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Simultaneous determination of leukotrienes B_4 and E_4 in whole blood and of leukotriene E_4 in urine of rabbit by reversed-phase high-performance liquid chromatography

Antonio Celardo*, Giuseppe Dell'Elba, Zeinab Mohamed Eltantawy, Virgilio Evangelista, Chiara Cerletti

Istituto di Ricerche Farmacologiche "Mario Negri", G. Bizzozero Laboratory of Platelet and Leukocyte Pharmacology, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy

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Abstract

Sulfidopeptide leukotrienes E_4 (LTE_4) and B_4 (LTB_4) were simultaneously extracted from rabbit whole blood with acetonitrile. LTC_4 and LTD_4 were converted to LTE_4 by γ-glutamyl transpeptidase and leucine-amino peptidase before extraction. LTE_4 was extracted from urine with C_{18} Sep-Pak cartridges. The compounds were resolved and quantitated by reversed-phase high-performance liquid chromatography (HPLC) with a diode-array detector; in selected cases the collected fractions were assayed for LTB_4 and LTE_4 by specific enzyme immunoassay (EIA). The correlation factor of the measured increase in LTE_4 concentrations and addition of incremental amounts of LTC_4 to blood was $r = 0.998$; slope of 1.05 ± 0.01 (mean ± S.D.). Concentrations of LTE_4 measured by HPLC correlated with those obtained with EIA ($r = 0.996$; slope $= 0.98 \pm 0.03$ and $r = 0.991$; slope $= 0.97 \pm 0.04$ in blood and urine, respectively). For blood LTB_4 the correlation of HPLC versus EIA was $r = 0.990$; slope $= 1.12 \pm 0.04$. The method described is accurate and reproducible, allowing measurement of both LTB_4 and LTE_4 in whole blood after a single extraction procedure. Simultaneous measurement of these metabolites after specific stimulation or in pathological conditions is recommended for in vivo investigations of LTs production.

1. Introduction

Leukotrienes (LTs) are lipid mediators synthesized through the 5-lipoxygenase pathway (Fig. 1). This enzyme catalyzes the conversion of arachidonic acid, released from the cell membrane by activated phospholipase A_2, to LTA_4. LTA_4 can be converted to LTB_4 by the action of LTA_4 hydrolase or conjugated with reduced glutathione by a specific glutathione-S-transferase to LTC_4 [1]. Subsequent cleavage of the peptide residues of LTC_4 by γ-glutamyltranspeptidase (GGTP) and leucine-amino peptidase (LAP) results in the formation of LTD_4 and its less potent metabolite LTE_4, respectively.

The metabolism and excretion of peptido-LTs have been recently investigated in humans [2–6] and other primates [7,8], in dogs [9] and rats [10,11]. Although similar metabolic pathways, such as omega and beta oxidation and N-acetyla-

* Corresponding author.

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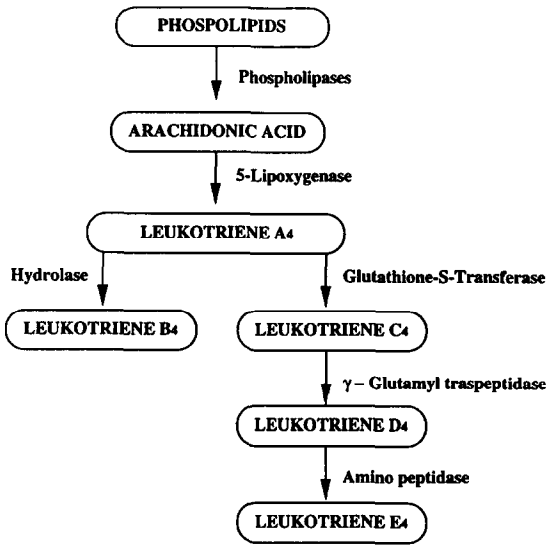

Fig. 1. Schematic presentation of leukotriene formation from arachidonic acid.

tion, are involved, the relative amounts of the single metabolites substantially differ between these species. In humans LTE_4 can be considered the major urinary metabolite of LTC_4 excreted in the urine in the first period following its release into the circulation. An increased urinary LTE_4 level has recently been shown in several pathological situations including asthma [12,13], myocardial ischemia [14] and adult respiratory distress syndrome (ARDS) [15]. Furthermore, peptido LTs production has been detected in sheep and dog models for ARDS [16,17]. Inhibitors of 5-lipoxygenase or LTs receptor antagonists showed beneficial effects in human asthma [18,19] and in experimental models of ARDS [16,20], and of myocardial reperfusion injury [21].

