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LTB4 as marker of 5-LO inhibitory activity of two new *N*-ω-ethoxycarbonyl-4-quinolones

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Abstract

The supposed 5-LO inhibitory activity of two *N*-ω-ethoxycarbonyl-4-quinolones was tested determining leukotriene B4 (LTB_4) in RBL-1 cell cultures, pretreated with the two compounds of interest. LTB_4, obtained by solid-phase extraction (SPE) from celle cultures supernatants, was determined by micellar electrokinetic chromatography (MEKC). The analysis was performed using an uncoated capillary, filled with borate buffer at pH 8.3, containing 12.5 mM SDS as micelles generator. Therefore, following the decreasing of LTB_4 it was possible to verify the 5-LO inhibitory activity of two quinolone derivatives. To asses the suitability of the use of LTB_4 as marker of the activity of the new compounds, the analysis was repeated using quercetin, a well known 5-LO inhibitor.

Keywords: LTB_4; Micellar electrokinetic chromatography; *N*-ω-carbethoxy pentyl-4-quinolones; 5-LO inhibitor activity

1. Introduction

[5*S*,12*R*]-Dihydroxy-[6*Z*,8*E*,10*E*,14*Z*]-eicosatetraenoic acid, well known as leukotriene B_4 (LTB_4), is a metabolite produced in the first step of arachidonic acid (AA) biosynthetic pathway, catalyzed by 5-lipoxygenase enzyme (5-LO) [1]. In human leukocites the 5-LO metabolizes the AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) which can be converted to a series of biologically active compounds called leukotrienes. One of them is LTB_4, released from specific cells involved in inflammatory diseases. Therefore a specific inibihition of LTB_4 biosynthesis [2,3], could influence the inflammation process.

In the recent years many 5-LO inhibitors [4–7], were synthesized and different trials were used to test their activity. One of these inhibitors, A64077 zileuton [8], was tested in man. A64077 zileuton is

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(±)-*N*-hydroxy-*N*-(1-benzo[b]thien-2-ylethyl)-urea [9–12]; its 5-LO inhibitor activity was investigated in a clinical trial, performed on healthy volunteers. The LTB_4 biosynthesis was induced in the whole blood by stimulation with calcium ionophore A23187. Then the activity of the drug was tested determining the decreasing of LTB_4 by immunoassay [13,14]. In our group two *N*-ω-ethoxycarbonyl-4-quinolones (Fig. 1, compounds **1** and **2**), a new class of LTB_4 biosynthesis inhibitors, were synthesized [15]. We tested their activity in vitro in the rat basophilic leukemia cell (RBL-1) cultures [16–18]. Cellular cultures, pretreated with compounds **1** or **2**, were stimulated with calcium ionophore A23187, the most effective activating agent of the LTB_4 biosynthesis [19]. The activity of compounds **1** and **2** was verified determining by MEKC the decreasing of LTB_4 concentration in the supernatants of the RBL-1 cell cultures pretreated or untreated, respectively.

In the past years many papers about the hydroxyeicosatetraenoic acids and leukotrienes determination have been published [20–25]. The majority of these papers described HPLC methods, but the capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC) are not yet used in the determination of LTB_4 in the biological media.

Capillary zone electrophoresis allowed the separation of LTB_4 and of eicosatetraenoic acids like 5-HETE, 15-HETE and 12-HETE in the biological medium, but the resolution was not completely satisfactory. The addition of a surfactant in the running buffer, like sodium dodecyl sulphate, increased the sensitivity and selectivity of the analysis. Therefore in this paper the use of a MEKC method to follow the possible decreasing of LTB_4 concentration in the supernatants of RBL-1 cell cultures, pretreated with **1** and **2** and stimulated with a Ca^{2+} ionophore, is reported. To verify the suitability of the proposed method the experiment was repeated using different concentration of quercetin, a well known 5-LO inhibitor activity (15), like other flavanoids.

The same samples were analyzed also by HPLC and the data obtained were compared with those obtained by MEKC.

