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BIO-Fully Automated Sample Treatment high-performance liquid chromatography and radioimmunoassay for leukotriene E_4 in human urine from asthmatics

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SUMMARY

BIO-Fully Automated Sample Treatment (BIO-FAST) high-performance liquid chromatography (HPLC) is a sophisticated column-switching technique in which a fresh pre-column is used for each sample prior to reversed-phase HPLC. The pre-columns, Varian Advanced Automated Sample Processor (AASP®) cartridges, are held and automatically advanced by the Varian AASP. A rapid and efficient extraction and separation for leukotrienes C_4 and E_4 from human urine has been developed using a C_8 cartridge and subsequent C_{18} analytical HPLC column. Quantitation of leukotriene E_4, accomplished by post-column radioimmunoassay, shows significantly increased leukotriene E_4 concentrations in urine samples from asthmatics after antigen challenge. This further confirms an active role for leukotrienes in the pathogenesis of bronchial asthma.

INTRODUCTION

Peptido-leukotrienes (LTs) are potent bronchoconstrictive inflammatory mediators [1] and may, with their metabolites, play an important role in the

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pathology of allergic disorders. One such disease is bronchial asthma, and leukotriene metabolites have been measured by reversed-phase high-performance liquid chromatography (RP-HPLC) and UV spectrometry, electrochemistry or radioimmunoassay (RIA) in lavage fluid from asthmatics [2–4].

As the technique of bronchial lavage in asthma may present clinical difficulties, some investigators have attempted to measure plasma leukotriene concentrations with the same techniques [5–7]. However, in such studies, the free circulating concentration is difficult to ascertain as leukotrienes might be released from leukocytes during blood or bronchial fluid sampling.

Measurement of urinary leukotriene metabolites may thus provide a more reliable method of investigating the involvements of leukotrienes in asthma, without the problems associated with obtaining plasma or lavage fluid. Of particular interest is LTE_4, the major metabolite of exogenous, intravenously administered [8] or inhaled leukotrienes [9].

We have recently demonstrated that LTE_4 can be measured in urine from cardiac ischaemia patients [10], a condition associated experimentally with 5-lipoxygenase activation. This study utilised octadecylsilyl silica (C_{18}) pre-column sample extraction and column switching, followed by C_{18} RP-HPLC and post-column RIA [10]. Similar on-line extractions and separations have been described for leukotrienes obtained from a variety of cellular sources [11–15]. Column-switching techniques reduce the time for sample preparation and allow a high degree of automation [16]. Unfortunately, repeated extractions of biological samples on a single pre-column or cartridge lead to changes in characteristics of the pre-column due to irreversible absorption of matrix components. This can cause high back-pressure, degradation of extraction efficiencies and cross-contamination between samples.

The BIO-Fully Automated Sample Treatment (BIO-FAST) HPLC system can be considered an ideal column-switching system because each sample is extracted using a fresh pre-column [17,18]. The pre-columns are Varian Advanced Automated Sample Processor (AASP®) cartridges [19] which are held by the AASP and are automatically changed for each sample, thus eliminating the problems associated with conventional column-switching techniques. Preliminary studies indicate that the previously described on-line C_{18} extraction and separation method for LTC_4 and LTE_4 [10] is readily adaptable to the BIO-FAST HPLC system with comparable results. Application of this system to the quantitation of LTE_4 in human urine from asthmatics after antigen challenge is presented.

EXPERIMENTAL

Reagents

Synthetic LTC_4 and LTE_4 were prepared in our laboratories. [^{3}H]LTC_4 (38.4 kCi/mol), [^{3}H]LTE_4 (31.8 kCi/mol) and scintillation fluid (Aquasol) were

supplied by NEN (Lachine, Canada). 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical (4-hydroxy-TEMPO) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and LC grade methanol from BDH (Montreal, Canada). Monoclonal antibodies against leukotrienes were obtained from the Weizmann Institute of Science (Rehovot, Israel). All other reagents were of analytical quality. Standard solutions of LTC_4 and LTE_4 (50–2500 ng/l) were prepared in 10-ml aliquots of ammonium acetate buffer (1 g/l, pH 5.4) containing 1 mmol/l Na_2EDTA in ultrapure water (Milli-Q system; Millipore, Bedford, MA, U.S.A.). The buffer was filtered through a 0.45-μm-pore Ultipor N-66 filter (Pall Trinity Micro, Cortland, NY, U.S.A.) before use.

