Increased urinary LTE₄ excretion following inhalation of LTC₄ and LTE₄ in asthmatic subjects


Increased urinary LTE₄ concentration was measured prior to and 1.5 and 3.5 h following inhalation of bronchoconstrictive doses of leukotriene C₄ (LTC₄) or LTE₄ in eight asthmatic subjects. Increasing doses of agonist were inhaled until a 35% fall in specific airways conductance (sGaw) was achieved.

There was no significant difference between the 53±3% (mean±SEM) fall in sGaw following inhalation of LTC₄ (63.1 ng geometric mean, GM, range 5.8–527.5 ng) and the 43±4% fall in sGaw following inhalation of LTE₄ 7.94 ng/GM (range 132–3701 ng). The LTE₄ excretion rate increased significantly from 2.95 (range 0.6–17.5) ng·h⁻¹ to 4.67 (range 0.8–20) ng·h⁻¹ at 1.5 h following LTC₄ inhalation; and from 1.8 (range 0.07–6.7) ng·h⁻¹ to 6.9 (range 2.9–27.3) ng·h⁻¹ at 1.5 h following LTE₄ inhalation; and had returned from baseline by 3.5 h. There was a correlation between the dose of LTC₄ inhaled and LTE₄ excreted in the urine (r=0.82 and r=0.72, respectively). The % recovery of LTE₄ in the urine, of the total dose of inhaled LTC₄ or LTE₄ administered, was 6.9±4.1% and 0.8±0.3%, respectively.

Thus, inhalation of bronchoconstricting doses of LTC₄ or LTE₄ alter urinary LTE₄ excretion in a dose-dependent fashion. This indicates that urinary LTE₄ can be used as a marker of sulphidopeptide leukotriene synthesis in the lungs of patients with asthma.


The sulphidopeptide leukotrienes (LTC₄, LTD₄ and LTE₄), previously recognized as slow-reacting substance of anaphylaxis (SRS-A) [1, 2], are derived from arachidonic acid by the action of 5-lipoxygenase which generates 5-hydroperoxy-eicosatetraenoic acid and then leukotriene A₄ (LTA₄). LTA₄ is metabolized by the addition of glutathione to LTC₄. LTC₄ may be converted by γ-glutamyl transpeptidase to generate LTD₄, which is converted by a dipeptidase to generate LTE₄ [3, 4].

In man, there is rapid metabolism of LTC₄ to LTD₄ and then to LTE₄. LTD₄ may be further metabolized to oxidation products which are excreted into bile and urine [5–7]. In vitro the sulphidopeptide leukotrienes LTC₄, LTD₄ and LTE₄ contract smooth muscle and enhance microvascular permeability [8–10]. In humans, they are potent bronchoconstrictor agents when inhaled and increase nonspecific bronchial hyperresponsiveness [10–12]. Combined reversed phase-high performance liquid chromatography (RP-HPLC) and radioimmunoassay (RIA) has enabled urinary LTE₄ concentration to be measured as an estimate of the production of sulphidopeptide leukotrienes in vivo [13]. Previous work has demonstrated an increase in LTE₄ excretion during an acute exacerbation of asthma compared to the recovery stage [14], and an increase in LTE₄ excretion following antigen challenge in asthmatic subjects [14, 15]. In a group of asthmatic subjects who were aspirin sensitive, baseline urinary LTE₄ concentration was raised with further release of LTE₄ following aspirin challenge [16]. There is no association between LTE₄ concentration and baseline lung function or degree of bronchial hyperresponsiveness to histamine [17, 18].

In normal healthy volunteers, the measurement of urinary LTE₄ following infusion of radiolabelled LTC₄ as either a bolus or infusion [19, 20], or inhalation of LTD₄ [21], suggests that urinary LTE₄ may act as a marker for whole body production of sulphidopeptide leukotrienes. No similar studies have been performed in asthmatic subjects and it is unknown how the local deposition and release of these mediators in asthmatic
airways alters urinary LTE₄ excretion. We have, therefore, determined the urinary LTE₄ excretion before and following inhalation of LTC₄ or LTE₄ in eight asthmatic subjects.