The method performed for the LTB_4 determination allowed also to analyze other AA metabolites like 5-HETE, 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*) eicosatetraenoic acid (15-(*S*)-HETE) and 12(*R*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-(*R*)-HETE).

2. Experimental

2.1. Chemicals

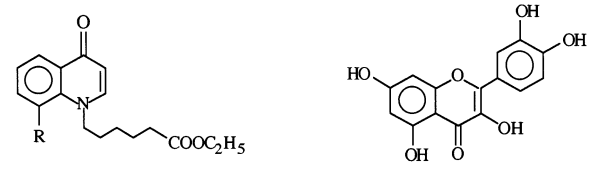
Boric acid, sodium tetraborate, sodium dodecyl sulphate (SDS), redistilled water were all of analytical or HPLC grade and were purchased from Merck (Darmstadt, Germany). Calcium ionophore A 23187, phorbolo-12-myristate-13-acetate, LTB_4, (15-(*S*)-HETE), (12-(*R*)-HETE) [(∓) 5-HETE] and prostaglandin B_2 (PGB_2), were purchased from Sigma–Aldrich (Italia S.r.l.).

2.2. Equipment

The electrophoretic experiments were carried out with a Spectra PHORESIS 1000 apparatus (Thermo Separation Products, Thermo Quest Group, San José, CA) equipped with a multi-wavelength UV-VIS detector SpectraFocus with deuterium lamp and cooling-air circulation by Peltier effect system (15–60°C).

The capillary electrophoresis was controlled and the data evaluated with the SpectraPhoresis CE v.1.05B software (Thermo Separation Products).

The separation of analytes was performed in a fused silica uncoated capillary (Supelco, Belle-



1) R = H
2) R = OH

QUERCETIN

Fig. 1. Chemical structures of *N*-ω-ethoxycarbonyl-4-quinolones (**1** and **2**) and quercetin.

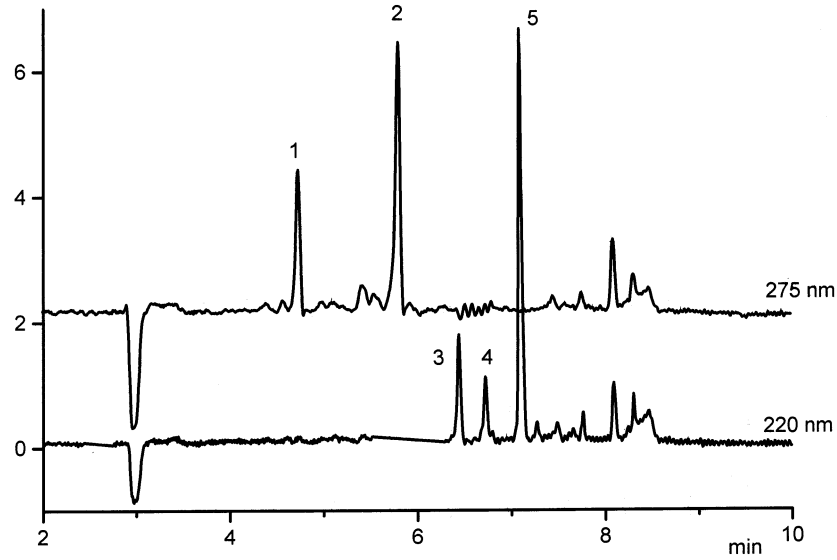


Fig. 2. Electropherogram of a standard solution containing: peak 1: PGB_2 (0.004 mg ml^{-1}); peak 2: LTB_4 (0.0025 mg ml^{-1}); peak 3: 12-HETE (0.004 mg ml^{-1}); peak 4: 15-HETE (0.006 mg ml^{-1}); peak 5: 5-HETE (0.003 mg ml^{-1}). Background Electrolyte: borate buffer (50 mM boric acid and 10 mM sodium tetraborate) at a pH 8.3 added with 12.5 mM SDS. Fused silica capillary with a total length of 42 cm (34 cm effective length) and 50 μ ID., The analyses conditions were: applied voltage 15 kV (15 μA), working temperature 20°C and injection time 6 s, detection wavelengths 270 nm for LTB_4 and 220 nm for HETEs.

fonte, PA) with a total length of 42 cm (34 cm effective length) and 50 μ I.D. The standard and sample solutions were introduced in the apparatus by hydrodynamic mode.