A pathogenetic role for these metabolites in pulmonary and vascular diseases was suggested on the basis of their stimulating effect on smooth muscle cell contraction [22,23], increase of vascular permeability [24], bronchial mucus secretion [25], and polymorphonuclear leukocytes (PMN) accumulation in ischemic tissue [26].

Rabbit is often used to develop models of diseases. For example infusion of a leukocyte agonist such as N-formyl-methionyl-leucyl-L-phenylalanine (fMLP) induces immediate leukopenia and subsequent thrombocytopenia as a consequence of cell sequestration in the lungs [27]. We have assayed the production of LTB_4 and LTC_4 in fMLP-challenged rabbits in order to assess the suitability of this model to investigate the synthesis and metabolism of LTs.

In this report we describe the methodology used to quantitate LTB_4 and LTE_4 in whole blood and LTE_4 and other possible metabolites in the urine of fMLP-challenged rabbits. Several HPLC-based methods have been recommended for the determination of lipoxygenase metabolites in aqueous samples from in vitro stimulated cells suspensions [28–32]. Some of them used solid-phase extraction of LTB_4 and LTC_4 from plasma [33] and of LTB_4, LTC_4 and LTD_4 from nasal secretion [34,35]. These methods require large elution volumes for a satisfactory recovery, and the use of toxic chemical solvents. Quantitation is usually performed by a combination of HPLC and radioimmunoassay/EIA techniques [36–40].

The HPLC methodology described here improves and simplifies the quantitation of LTC_4 after transformation to LTE_4 by GGTP and leucine-amino peptidase (LAP) in whole blood. Our method has been validated by using EIA and utilizes only a few steps for extraction of LTB_4 and LTE_4 from whole blood, requiring limited time and cost for sample processing and analysis.

2. Experimental

2.1. Chemicals

Standards of LTB_4, LTC_4, LTD_4, LTE_4, LTF_4, 20-carboxy LTE_4, and LTB_4 and LTE_4 EIA kits were purchased from Cayman Chemicals (Ann Arbor, MI, USA). N-Acetyl-LTE_4 ($NAcLTE_4$), prostaglandin B_2 (PGB_2), 4-hydroxy-TEMPO (HTMP), fMLP, GGTP, LAP and indomethacin were from Sigma Chemical Co. (St. Louis, MO, USA), heparin from Roche (Milan, Italy), ethylenediaminetetraacetic acid (EDTA) from Merck (Darmstadt, Germany). All other chemicals were reagent grade; sol-

vents, acetonitrile, methanol (Farmitalia Carlo Erba, Milan, Italy) were UV grade.

2.2. Equipment

The automated reversed-phase high-performance liquid chromatograph (HPLC) System Gold (Beckman Instruments, San Ramon, CA, USA) consisted of a dual-pump programmable solvent module (Model 126) and a diode-array detector module (Model 168) equipped with a PS/2 IBM model 55/65. A 4-μm Superspher 100 RP 18 LichroCART (250 × 4.6 mm I.D.) column from Merck (Darmstadt, Germany) was used. The mobile phase consisted of methanol–acetic acid (0.1% in deionized water from MilliQ water purification system; Millipore, Bedford, MA, USA) (adjusted to pH 5.6 with NH_4OH)–acetonitrile (60:35:5, v/v, for blood and 57:33:10, v/v, for urine samples). Solvents were filtered through a Millipore 0.4-μm filter. LTB_4 and LTE_4 from blood were eluted at flow-rate of 0.5 ml/min and analyzed using the Model 168 diode-array detector, set at 280 nm, the maximum absorbance for LTs. For extracted urine samples the pH of the mobile phase was adjusted to 5.4 with H_3PO_4 and the elution flow-rate to 0.8 ml/min.