Apparatus

A schematic diagram of the BIO-FAST HPLC system used for this study is given in Fig. 1. The system comprises a Vista 5500 LC (pump A) (Varian, Walnut Creek, CA, U.S.A.), a manually operated Rheodyne 7010 valve with 10-ml sample loop (Rheodyne, Cotati, CA, U.S.A.), an AASP and AASP injector valve (Varian), a Model 510 pump (pump B) (Waters Assoc., Milford, MA, U.S.A.), a Model 1040A photodiode array detector and 79994A HPLC Chem Station (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a Frac-100 frac-

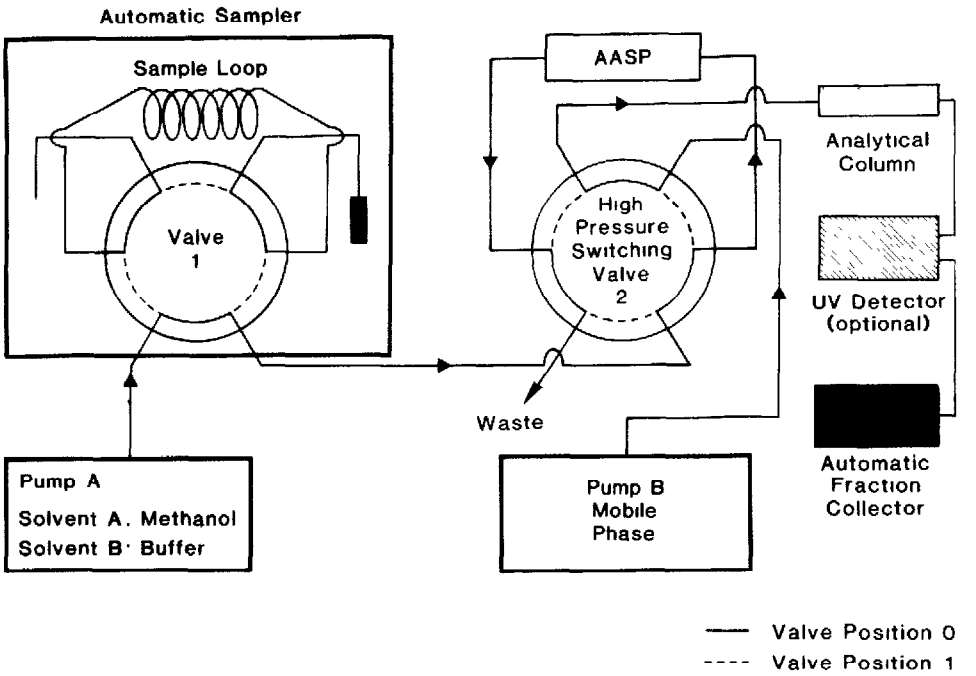

Fig. 1. Configuration of the BIO-FAST HPLC system. For this study a manual injection valve was substituted for the automatic sampler.

tion collector (Pharmacia, Uppsala, Sweden). Liquid scintillation counting was conducted with a Model 1219 spectrometer (LKB, Turku, Finland).

Urine samples

Baseline urine samples were obtained from seven allergic, mildly asthmatic males aged 19–31 years, at times outside the occurrence of seasonal allergens. All patients were either drug-free or controlled by inhaled β-agonists alone, the latter having no treatment for at least 10 h before urine sampling. After baseline urine collections, four subjects were challenged with inhaled antigen (birch or timothy pollen, or dog or cat dander) at a dose previously found to cause greater than 15% reduction in bronchial function. This was measured by the forced expiratory volume in 1 s, and compared with baseline values (% FEV_1), which ranged from 3.84 to 4.82 l and comprised $92.0 \pm 12.2\%$ (mean $\pm$ S.D.) of the volumes predicted from actuarial data. Urine was then collected during the early asthmatic response (between 0 and 3 h post-challenge). Samples were separated into 10-ml aliquots, endogenous leukotrienes stabilised by the addition of sodium hydroxide (to a final concentration of 0.1 *M*) and 4-hydroxy-TEMPO (to a final concentration of 1 m*M*). They were stored at $-70°C$.

Sample preparation for HPLC

For method development, standard reference solutions were prepared daily from synthetic LTC_4 and LTE_4. Solutions of 1.0 μg/ml were prepared in ammonium acetate buffer. Solutions of 2.0 ng/ml were prepared in water, ammonium acetate buffer or 5% or 10% methanol in ammonium acetate buffer, depending on the experiment.