The separation of analytes by HPLC was made with a chromatograph Hewlett-Packard Series 1050 equipped with an HP 1040 M linear photodiode-array detector controlled by an HP 9000 Model 310 computer (Hewlett-Packard, Palo Alto, CA).

Sample solutions were injected via a Rheodyne Model 7125 valve using a 20 μl sample loop.

2.3. Samples preparation

LTB_4 concentration and then the 5-LO inhibitor activity of 1 and 2 was evaluated in the supernatants of the following cell cultures:

(a) a cell culture to check the usual cellular metabolites (blank);

(b) a cell culture stimulated with calcium ionophore A 23187;

(c) a cell culture preincubated for 15 min with quercetin and then stimulated with calcium ionophore A 23187;

(d) a cell culture preincubated for 15 min with the compound **1** or **2** and then stimulated with calcium ionophore A 23187.

All cell cultures were prepared using RBL-1 cell line (rat basophilic leukemia), grown in Eagle's minimal essential medium (EMEM) supplemented with 10% of fetal calf serum and 2 mM L-glutamine and penicillin-streptomycin. For the in vitro assay the cells were suspended, after washing with phosphate buffer saline pH 7.4, in EMEM without serum at a final concentration of 1.5×10^7 cell ml^{-1}. This suspension was preincubated at 37°C with test compound (50 or 100 μM) and dissolved in DMSO immediately before use. DMSO alone was added to cell suspension as negative control, while preincubation of cells with quercetin (6 μM) represented the positive control of 5-LO inhibition. After 15 min. Ca^{2+} ionophore A23187 and phorbolo-12myristate-13-acetate were added at a final concentration of 5 μg ml^{-1} and 40 ng ml^{-1}, respectively. The incubation was

stopped after 5 min at 37°C by addition of a mixture of 700 μl acetonitrile and methanol (1:1). The samples were than cooled at +4°C, centrifuged at 400 g min^{-1} for 15 min and the supernatants were collected and stored at −80°C until the analysis time.

The extraction of AA metabolites was performed using an octadecyl C18 cartridge (Sep-Pak C18, Millipore Waters Chromatography, Milford MA). The C18 cartridge was conditioned with methanol and water according to the manufacture's instruction. The supernatant of each cell cultures, added with convenient amount of PGB_2 as internal standard, was applied to the cartridge and washed with water (approx. 10 ml). The AA metabolites, adsorbed to the cartridge, were then eluted with methanol (about 4 ml). The recovery has been determined by the extraction of LTB_4 and PGB_2 standards from the supernatants of cell culture (blank), where they were added in known amounts. The average value of the recovery, obtained from five replicated extractions, was 81.7% ± 1.5 for the LTB_4 and 82.1% ± 1.3 for PGB_2.

2.4. Micellar electrokinetic chromatography (MEKC) conditions

The micelles were generated by adding SDS 12.5 mM to the borate buffer (50 mM of boric