2.3. Standard solutions

Stock solutions of the standard compounds (10 μg/ml) were prepared in deionized water for LTB_4, PGB_2 and LTC_4, in methanol–water (60:40, v/v) and ethanol–water (60:40, v/v) for LTE_4 and stored at −80°C. Standards were added to blank pools of blood and urine to give final concentrations of 40 and 80 ng/ml for LTB_4 and LTE_4, respectively, in blood and of 40 ng/ml for LTE_4 in urine. These were further diluted with the same blank pool of blood and urine used above to provide the material for quality control tests. The blood pool was divided into 1.0-ml aliquots and frozen at −80°C until analysis. We prepared two sets of blood samples containing 10, 40 and 20, 80 ng/ml for LTB_4 and LTE_4, respectively. The urine pool was divided into 5-ml aliquots and frozen at −80°C until analysis. We prepared two sets of samples of 10 and 40 ng/ml of LTE_4. The samples obtained were analyzed over a three-months period, obtaining ten replicates for each set.

2.4. Animal treatment

New Zealand male rabbits HY/Cr (3–4 kg body weight) were purchased from Charles River (Calco, Italy) and acclimatized to the research facilities, receiving standard laboratory chow and water ad libitum. The animals were housed separately in stainless-steel cages at controlled temperature (20°C), humidity (50%) and light cycle (12/12 light/dark). Animals were cannulated in the jugular vein and carotid artery, according to Popovich and Popovich [41] for infusion of fMLP and blood sampling. Twenty-four hours before and after fMLP infusion urine samples were collected from rabbits housed in metabolic cages.

This work was performed according to the "Guidilines for the use of animals in biomedical research" [42].

2.5. Blood collection and extraction of LTB_4

Rabbit blood samples (1 ml) were collected in 10 μl of an inhibitory mixture containing HTMP 0.5 mmol, EDTA 0.1 mmol, indomethacin 0.1 mmol and 5 U of heparin to prevent ex vivo metabolism, incubated with 0.2 unit each of GGTP and LAP at 37°C for 3 h and extracted. LTB_4 and LTE_4 were simultaneously extracted from blood by adding two volumes of acetonitrile containing the internal standard PGB_2 (50 ng). The first volume of acetonitrile was slowly added to blood gently mixed by hand to avoid immediate clotting of proteins and red blood cells. After addition of the second volume of acetonitrile blood was vortex-mixed for 30 s, centrifuged at room temperature at 4500 *g* for 10 min, the supernatant transferred and dried under a gentle stream of nitrogen in bath at 45°C. The dried residue, which could be stored at 4°C, was reconstituted in the mobile phase, injected onto the HPLC system and analysis was performed by isocratic elution at room temperature.

2.6. Conversion of sulfidopeptide LTS to LTE_4 in blood

Considering that LTC_4 is rapidly metabolized by peptidase activities normally present in plasma [43] we decided to evaluate LTC_4 as its stable end-product LTE_4. Increasing amounts of LTC_4 were added to 1 ml of rabbit blood, incubated for 3 h at 37°C with GGTP and LAP (0.2 unit each). Blood without added LTC_4 was also analyzed to evaluate the endogenous sulfidopeptide LTs concentration. Extraction was performed as described and samples injected onto the HPLC system. LTC_4, LTD_4 and LTB_4 were eluted with different retention times (10.9, 20.0 and 23.8 min, respectively), and resolved from LTE_4 (retention time = 27.2 min). When specified, fractions were collected at the retention time of LTE_4 to be analyzed by EIA.

2.7. Urine collection and extraction of LTE_4

Urine was collected in a cylinder stored at −20°C, containing 1 m*M* of HTMP and 0.5 m*M* EDTA per 10 ml urine and stored at −80°C until analysis. Urine were centrifuged at 1600 *g* at −4°C for 10 min, 5 ml were mixed with methanol (1:3, v/v) and kept at −20°C for 3 h. Samples were then centrifuged for 15 min at 2900 *g* at −4°C and supernatants were evaporated to ca. 5 ml in bath at 45°C under nitrogen. After addition of 5 ml of bidistilled water the samples were mixed by vortex-mixing and passed through C_{18} Sep-Pak cartridges (Waters), pretreated with 10 ml of methanol and 10 ml of 0.1% acetic acid pH 5.6. The samples were washed with bidistilled water (20 ml), 0.1% acetic acid pH 5.6 (20 ml), *n*-hexane (20 ml), chloroform (20 ml), bidistilled water (10 ml) and finally LTE_4 was eluted with 8 ml of methanol, evaporated under gentle stream of nitrogen at 45°C. The dried residue, which could be stored at 4°C, was reconstituted in the mobile phase and injected onto the HPLC system.