Aliquots of urine (10 ml) were thawed and centrifuged at 9000 *g* for 10 min at 4 °C to precipitate particulates. The supernatant was acidified (pH 5.4) with glacial acetic acid, and the appropriate synthetic leukotrienes were added for estimation of recoveries.

[^{3}H]LTC_4 (10 nCi, 162.3 pg) was added to all 10-ml aliquots of urine samples as an internal standard for estimation of recovery from the chromatographic system. Similarly, [^{3}H]LTE_4 (10 nCi, 115.8 pg) or synthetic LTE_4 (0.5, 2.0 or 10.0 ng per 10 ml of urine) was added immediately before sample processing for estimation of LTE_4 recovery.

BIO-FAST HPLC procedure

The configuration of the BIO-FAST HPLC system and time programming of the master pump (pump A) are shown in Fig. 1 and Table I. AASP phenyl, C_{18} and C_8 cartridges (Analytichem International, Harbor City, CA, U.S.A.) were used in this study. The AASP washing and conditioning solvents were methanol and ammonium acetate buffer (see *Reagents* section). The analytical column was a Nova-Pak® C_{18}, 4 μm particle size, 150 mm $\times$ 3.9 mm I.D. (Waters Assoc.). The mobile phase consisted of 60:40 (v/v) methanol–am-

TABLE I

SEQUENCE OF VALVE SWITCHING AND PUMP A OPERATION

Time (min)	Operation	Effect
0.0	Valve 1: position 0 Valve 2: position 0	Load fresh C_8 cartridge
0.10	Pump: solvent A (4.0 ml/min)	Methanol wash of C_8 cartridge
0.75	Pump: solvent B (4.0 ml/min)	Buffer conditioning of C_8 cartridge
1.50	Pump: solvent B (2.0 ml/min)	Adjust flow to application rate
2 0	Valve 1: position 1	Inject sample and carry through C_8 cartridge
8.0	Valve 2: position 1	C_8 on-line with column: mobile phase elutes analytes onto column
8.1	Pump: 0.0 ml/min	Stop pump
9.0	Valve 2: position 0	End of on-line time for C_8 cartridge with column
9.1-30 0	Valve 1: position 0	Rinse sample loop
30.0	Automated advance to next cassette	Re-initiate cycle

monium acetate buffer, pH 5.4 (see above). Thus 60 volumes of methanol were diluted to 100 volumes with buffer. The mobile phase was sparged with helium for 15 min before use and ran continuously at 1.0 ml/min. In the method development experiments, eluting leukotrienes were monitored by a photodiode array detector set at 280 nm.

Radioimmunoassay

A 1-ml volume of appropriately eluting fractions of column effluent were collected in polypropylene tubes and evaporated under a stream of nitrogen gas. The residue was redissolved in 0.1–0.3 ml of an assay buffer consisting of 140 m*M* NaCl with 20 m*M* NaH_2PO_4 containing 0.1% gelatin and 0.02% sodium azide, adjusted to pH 7.2. RIA for LTE_4 was performed on 0.1-ml aliquots, as previously described [10,20]. LTE_4 immunoreactivity was quantified by comparison with authentic standards (10 p*M* to 100 n*M*). The identity of endogenous LTE_4 immunoreactivity was confirmed by its co-elution with added synthetic LTE_4.

Recoveries

For method development studies, recoveries were assessed by comparing peak areas of 20-μl injections of LTC_4 and LTE_4 (1.0 μg/ml each) made directly onto the analytical column with 10-ml samples of LTC_4 and LTE_4 (2.0 ng/ml each) injected onto the BIO-FAST HPLC system.

Analytical recovery of radiolabelled leukotrienes added to urine samples (see

Sample preparation for HPLC section) was assessed by liquid scintillation spectrometry. Column effluent was collected at 1-ml intervals into scintillation vials and mixed with 5 ml scintillation fluid. Radioactivity in appropriately eluting fractions was measured and compared with that of standard solutions dissolved in equivalent amounts of column effluent.

The recovery of exogenous LTE_4 was assessed by evaluating patients' baseline samples with or without addition of synthetic LTE_4. The LTE_4 content of fractions which eluted at the retention time of synthetic [^{3}H]LTE_4 was measured by RIA in blank urine samples and in the samples to which synthetic LTE_4 had been added. For each patient, the former result was subtracted from the latter to give the amount of exogenous LTE_4 recovered.