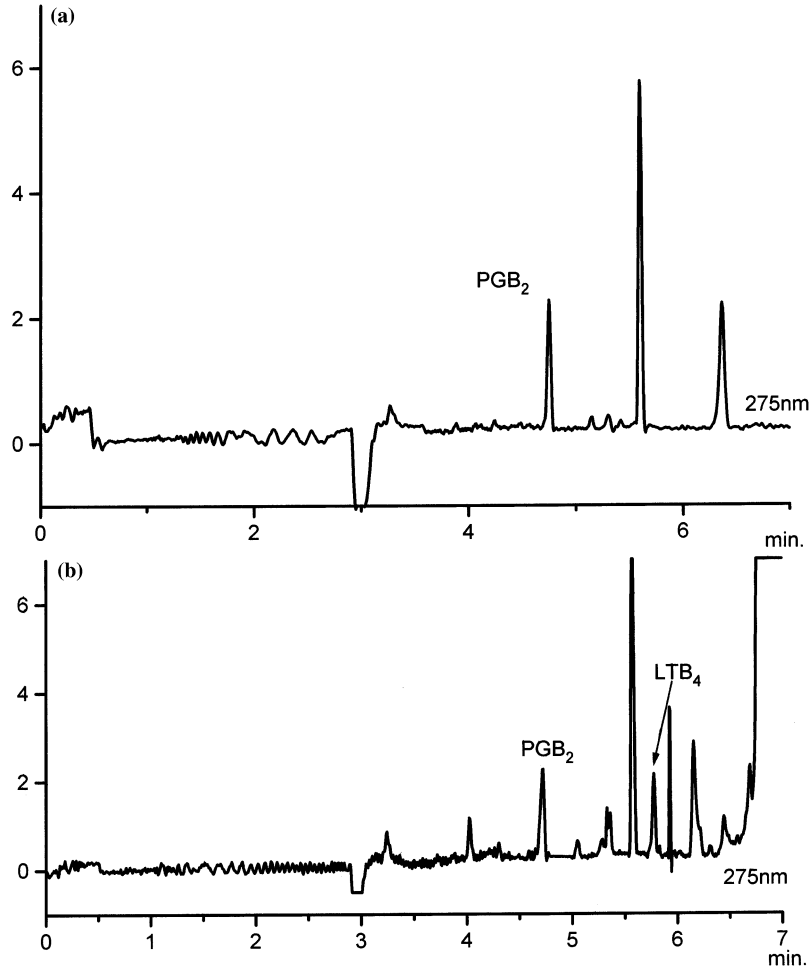


Fig. 3. Electropherograms of supernatants of: (a) blank and (b) stimulated cell cultures. Analysis conditions as in Fig. 2.

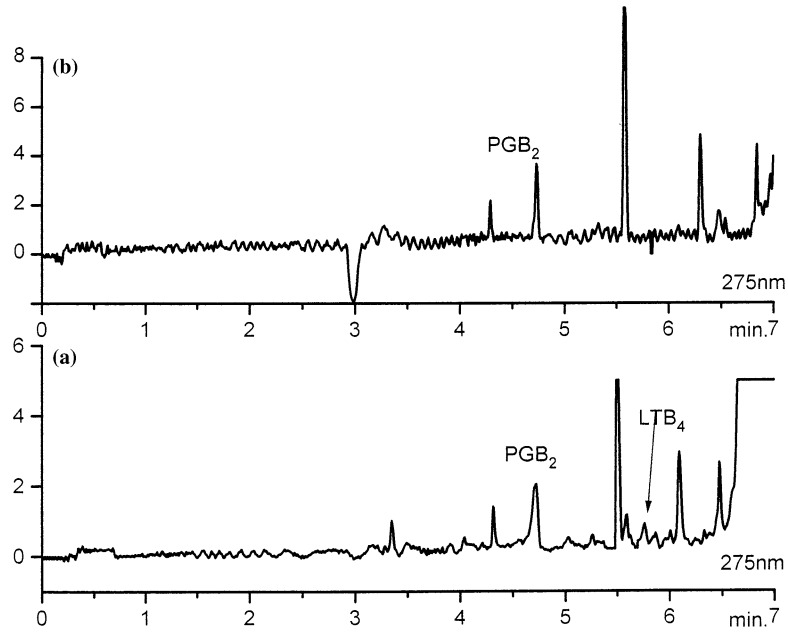


Fig. 4. Electropherograms of the supernatants of: cell cultures preincubated with quercetin and then stimulated with Ca^{2+} ionophore: (a) quercetin 3 μM and (b) quercetin 6 μM. Analysis conditions as in Fig. 2.