2.8. Enzyme immunoassay (EIA)

Fractions eluted from the HPLC system were dried at 45°C under nitrogen and resuspended in 1 ml of EIA buffer. Each sample was assayed at least at two different dilutions according to the instructions of the commercial kit. The specificity of the EIA assay for LTB_4 was 100% with less than 0.01% cross reactivity with other LTs. The specificity of the EIA assay for LTE_4 was 100, 20, 10, 9, and $<0.01\%$ for LTE_4 $NacLTE_4$, LTC_4, LTD_4 and LTB_4, respectively. An automatic plate reader (Titertek Multiskan MC/340, Flow Laboratories, Helsinki, Finland) was used to detect the 5-thio-2-nitrobenzoic acid production at 412 nm.

2.9. Identification of compounds

LTs were identified on the basis of retention time and UV spectra (scanned from 220 to 320 nm every 2 s) compared to authentic standards; HPLC fractions were collected to determine immunoreactive LTB_4 and LTE_4 by the EIA kit.

2.10. Calculations

Quantitation of LTB_4 and LTE_4 in blood was performed utilizing a plot of the ratio between the peak area of the analyte and the peak area of the internal standard versus concentration of analyte of a standard curve. Quantitation of LTE_4 in urine was achieved by a standard curve obtained by plotting the peak area of the compound versus its concentration.

The endogenous concentrations of LTB_4 and LTE_4 in blood (0.05 ± 0.03 and 0.16 ± 0.07 ng/ml, respectively, mean ± S.D., $n = 3$) and LTE_4 in urine (1.04 ± 0.96 ng/ml, mean ± S.D., $n = 3$) were evaluated in the corresponding HPLC fractions by EIA and subtracted from the concentrations measured in spiked samples. Results are expressed as mean, standard deviation (S.D.) and coefficient of variation (C.V.%). Fitting of standard curves and calculation of LTB_4 and LTE_4 concentrations in samples by EIA were computed using the Mac ALLFIT program [44].

3. Results and discussion

The data obtained with the described extraction procedure and HPLC method are reported

Table 1
Performance data for the determination of LTB_4 and LTE_4 in blood and LTE_4 in urine by HPLC

Compound	Reproducibility	ng added	Inter-assay precision	Accuracy	Linearity[a]	Recovery	HPLC detection limit (ng/ml)
Blood							
LTB_4	0.025 ± 0.003 (12.0)	10	10.70 ± 0.14 (1.30)	7.00 ± 1.41	$y = 0.024x - 0.062$ $r = 0.996$	76.3 ± 11.1	2
		40	38.50 ± 0.98 (2.55)	4.00 ± 2.82			
LTE_4	0.009 ± 0.001 (4.3)	20	21.60 ± 0.57 (2.60)	8.00 ± 2.83	$y = 0.010x + 0.002$ $r = 0.992$	84.9 ± 11.1	5
		80	77.50 ± 0.71 (0.91)	3.13 ± 0.10			
Urine							
LTE4	0.058 ± 0.007 (8.1)	10	9.90 ± 0.14 (1.41)	2.25 ± 3.30	$y = 0.047x + 0.133$ $r = 0.992$	76.3 ± 8.7	2
		40	42.20 ± 0.42 (9.90)	4.02 ± 2.76			

Values are means ± S.D. of $n = 10$ replications, except for recovery ($n = 16$); in parentheses C.V. percent are reported.
[a] Representative equation of a standard curve.

in Table 1. Reproducibilities are expressed as the coefficient of variation percent [C.V.(%) = (standard deviation/mean) · 100] of the slopes of the calibration curves run daily in duplicate at 5, 10, 20 and 40 ng/ml for blood and urine after extraction: the curves passed through the origin and were linear up to at least 80 ng/ml for all analytes. The between-day assay (inter-assay) precision over three months was assessed by determining the mean ± S.D. and C.V. at 10 and 40 ng/ml for LTB_4, 20 and 80 ng/ml for LTE_4 in blood and 10 and 40 ng/ml for LTE_4 in urine ($n = 10$ for each concentration). Recovery was calculated at 5, 10, 20 and 40 ng/ml for LTB_4 and LTE_4 in blood and 10, 20, 40 and 80 ng/ml for LTE_4 in urine (four replicates for each concentration) by comparing chromatographic peak areas with peak areas obtained by direct injection of equal amounts of LTs and reported as the mean ± S.D. for all values. Precision, accuracy, analytical recovery and the detection limit at a signal-to-noise ratio of 3:1 are also reported in Table 1.