**Methods**

**Subjects**

Eight asthmatic subjects were studied (4 males, 4 females) aged 18–48 yrs, of whom three were atopic (table 1). Asthma was defined by a history of episodic wheezing and a greater than 20% reversibility of resting forced expiratory volume in one second (FEV₁) following 400 µg inhaled albuterol. Extrinsic asthmatic subjects demonstrated a greater than 3 mm wheal, as compared to the diluent control, in response to skin-prick tests to at least two common aeroallergens: grass pollen, tree pollen, cat dander, dog hair. *Dermatophagoides pteronyssinus* and *D. farinae*. Subject medication included inhaled albuterol and inhaled beclomethasone. Medication was withheld 8 h prior to provocation on each study day. Subjects had not taken antihistamines or cromolyn in the month prior to the study, and no subject had experienced an upper respiratory tract infection in the preceding month or during the study. The study protocol was approved by the Hochgebirgs-klinik, Davos-Wolfgang Ethics Committee and each subject gave informed consent.

**Study protocol**

Subjects attended the laboratory on two occasions, separated by at least 10 days, when inhalation challenge with inhaled LTC₄ or LTE₄ was performed in a single-blind, randomized fashion. On attending the laboratory on the second occasion, subject No. 6 refused LTC₄ inhalation challenge. Urine samples were collected at baseline, 1.5 and 3.5 h following inhalation of LTC₄ or LTE₄.

**Measurements of airway calibre**

Measurements of specific airways conductance (sGaw) were made in a total body plethysmograph linked to a digital computer (Bodytest, Jaeger Ltd).

**Inhalation challenge**

Inhalation challenges were performed using the Asthma Provocation System (APS) Jaeger dosimeter which delivers compressed air at a pressure of 1.6 bar (22.8 psi) for a duration of 0.6 s from the start of each breath. Under these conditions, the nebulizer delivers droplets with a mass median aerodynamic diameter of 1.9 µ, and the output of the nebulizer is 5.8 µl·breath⁻¹. The mean of five measurements of sGaw were recorded at baseline. Provided baseline sGaw was >0.7 s·kPa⁻¹, inhalation challenge with agonist proceeded. Subjects inhaled control solution (10 breaths of phosphate buffered saline) (PBS). Each inhalation started at functional residual capacity and terminated at approximately 70% baseline vital capacity. A 5 s breathhold was maintained at the end of each inhalation. If the decrease in sGaw was <10% from baseline value, subjects underwent inhalation challenge with LTC₄ or LTE₄.

**Inhalation challenge with LTC₄ and LTE₄**

LTC₄ and LTE₄ were prepared by total chemical synthesis, as described previously, and frozen under argon at -70°C [1]. Each leukotriene was analysed before inhalation challenge by RP-HPLC on a 10 µC₁₈ ultrasil-ODS column (4.6 x 250 mm; Beckman Instruments Inc., Berkley, CA, USA), at a flow rate of 1 ml·min⁻¹.

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**Table 1. – Characteristics of patients studied**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age yrs</th>
<th>Sex</th>
<th>Atopy</th>
<th>Treatment</th>
<th>FEV₁ % pred</th>
<th>PD₁₃₅sGaw LTC₄ nmol</th>
<th>PD₁₃₅sGaw LTE₄ nmol</th>
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<tr>
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<td>22</td>
<td>F</td>
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<td>A</td>
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<td>0.04</td>
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<td>+</td>
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<td>ND</td>
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</tr>
<tr>
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<td>F</td>
<td>+</td>
<td>A</td>
<td>89.0</td>
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<tr>
<td>8</td>
<td>46</td>
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<td>+</td>
<td>AB</td>
<td>112.0</td>
<td>0.004</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Mean: 36 SEM: 4.5