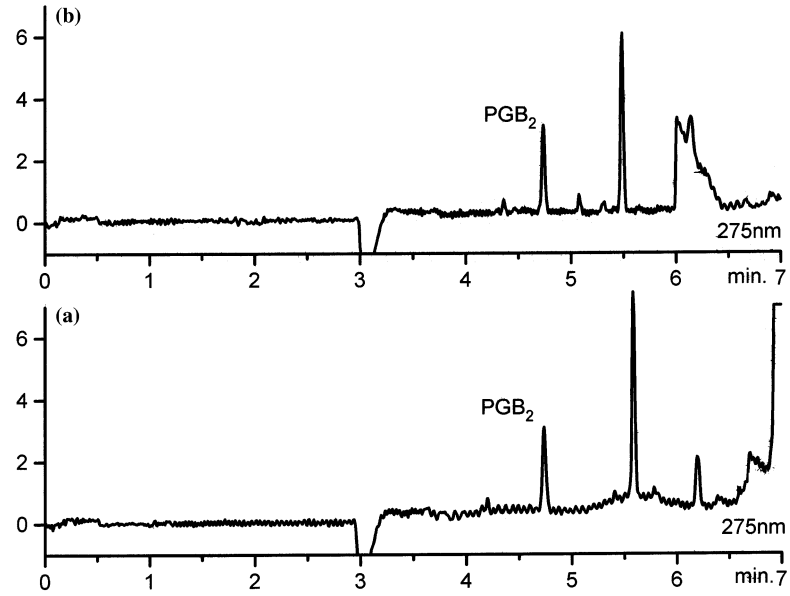


Fig. 5. Electropherograms of the supernatants of: cell cultures pre incubated: (a) with compound **1** 100 μM and (b) compound **2** 100 μM. After incubation both cultures were stimulated with Ca^{2+} ionophore. Analysis conditions as in Fig. 2.

acid and 10 mM of sodium tetraborate) at a pH 8.3.

Capillary conditioning was done every day (washing with 0.1 M sodium hydroxyde for 30 min) and the subsequent conditioning cycles were done before each run (washing with water for 10 min and buffer for 3 min).

The analyses conditions were: applied voltage 15 kV (15 μA), working temperature 20°C, injection time 6 s, UV detection at 220 and 275 nm.

2.5. HPLC conditions

Column: Inertsil ODS 5 μ (250 × 4 mm^2 I.D).
Eluent: acetonitrile: water acidified with 0.08% of acetic acid (65:35).
Flow rate 0.7 ml min^{-1}.
Detection at 273 nm and 230 nm for LTB_4 and HETEs, respectively.
Acquisition rate of spectra: 1.280 ms.
Bandwidth for each channel: 4.
Sensitivity range 50.
Reference wavelength 450 nm and reference bandwidth 50.

2.6. 5-HETE, 12-HETE, 15-HETE and LTB_4 determination

2.6.1. Working standard

To check the suitable operating conditions and the linearity of calibration curve a working standard solution was prepared by dissolving 5-HETE (0.003 mg ml^{-1}), 12-HETE (0.004 mg ml^{-1}), 15-HETE (0.006 mg ml^{-1}) and LTB_4 (0.006 mg ml^{-1}) in methanol. PGB_2 (0.004 mg ml^{-1}) as internal standard has been used.

Since LTB_4 and HETEs show different maximum absorbance values, the analyses have been carried out at 275 and 220 nm for CE and 273 or 230 nm for HPLC, respectively.

2.6.2. Samples analyses

Each methanolic fraction, obtained by solid-phase extraction from the supernatants of the cell cultures, was concentrated under vacuum until dryness, added with 500 μl of methanol and then analysed by MEKC or HPLC.