Although not reported here, similar performance data were obtained for LTF_4 and $NAcLTE_4$ in rabbit urine.

The conversion of LTC_4 to LTE_4 in rabbit whole blood by exogenous GGTP and LAP was evaluated at the following LTC_4 concentrations: 0.2, 1, 10, 20, 40 and 80 ng/ml ($n = 4$ for each point). At LTC_4 concentrations of 0.2 and 1 ng/ml the resulting LTE_4 was measured by EIA in the fraction collected from HPLC. We obtained a significant correlation between added LTC_4 and measured LTE_4 ($r = 0.998$; slope = 1.05 ± 0.01 and intercept = -1.86 ± 0.80, mean ± S.D.).

In order to assess the specificity and the accuracy of the HPLC separation and the quantitation, we measured both LTB_4 and LTE_4 from 2 to 20 and 10 to 50 ng/ml, respectively in blood samples by both HPLC and EIA, obtaining a coefficient of correlation $r = 0.990$, slope of 1.12 ± 0.04 and intercept of -0.33 ± 1.90 for LTB_4 and $r = 0.996$, slope of 0.99 ± 0.03 and intercept of 0.33 ± 1.80 for LTE_4. For LTE_4 in the urine HPLC vs. EIA gives $r = 0.991$, slope = 0.97 ± 0.04 and intercept of 0.92 ± 2.10 (S.D.).

Figs. 2 and 3 show representative chromatograms obtained from various untreated and spiked blood and urine samples. All peaks were analyzed on the basis of retention times and UV