The mean is the arithmetic mean for age and % predicted FEV₁; and the geometric mean for PD₁₃₅sGawLTC₄ and PD₁₃₅sGawLTE₄. LTC₄: leukotriene C₄; LTE₄: leukotriene E₄; sGaw: specific airways conductance; A: inhaled albuterol 200 µg b.d. p.r.n.; B: inhaled beclomethasone dipropionate 200 µg b.d.; ND: LTC₄ inhalation challenge not performed in this subject; FEV₁: forced expiratory volume in one second; PD₁₃₅: provocative dose producing a 35% fall in sGaw.
with 65% methanol (BDH), 34.9% water, 0.1% acetic acid, pH 5.6, as solvent. Absorbance was monitored with an on-line spectrophotometer at 280 nm, linked to an integrator (Spectraphysics, Mountain View, CA, USA model SP 4270). The purity of each leukotriene was confirmed before challenge by its co-elution as a single peak at the identical retention times of the respective synthetic standards. The concentration of each leukotriene solution was assessed by ultraviolet scanning at 280 nm, assuming an extinction coefficient of 40,000 cm⁻¹·M⁻¹, and dilutions of each leukotriene were prepared in PBS.

For LTC₄ and LTE₄ challenges, each subject inhaled 10 breaths of geometrically increasing concentrations starting at 4×10⁻⁴ M and 4×10⁻⁵ M up to a maximum concentration of 1×10⁻³ M and 1×10⁻⁴ M for LTC₄ and LTE₄, respectively. The initial concentration of each inhaled leukotriene was determined from previous studies [22, 23]. sGaw was measured at 2 and 5 min, and then at 5 min intervals for 15 min. If a 35% decrease in sGaw was not achieved, the concentration of leukotriene in the nebulizer was increased by three fold and the protocol was repeated.

**Measurement of urinary LTE₄**

Urine was collected prior to and at 1.5 and 3.5 h following inhalation of LTC₄ and LTE₄. The volume of urine was recorded and a 50 ml aliquot saved. The free radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidino-oxy free radical (4-Hydroxy TEMPO; Aldrich Chemical Co., Milwaukee, WI) was added at a final concentration of 1 mM, and the samples adjusted to pH 9.0 with NaOH to stabilize endogenous leukotriene metabolites. The samples were coded and stored at -70°C until measurement of LTE₄ as described previously [13]. [3H] LTC₄, I.14 nCi (38.4 kCi·mol⁻¹; NEN, Lachine, Quebec, Canada) was added per milliliter of thawed urine samples, and the pH of the samples was adjusted to pH 5.4 with acetic acid. [3H] LTC₄, and endogenous LTE₄ were then extracted from 10 ml aliquots of urine using an "in-line" reversed-phase precolumn (C₁₈ Adsorbosphere, 5 µm diameter packing material; Alltech, Mandel Scientific, Lachine, Quebec, Canada). Leukotrienes were retrogradely eluted, via twin switching valves, onto a reversed-phase 10 mm diameter, 15 cm long, analytical column (C₁₈ HS, 3 µm diameter packing material; Alltech, Deerfield, III, USA) with a mobile phase consisting of MeOH:ammonium buffer (0.1% containing 1 mM disodium ethylenediaminetetra-acetic acid (EDTA); pH 5.4) in the proportions 70:30 (v:v) at a flow rate of 1 ml·min⁻¹. The column was calibrated for the retention times of synthetic LTC₄ (5.33±0.05 min, n=18) and LTE₄ (14.01±0.15 min, n=18). The radioactivity of the fractions eluting with retention time of synthetic LTC₄ was assessed by liquid scintillation spectrometry to determine leukotriene recovery. Fractions eluting with retention time of synthetic LTE₄ were evaporated to dryness under vacuum, and the LTE₄ concentration was measured by specific radioimmunoassay as described previously [13]. The sensitivity of the assay is 8 pg·ml⁻¹. The intra- and interassay coefficients of variation were 12 and 16%, respectively.