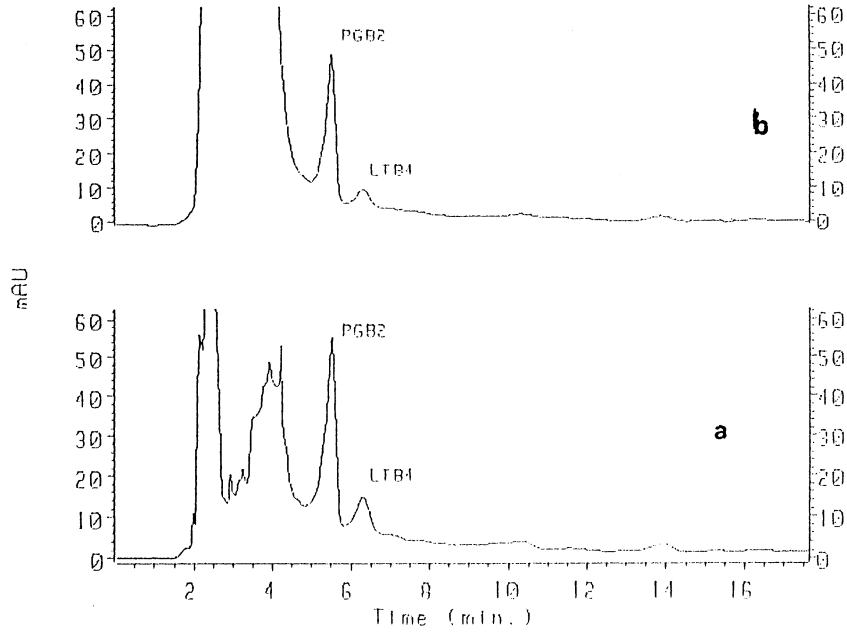


Fig. 6. Chromatograms of supernatants of: cell cultures: (a) stimulated with Ca^{2+} ionophore; and (b) preincubated with quercetin 3 μM before the stimulatiuon with Ca^{2+} ionophore. The chromatographic separations have been carried out on a column Inertsil ODS 5 μ (250 × 4 mm^2 I.D.), using as eluent a mixture of acetonitrile: water (65:35) containing 0.08% of acetic acid at a flow rate 0.7 ml min^{-1}. Detection at 273 and 230 nm.

Table 1
LTB_4 found in the supernatants of RBL-1 cells cultures[a]

Blanks and samples	ng LTB_4 by CE	ng LTB_4 by HPLC
Blank	–	–
Stimulated with Ca^{2+} ionophore A23187 (blank2)	182	189
Stimulated and preincubated with quercitin 3 μM	63	60
Stimulated and preincubated with quercitin **6** μM	–	–
Stimulated and preincubated with compound **1** 50 μM	81	82
Stimulated and preincubated with compound **1** 100 μM	–	–
Stimulated and preincubated with compound **2** 50 μM	70	65
Stimulated and preincubated with compound **2** 100 μM	–	–

[a] The amount of LTB_4 in the samples preincubated with compounds 1 and 2 100 μM was lower then LOQ, while the other metabolites were completely absent.

3. Results and discussion

The analytical conditions to quantitate LTB_4 in the biological fluids were optimized with a standard mixture containing LTB_4, 5-HETE, 12-HETE, 15-HETE and PGB_2 (the internal standard). As can be seen in Fig. 2 a good resolution of all used AA metabolites was obtained by adding 12.5 mM SDS to the borate buffer (pH 8.3). HETEs and LTB_4 have two different maximum absorbance values, therefore the electropherograms were recorded at 275 nm for LTB_4 and at 220 nm for HETEs.

The influence of 1 and 2 on LTB_4 biosynthesis was evaluated by the determination of this AA metabolite in the supernatants of following RBL-1 cell cultures:

1. Culture as blank (blank 1)
2. Culture, grown in the same conditions of blank1, but stimulated with calcium ionophore A23187 (blank 2)
3. Culture, preincubated with two different concentrations of quercetin before the stimulation with Ca^{2+} ionophore (sample 1a and 1b)
4. Culture, preincubated with two different concentration of drug **1** and then stimulated with Ca^{2+} ionophore (samples 2a and 2b)
5. Culture, preincubated with two different concentration of drug **2**, and then stimulated with Ca^{2+} ionophore (sample 3a and 3b)

All cultures, blanks and samples, were mixed with a known amount of PGB_2, as internal standard, before the extraction by Sep-Pack C_{18}.

The electropherogram reported in Fig. 3a, shows that LTB_4 was not in the supernatant of blank1 where only two unidentified peaks are present together with PGB_2. Recording the electropherogram at 220 nm the presence of the other AA metabolites, related to 5-LO, 12-LO or 15-LO biological pathway, was also excluded.

