 6$) for 200 pg of LTB_4 and ± 0.4 ($n = 6$) for the same amount of the metabolite. Because of the same pretreatment of the electrode at the start of every day's work the inter-assay variability was very low.

The detection limit of LTB_4, based on peak height versus baseline noise ratio of 3:1, was determined to be 50–60 pg at $k' = 3.0$. Even 40–50 pg of the 20-hydroxy derivative ($k' = 0.6$) were detectable. Since the noise level generally depended on a constant solvent flow-rate, this was the restricting factor for trace determinations of LTB_4.

Using the HPLC–UV equipment described here, amounts of LTB_4 as low as 0.5–1.0 ng could be measured. The results were in accord with those reported by Mathews et al. [14]. Thus, ED appears to be ten times more sensitive than UV detection.

An extract of PMNLs was chosen to demonstrate the detectability of diastereoisomers and metabolites of LTB_4 by this new method. Thus, Fig. 2 shows an HPLC–ED profile of such an extract obtained as described in the Experimental section. The numbers indicate the retention times of the standard. Close to the 20-OH-LTB_4 peak another peak is observed, which corresponds to another LTB_4 metabolite (20-carboxy-LTB_4), also formed in the PMNLs. The two peaks with the retention times 11.5 and 12.5 min correspond to the 12-*S*/*R* diastereoisomers of 6-*trans*-LTB_4. For both ED and UV detection, the amount of LTB_4 determined by external standard quantification was identical. Since prostaglandin B_2 (PGB_2) is active at an oxidative voltage above +1.60 V, it cannot be used as internal standard. Therefore we are considering other compounds that would be oxidized in the same manner as LTB_4 and separate quite well from LTB_4 and its two isomers. LTB_5 or synthetic LTB_3 might fulfil the requirements to be used as

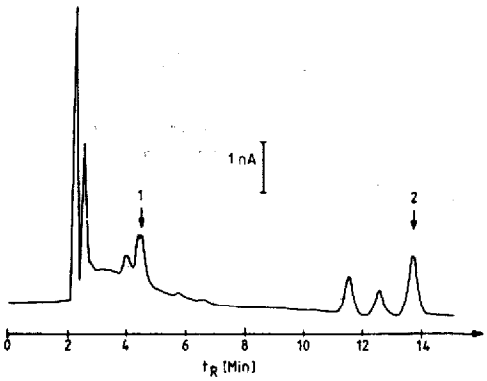

Fig. 2. Chromatogram of 10 μl PMNL extract obtained as described in the Experimental section. Eluent, methanol–water (70:30, v/v) containing 1 g/l lithium chlorate and 0.05 g/l sulphuric acid; flow-rate, 1 ml/min; detector potential, +1.3 V (vs. Ag/AgCl). Peaks: 1 = 20-OH-LTB_4; 2 = LTB_4.

internal standard and to exclude the inter-assay variability caused by the electrode performance.

In summary, we report here studies of a new method of detection for extremely potent biological mediators represented by LTB_4, its 20-hydroxy metabolite and 5-HETE. The HPLC–ED approach provides a rapid, sensitive and selective method of estimation, without the need for derivatization of the samples. Since ED is about ten times more sensitive than UV detection, it could become a powerful tool in the analysis of LTB_4, particularly in clinical and experimental medicine. Further, this method is applicable to extracts of cells. It was observed during our studies with this method that no interfering substances were present after the usual work-up of the extract. This could be of particular significance when biological fluids are employed. Sulphidopeptide leukotrienes require a different extraction method. The conditions of HPLC separation are also different from those described here, and they will be part of a separate report. Applications to other arachidonic acid metabolites, including lipoxins and hydroxyeicosanoids, especially 15-HETE, 12-HETE, 5-HETE and also hydroxy metabolites from other precursors, are currently being investigated.

REFERENCES

1 P. Borgeat and B. Samuelsson, J. Biol. Chem., 254 (1979) 2643–2646.
2 P. Borgeat and B. Samuelsson, J. Biol. Chem., 254 (1979) 7865–7869.
3 M.A. Bray, Br. Med. Bull., 39 (1983) 249–254.
4 J.A. Salmon, P.M. Simmons and R.M.J. Palmer, Prostaglandins, 24 (1982) 225–235.
5 P.T. Kissinger, Anal. Chem., 49 (1977) 447A–456A.
6 G. Hansson, J.A. Lindgren, S.E. Dahlen, P. Hedqvist and B. Samuelsson, FEBS Lett., 130 (1981) 107–112.
7 A.H. Anton, Life Sci., 35 (1985) 79–85.
8 J. Zak and T. Kuwana, J. Am. Chem. Soc., 104 (1982) 5514–5515.
9 B. Kazee, D.E. Weisshaar and T. Kuwana, Anal. Chem., 57 (1985) 2736–2739.
10 R. Hjorth, A.K. Jonsson and P. Vretblad, Immunol. Methods, 43 (1981) 95–101.
11 B. Samuelsson, Science, 220 (1983) 568–575.

12 J.R. Luderer, D.L. Riley and L.M. Demers, J. Chromatogr., 273 (1983) 402–409.
13 W.S. Powell, Anal. Biochem., 148 (1985) 59–69.
14 W.R. Mathews, J. Rokach and R.C. Murphy, Anal. Biochem., 118 (1981) 96–101.