**Statistical analysis**

At baseline and at 1.5 and 3.5 h following inhalation of LTC₄ or LTE₄, urinary volume, creatinine and leukotriene concentrations were measured, and urinary LTE₄ was expressed as pg·mg⁻¹ creatinine. The LTE₄ excretion rate in ng·h⁻¹ was calculated. The baseline LTE₄ excretion rate was determined from the LTE₄ concentration in the urine at baseline and the mean rate of creatinine excretion over the 3.5 h period, since we have previously shown that creatinine excretion rate does not vary significantly over this period of time [24]. The increase in LTE₄ concentration was determined as the difference in LTE₄ concentration between the value at baseline and 1.5 h, and 1.5 and 3.5 h. The percentage nanogram recovery of LTE₄ in the urine was determined as the nanogram increase in urinary LTE₄ concentration, compared to baseline urinary LTE₄ divided by total nanogram amount of LTC₄ or LTE₄ inhaled at the mouth. Values of LTE₄ concentration were log transformed prior to analysis. The "t-test" for paired observations was used to compare baseline sGaw on the separate study days and percentage change in sGaw following bronchoconstriction with LTC₄ or LTE₄.

Comparison of urinary LTE₄ excretion rate and increase in urinary LTE₄ at baseline, 1.5 and 3.5 h following inhalation of LTC₄ or LTE₄ was performed using the Wilcoxon matched pairs test. The correlation between the increase in urinary LTE₄ excretion and the total inhaled dose of LTC₄ or LTE₄ at the mouth was calculated by least squares linear regression.

**Results**

**LTC₄ study day**

The cumulative dose of LTC₄ inhaled was 63.1 ng (GM), (range 5.8–527.5 ng) and was accompanied by a 53±3% (mean±SEM) fall in sGaw (table 2). The baseline urinary LTE₄ concentration on the LTC₄ inhalation study day was 30.1 gm (range 5.8–150) pg·mg⁻¹ creatinine. Following inhalation of LTC₄ there was a significant increase in urinary LTE₄ concentration at 1.5 h to 65.1, (range 13.9–154) pg·mg⁻¹ creatinine (p<0.05). At 3.5 h the urinary LTE₄ concentration was 45.1 gm, (range 0–93) pg·mg⁻¹ creatinine which was similar to that of baseline urinary LTE₄ concentration (p>0.05) (fig. 1). There was no correlation between baseline urinary LTE₄ concentration and the provocative dose of LTC₄ producing a 35% fall in sGaw (PD₃₅ sGaw LTC₄), which was 0.05 gm, (range 0.004–0.46 nmol (p>0.05).
Following inhalation of LTC₄, the increase in LTE₄ excretion at 1.5 h was 1.54 GM (range 0.27–5.5) ng (p<0.05) (table 2). There was a positive correlation between the cumulative dose of LTC₄ inhaled at the mouth and the increase in LTE₄ over baseline values at 1.5 h following inhalation of LTC₄ (r=0.82; p<0.05) (fig. 2).

The recovery of LTE₄ in the urine at 1.5 h following inhalation of LTC₄ was 6.9±4.1% (mean±SEM) (table 2). There was no significant recovery of LTE₄ in the urine at 3.5 h following inhalation of LTC₄.

Table 2. – The doses of LTC₄ or LTE₄ inhaled, increases in LTE₄ and the percentage recovery of LTE₄ in the urine at 1.5 and 3.5 h after leukotriene inhalation

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Inhaled dose ng</th>
<th>% fall in sGaw</th>
<th>Increase in LTE₄ ng 1.5 h</th>
<th>Increase in LTE₄ ng 3.5 h</th>
<th>% recovery LTE₄ 1.5 h</th>
<th>% recovery LTE₄ 3.5 h</th>
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<td>0.46</td>
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<tr>
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<td>0.24</td>
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</table>

Means are the geometric means except for the % recovery of LTE₄ in the urine and % fall in sGaw which is the arithmetic mean±SEM. For further abbreviations see legend to table 1.

Fig. 1. – The increase in urinary leukotriene E₄ (LTE₄) excretion in asthmatic subjects prior to and at 1.5 and 3.5 h post-inhalation of leukotriene C₄ (LTC₄). Symbols denote inhaled dose of LTC₄: ●: 520 ng; ▲: 58 ng; △: 18 ng; □: 5 ng. Bars represent geometric means.