RESULTS AND DISCUSSION

The BIO-FAST HPLC system is a sophisticated column switching technique which comprises two sub-systems as shown in Fig. 1 [17,18]. The preparative sub-system consists of a programmable master pump (pump A), an automatic sampler and the AASP. The analytical sub-system consists of a pump (pump B), the analytical column, a detector and, in this case, a fraction collector. The sub-systems are linked by a fully automatic high-pressure pneumatic switching valve which is controlled via the master pump. Under normal operation, the biological sample containing internal standard is automatically injected into a stream of solvent which carries the solution through a pre-conditioned cartridge (held by the AASP) where the analyte and internal standard are retained. Following a wash to remove endogenous contaminants, the cartridge is placed on-line with the analytical sub-system via the fully controlled switching valve. The mobile phase transfers the analytes and internal standard to the analytical column in a forward flow fashion. While the chromatographic analysis is running, the switching valve is reset, a new cartridge is loaded and the system begins to prepare the next sample.

Since a fresh cartridge is used for each sample, pressure build-up, changes in extraction efficiency and sample cross-contamination do not occur. The cartridges are available in a wide variety of solid phases [19] at a relatively low cost. The overall system has minimal dead volume which reduces band broadening. It can be totally automated, allowing continuous operation of up to 100 samples with no human intervention.

Method development

Development of a column-switching method involves balancing the interactions between the sample matrix, solid-phase extraction and analytical chromatography conditions. During development of the BIO-FAST HPLC extraction and separation of leukotrienes from human urine, many of the parameters from the previous C_{18} method were retained [10]. For example, sample prep-

aration and chromatographic conditions were not significantly modified. Development centered around optimization of the AASP cartridge extraction.

Several studies have assessed the efficiency of solid-phase C_{18} cartridges for extracting leukotrienes from biological media such as plasma or cell incubates, and values ranging from 51 to 90% have been reported [21–23]. Ideally, for forward elution column-switching techniques, the pre-column sorbent should have a lower affinity for the analytes than the analytical column. This causes the analytes to elute in a narrow band with the analytical mobile phase, increasing the sensitivity by minimizing band broadening. Because the analytes are transferred quickly, the valve on-line time is short and this limits the transfer of endogenous compounds which cause late-eluting peaks.

Method development was conducted using reference solutions containing 2 ng/ml each of LTC_4 and LTE_4. Leukotriene recoveries and peak widths are indicated in Table II for the various parameters which were evaluated. C_8, C_{18} and phenyl cartridge types were tested, with C_8 cartridges giving the best overall performance. Use of phenyl cartridges resulted in narrow peaks, whereas C_{18} cartridges yielded the broadest peaks. C_8 cartridges gave intermediate peak shapes and the highest recovery values. Conditioning of the cartridge and application of the sample were evaluated with water, ammonium acetate buffer and 5 and 10% methanol in ammonium acetate buffer, all with comparable results. Buffer was chosen as it most closely resembled the analyte matrix.

TABLE II

EVALUATED METHOD DEVELOPMENT PARAMETERS

	LTC_4		LTE_4	
	Recovery (%)	Peak width at half-height (s)	Recovery (%)	Peak width at half-height (s)
Cartridge type (buffer conditioning solvent, 2 0 ml/min)				
C_8	70.2	16	62.4	32
C_{18}	52.0	20	54.7	39
Phenyl	62.9	12	30.3	20
Conditioning solvent (C_8 cartridge, 2.0 ml/min)				
Water	71.5	16	59.7	32
Ammonium acetate buffer	70.2	16	62.4	32
5% Methanol in buffer	70.7	16	62.6	33
10% Methanol in buffer	71 2	16	61.2	33
Application flow-rate (C_8 cartridge, buffer conditioning solvent)				
2 0 ml/min	70.2	16	62.4	32
1.0 ml/min	59.3	16	59.2	30
0.5 ml/min	40.6	16	44.4	30

Using buffer as the application solvent, flow-rates of 2.0, 1.0 and 0.5 ml/min were evaluated. The lower rates compromised leukotriene recoveries.

Previously reported methods have utilised an extensive post-application wash of the pre-column. However, a 20-ml buffer (4 ml/min for 5 min) wash did not clean-up the chromatogram of blank urine significantly and caused reduced recoveries of leukotrienes from the reference solution. Accordingly, in the method reported above, a minimal 2-ml wash was accomplished since the 10-ml sample was passed through the cartridge at 2 ml/min for 6 min.