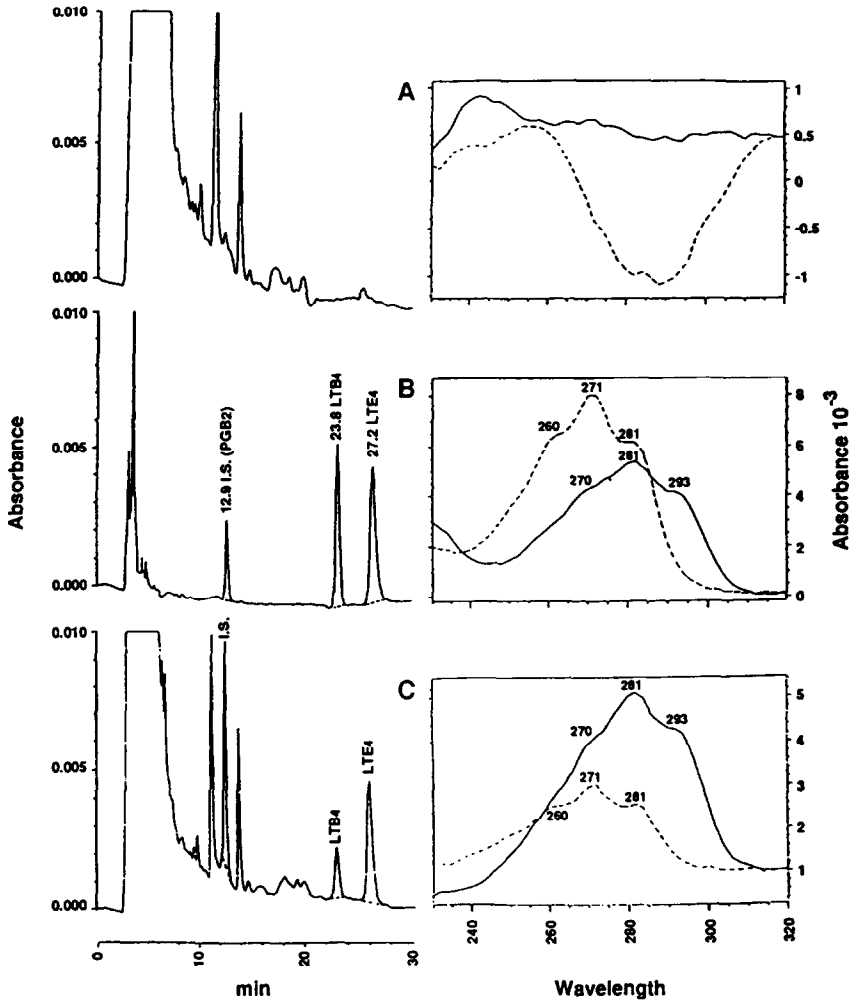

Fig. 2. Chromatograms and UV spectra of leukotrienes in rabbit blood. (A) Blank blood (1 ml) extracts. (B) HPLC analysis of LTB_4 (5 ng, dotted line) and of LTE_4 (10 ng, continuous line) and UV spectra of the peaks. Standards were injected directly into HPLC, together with the internal standard PGB_2 (10 ng). Retention times were 12.9, 23.8 and 27.2 min for PGB_2, LTB_4 and LTE_4, respectively. (C) Extracts of a representative rabbit blood sample (1 ml), after fMLP infusion. The concentrations of LTB_4 and LTE_4 were 11.9 and 80.3 ng/ml, respectively. UV spectra of the peaks are shown.

spectra compared to those of authentic standards of LTB_4 and LTE_4. No interfering peaks were found in the HPLC chromatograms at retention times close to those of LTs (capacity factor $k'_{LTB4} = 6.69$, $k'_{LTE4} = 7.78$ in blood and $k'_{LTE4} = 6.91$ in urine).

Although not reported here, similar performance data for LTB_4 and LTE_4 were obtained with other matrices, such as plasma, human whole blood, red blood cells, and washed white cell suspensions. The methods we utilized are based on simultaneous extraction from whole blood and HPLC separation and quantitation of LTB_4 and LTE_4 on the basis of retention times and UV spectra of the peaks. Using the described procedure the lifespan of the analytical column averaged 1000 h. Specific LTB_4 and LTE_4 EIA kits commercially available were used when the levels were below the HPLC detection limit.