Fig. 2. – The correlation between the cumulative dose of leukotriene C₄ (LTC₄) inhaled and the increase in urinary leukotriene E₄ (LTE₄) above baseline LTE₄ at 1.5 h post-inhalation in asthmatic subjects.
The basal LTE\(_4\) excretion rate on the LTC\(_4\) inhalation day was 2.95 \(\text{GM (range 0.6–17.5) ng·h}^{-1}\) (table 3). Following inhalation of LTC\(_4\) there was a significant increase in LTE\(_4\) excretion rate to 4.67 \(\text{GM (range 0.8–20) ng·h}^{-1}\) at 1.5 h (\(p=0.02\)), which had returned toward baseline by 3.5 h.

**LTE\(_4\) study day**

The dose of LTE\(_4\) inhaled was 794 \(\text{GM (range 132.6–3701) ng}\), which was accompanied by a 43±4\% (mean±SEM) fall in sGaw (table 2).

The baseline LTE\(_4\) concentration on the LTE\(_4\) inhalation study day was 18.3 \(\text{GM (range 0.6–68) pg·mg}^{-1}\) creatinine, which did not differ from the baseline LTE\(_4\) concentration on the LTC\(_4\) inhalation study day (\(p=0.4\)). Following inhalation of LTE\(_4\), there was a significant increase in urinary LTE\(_4\) concentration at 1.5 h to 84.8 \(\text{GM (range 18.9–209) pg·mg}^{-1}\) creatinine (\(p<0.05\)) (fig. 3). At 3.5 h the urinary LTE\(_4\) concentration was 55 \(\text{GM (range 6.4–232) pg·mg}^{-1}\) creatinine, which was similar to that of baseline urinary LTE\(_4\) concentration (\(p>0.05\)). There was no correlation between baseline urinary LTE\(_4\) concentration and the PD\(_{35}\) sGaw LTE\(_4\), which was 1.27 \(\text{GM (range 0.20–8.13 nmol)}\).

Following inhalation of LTE\(_4\), the increase in LTE\(_4\) excretion over baseline at 1.5 h was 3.7 \(\text{GM (range 0.2–40.8) ng}\) (\(p<0.05\)), which had returned toward baseline value by 3.5 h (table 2). There was a positive correlation between the cumulative dose of LTE\(_4\) inhaled at the mouth and the increase in LTE\(_4\) over baseline LTE\(_4\) at 1.5 h (\(r=0.72; p<0.05\)) (fig. 4).

The basal LTE\(_4\) excretion rate on the LTE\(_4\) inhalation day was 1.8 \(\text{GM (range 0.07–6.7) ng·h}^{-1}\) which did not differ significantly from that of the LTC\(_4\) inhalation day (\(p>0.05\)). Following inhalation of LTE\(_4\), there was a significant increase in LTE\(_4\) excretion rate to 6.9 \(\text{ng·h}^{-1}\) \(\text{GM (range 2.9–27.3) ng·h}^{-1}\) at 1.5 h (\(p<0.05\)), which had returned toward baseline by 3.5 h (table 3).

The recovery of LTE\(_4\) in the urine 1.5 h following inhalation of LTE\(_4\) was 0.82±0.33\% (mean±SEM)
E4 (LTE4) inhaled and the increase in urinary LTE4 above baseline.

Fig. 4. – The correlation between the cumulative dose of leukotriene LTC4 or LTE4 inhaled and the increase correlated with the dose of LTC4 or LTE4 inhaled in asthmatic subjects. This study has demonstrated that inhalation of LTC4 or LTE4 in asthmatic subjects resulted in an increase in the LTE4 excreted into the urine, and the increase correlated with the total dose of LTC4 or LTE4 inhaled. This suggests that urinary LTE4 excretion can reflect changes in LTE4 excretion. Although the dose of LTE4 or LTC4 inhaled at the mouth may differ from that administered to the bronchi, the observation that increases in LTE4 excretion correlated with the dose of LTC4 or LTE4 administered at the mouth suggests that even small changes in pulmonary levels of leukotriene are reflected by changes in LTE4 excretion. Although subject medication including inhaled albuterol and inhaled beclomethasone were withheld 8 h prior to inhalation challenge, one cannot exclude the possibility that these drugs may have an effect on leukotriene release, metabolism or excretion.