The valve on-line time was set at 1.0 min, during which time transfer of the leukotrienes was complete. Late-eluting peaks were limited which allowed the use of an isocratic mobile phase. With the reference solution, retention times were approximately 3.8, 8.2 and 10.4 min for LTC_4, LTD_4 and LTE_4, respectively (Fig. 2). With one batch of mobile phase and column the retention times for LTC_4 and LTE_4 were as long as 6.2 and 16.1 min, respectively, in urine samples. Patient urine samples gave a late-eluting peak at approximately 25 min.

The overall BIO-FAST HPLC procedure is summarized in Table I. The total processing time for a given sample is 38 min, but a new sample is processed every 30 min because preparation of a subsequent sample is in progress while chromatography of the previous one continues. With full automation, 48 samples could be processed in a 24-h analytical day.

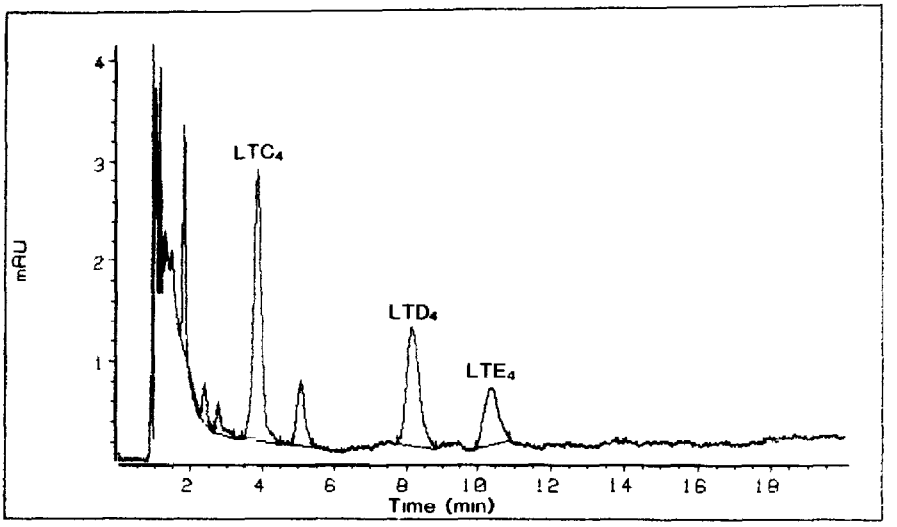

Fig. 2. BIO-FAST HPLC of synthetic LTC_4, LTD_4 and LTE_4, obtained from a 10-ml injection of a reference solution containing 2 ng/ml of each leukotriene in buffer with UV detection at 280 nm. The peaks at approximately 2 and 5 min are due to components of the buffer solution which were co-extracted with the leukotrienes using a C_8 cartridge. (For LC conditions, see Experimental.)

Validation

This method development was conducted using a manual injection valve. Preliminary validation was conducted in order to evaluate the BIO-FAST HPLC potential before investing in a large volume automatic sampler and fully automated fraction collector. This validation focused on recovery, reproducibility, inter-subject matrix evaluation and a cross-validation of patient samples with both the C_{18} extraction method [10] and the BIO-FAST method using C_8 cartridges.

The recoveries (mean ± S.D.) of radioactive leukotrienes [^{3}H]LTC_4 and [^{3}H]LTE_4 from urine samples of asthmatics were 71.1 ± 4.4% (10 nCi per 10 ml or 162.3 pg per 10 ml, $n=4$) and 81.9 ± 2.4% (10 nCi per 10 ml or 115.8 pg per 10 ml, $n=4$), respectively, as shown in Fig. 3. This demonstrates the reproducible recovery of the internal standard, [^{3}H]LTC_4. The high reproducibility for both leukotrienes in this inter-subject matrix evaluation suggests that the recoveries will be consistent throughout a population study.