The stimulation of RBL-1 cells culture with Calcium ionophore A23187 (blank 2) induced the biosynthesis of LTB_4 (Fig. 3b), while was not observed any HETEs production.

The inhibitor activity of **1** and **2** was checked determining by MEKC the concentration of LTB_4 in the supernatants of sample 4 and 5. To assess the suitability of the use of LTB_4 as marker of 5-LO inhibitory activity of **1** and **2**, quercetin, a well known inhibitor of the 5-LO activity, was used. Therefore two RBL-1 cell cultures were preincubated with quercetin, 3 and 6 μM, respectively, then stimulated with Calcium ionophore A 23187. The electropherograms clearly showed that the lower quercetin concentration reduces the peak height related to LTB_4 which falls below the limit of detectability with the highest one (Fig. 4a,b).

The LTB_4 inhibition test, made with drugs **1** and **2**, showed that LTB_4 began to decrease when the samples were pretreated with a concentration of 50 μM of **1** or **2**. The complete disappearance of LTB_4 in the supernatant was obtained with a 100 μM concentration (Fig. 5a,b). The inhibition power on LTB_4 biosynthetic pathway seems to be the same for both drugs.

Table 2
HPCE method validation

Compound	Range of linearity (mg ml^{-1})	Correlation coefficent	LOD (mg ml^{-1}) (%RSD)	LOQ (mg ml^{-1}) (%RSD)	RSD of migration time intra day	RSD of migration time inter day
LTB_4	0.0009–0.004	0.9986	0.0008 (2.4%)	0.0009 (2.5%)	1.2%	1.4%
5-HETE	0.001–0.004	0.9961	0.0009 (2.6%)	0.001 (2.8%)	1.3%	1.6%
12-HETE	0.001–0.004	0.9950	0.0009 (2.6%)	0.001 (2.6%)	1.4%	1.6%
15-HETE	0.001–0.004	0.9952	0.0009 (2.5%)	0.001 (2.6%)	1.4%	1.5%
PGB_2	0.0008–0.004	0.9998	0.0007 (2.4%)	0.0008 (2.4%)	1.3%	1.5%

In the electropherograms the peaks identity was proved by:

(a) the increase of each peak height when the supernatants were sequentially enriched with a fixed amount of LTB_4 (or 15-HETE or 12-HETE) standard solution.

(b) the comparison of the UV spectra of the peaks with those of AA metabolites standard solutions.

The high LTB_4 absorptivity coefficient and a long injection time (6 s), allowed to have a very sensitive method and then to follow the decreasing of LTB_4 concentration in the samples 3, 4 and 5.

The suitability of the proposed method was evaluated repeating the determination of LTB_4 in the same samples by RP-HPLC.

The chromatogram of Fig. 6a clearly shows the presence of LTB_4 in the supernatant of a RBL-1 cells culture, stimulated with Calcium ionophore A23187. Also the decreasing of LTB_4 in the sample preincubated with quercitine 3 μM (Fig. 6b) is well visible. The results obtained by HPLC and MEKC seem to be comparable.

The results obtained analysing the supernatants of RBL-1 cells cultures preincubated with compounds **1** and **2** have been summarized in Table 1.

The method was validated by determining the range of calibration graph linearity, the limits of detection (LOD) and quantitation (LOQ) from 6 replicate injections, the accuracy and the repeatibility of migration time (Table 2).

In conclusion the determination of LTB_4, used as marker of 5-LO inhibition, seems to be a suitable way to test the activity of two *N*-ω-ethoxycarbonyl-4-quinolones. The suitability of MEKC method to follow the possible decreasing of LTB_4 in the supernatants was confirmed by analysis of supernatants of RBL-1 cell cultures were different amounts between 200 and 50 ng of LTB_4 were added together with a fixed amount of PGB_2. The MECK method resulted to be suitable for the determination of LTB_4; actually the comparison with the data obtained from HPLC analysis (LOD 0.7 mg ml^{-1} and LOQ 0.9 mg ml^{-1} for LTB_4) showed the same sensitivity and selectivity.

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