The timing of urine samples at 1.5 and 3.5 h was chosen from studies in asthmatic subjects where maximal LTE4 excretion occurs within 4 h following antigen challenge [14]. The increase in urinary LTE4 excretion following inhalation of LTC4 or LTE4 occurred within 1.5 h and returned towards baseline by 3.5 h. These results are consistent with rapid plasma clearance and local action of the sulphidopeptide leukotrienes.

There was no significant difference in the degree of bronchoconstriction following inhalation of LTC4 or LTE4 in the asthmatic subjects studied. There was a correlation between the dose of LTC4 or LTE4 inhaled and the increase in leukotriene excretion, such that a small increase in LTE4 excretion occurred in subjects who had the greatest airway responsiveness to inhaled LTC4 or LTE4. Thus, release of endogenous pulmonary leukotrienes, which could play a role in bronchoconstriction, may be reflected by alterations in urinary LTE4 concentration.

Approximately 7% of the total dose of LTE4 inhaled in asthmatic subjects was recovered in the urine at 1.5 h. This finding is similar to that in a study by Verhagen et al. [21], where the fractional conversion of inhaled LTD4 to urinary LTE4 in normal subjects was 3% [21]. Following infusion of radiolabelled LTC4 into healthy volunteers, 4–6% appeared in the urine as LTE4 [19, 20]. Subsequent metabolism of LTE4 to oxidative analogues, and biliary excretion of the sulphidopeptide leukotrienes, which were not assessed in this study, probably accounts for the remaining excretion. In two subjects (Nos. 4 and 8), there was an increased percentage recovery of LTE4 following LTC4 inhalation at 3.5 h. There were no clinical features distinguishing these two subjects and, whilst one cannot exclude the possibility that there was altered leukotriene metabolism in these subjects, it is more likely that an overestimation of the degree of excretion occurred due to the smaller dose of LTC4 inhaled in these subjects.

The more rapid cellular uptake of LTE4, compared to LTC4, and subsequent metabolism of LTE4 to metabolites not measured in this study, may account for the decreased recovery of LTE4 in the urine at 1.5 h following LTC4 inhalation. Uehara et al. [25] suggested that there are energy-dependent uptake processes for the transport of sulphidopeptide leukotrienes into cells, with a rank order of LTE4 > LTD4 > LTC4.

The baseline urinary LTE4 level in the asthmatic subjects studied was similar to that reported previously [14–16], and the 2–3 fold increase in LTE4 excretion following inhalation of LTC4 or LTE4 was similar to that observed following antigen challenge [14, 15]. We were unable to demonstrate a correlation between urinary LTE4 excretion and the degree of LTC4 or LTE4-induced bronchoconstriction consistent with prior studies [16, 17]. This is probably explained by the large variation in sensitivity of the airways to inhaled leukotrienes. Cumulative inhalations of 132–3701 ng LTE4 (an approximate 40 fold range) produced similar changes in sGaw (table 2).

In summary, we have demonstrated that a wide range of bronchoconstrictive doses of cysteinyl leukotrienes can be quantitatively recovered in the urine of asthmatic subjects. These results do not differ from previous studies in normal subjects [19–21]. The data suggest that the leukotriene-driven "tone" of asthmatic airways, as demonstrated by the bronchodilatory effects of LTD4 receptor antagonists [26, 27], is not caused by a defect in the metabolism or transfer of LTC4 from
the lung. The ability of urinary LTE\textsubscript{4} determinations to detect sub-nanogram changes in pulmonary leukotriene levels suggests that such measurements may accurately reflect alterations in the sulphidopeptide leukotriene status of the asthmatic lung [14–17].

References