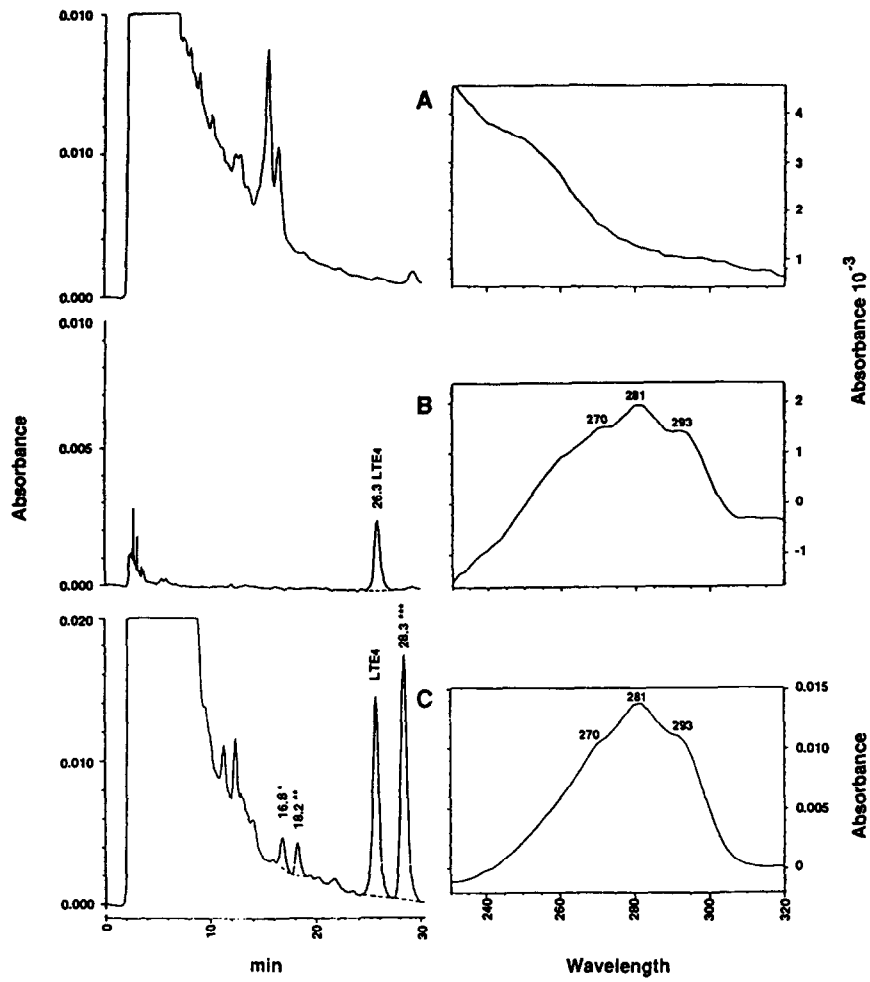

Fig. 3. Chromatograms and UV spectra of leukotrienes in rabbit urine. (A) Blank urine (5 ml) extracts. (B) HPLC analysis of LTE_4 (40 ng injected) standard with UV spectra of the peak. The retention time was 26.3 min. (C) Extracts of a representative urine sample (5 ml) after fMLP infusion, containing 66.4 ng/ml of LTE_4 with relative UV spectra on the peak. Peaks indicated with asterisks (*, **, ***) at retention times of 13.1, 18.1 and 27.5 min, respectively, were not characterized, but UV spectra were very similar to those of other LT metabolites (not shown).

Fig. 4 summarizes preliminary data of the kinetic profile of LTB_4 and LTE_4 in rabbits during and after 15 min of intravenous infusion by an Harward pump of the chemotactic peptide fMLP (2.5 nmol/kg/min). HPLC analysis of urine collected during 24 h after fMLP infusion showed detectable amounts of LTE_4 averaging 27.08 ± 2.08 ng/kg/h (mean $\pm$ S.D., $n = 3$). The chromatograms of urinary metabolites revealed at least three different components besides LTE_4 (designated with asteriks; see Fig. 3C). LTE_4 and component *** were predominant. The retention times of the above compounds were different from authentic standards of LTF_4, 20-carboxy-LTE_4 and $NAcLTE_4$ chromatographed and reported to represent potential metabolites excreted in urine in different animal species [4,7–11]. Structural characterization of these compounds remains to be established.

The concentration of LTE_4 was below the HPLC detection limit in the urine collected 24 h before surgery or before fMLP infusion. EIA

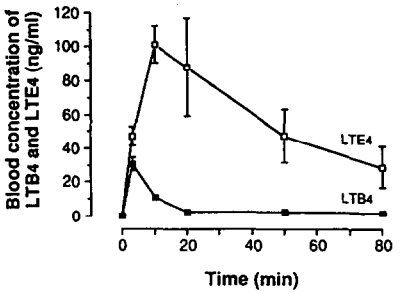

Fig. 4. Blood concentrations (ng/ml) of LTB_4 and LTE_4 at different times after intravenous infusion of fMLP (2.5 nmol/kg/min) in rabbit.

measurements of LTE_4 generation under basal conditions in the corresponding HPLC fractions showed an excretion rate of this metabolite of 0.84 ± 0.95 (mean $\pm$ S.D., $n = 3$) ng/kg/h. Fractionated urine collection indicated that LTE_4 was maximally excreted in the urine during the first 5-h period following fMLP infusion (not shown).

Urinary excretion of LTE_4 in rabbits can be an appropriate index for the in vivo generation of 5-lipoxygenase products in this species. Although rabbit is often used for experimental models of diseases in which LTs may be involved, no data are available, to our knowledge, on the synthesis and metabolism of LTB_4 and peptido-LTs in this animal species.

The method described, routinely used in our laboratory, offers the possibility to analyze in vivo production of LTB_4 and LTC_4 (from LTE_4 concentration) measuring these arachidonic metabolites directly in the blood stream and the production of peptido-LTs by measuring their major urinary metabolite after challenge of the animal with leukocyte agonist. This model might be useful to investigate the origin, the metabolism and the possible pharmacological modulation of LTs following in vivo stimulation by inflammatory mediators or in experimental disease models.

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