Recovery of LTE_4 was assessed by evaluating patient samples to which synthetic LTE_4 was added at concentrations of 0.050, 0.200 and 1.000 ng/ml. Initially, endogenous LTE_4 immunoreactivity was evaluated in all baseline samples [0.034 ± 0.037 ng/ml (mean ± S.D.), seven samples, seven subjects]. The baseline LTE_4 concentration of each sample was subtracted from the total LTE_4 concentration determined for each spiked sample. The mean recoveries

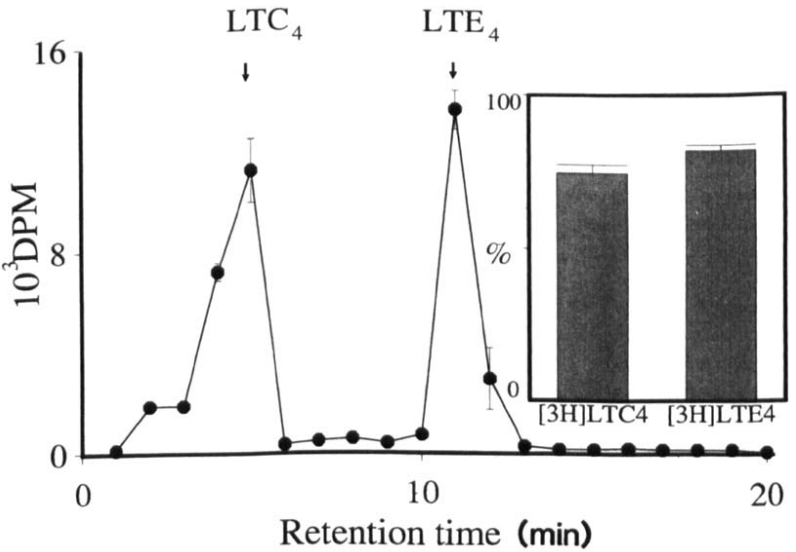

Fig. 3. BIO-FAST HPLC of [^{3}H]leukotrienes added to urine from asthmatic patients. ^{3}H was assessed by liquid scintillation spectrometry of fractions eluting after RP-HPLC of urine samples (10 ml, $n=4$) containing 10 nCi of [^{3}H]LTC_4 (left peak) and [^{3}H]LTE_4 (right peak). The analytical recoveries (mean ± S.D.) were 71.1 ± 4.4 and 81.9 ± 2.4%, respectively

were found to be in the range 50.7–74.4% with relative standard deviations of 24.9–31.5%. The results are summarized in Table III and Figs. 4 and 5. There was a wider variation when LTE_4 recoveries were assessed by post-column RIA, compared with scintillation spectrometry of fractions containing [^{3}H]LTE_4. This suggests that post-HPLC sample processing may be a source of variability. Nevertheless, results suggest that reproducible assays may be performed over the rather wide range of LTE_4 concentrations expected in urine from asthmatics [24]. These recoveries were comparable to those obtained from spiked urine samples from cardiac ischaemia patients [10].

TABLE III

RECOVERY OF EXOGENOUS LTE_4 FROM BASELINE URINE SAMPLES OF ASTHMATIC PATIENTS, ASSESSED BY BIO-FAST HPLC–RIA

Concentration of added synthetic LTE_4 (ng/ml)	Recovery (mean ± S.D.) (%)	n
0.050	74.4 ± 29.3	5
0 200	71.8 ± 31.5	7
1.000	50.7 ± 24.9	3

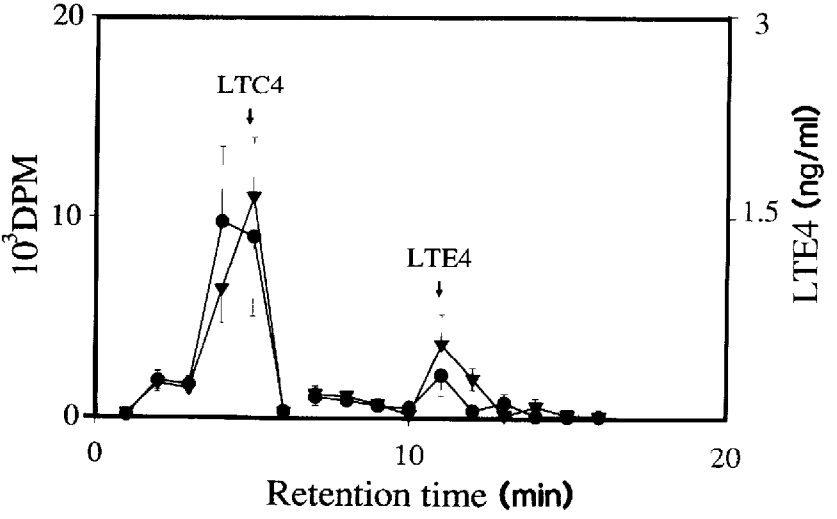

Fig. 4. BIO-FAST HPLC of endogenous LTE_4 (●) and endogenous plus synthetic LTE_4 (▼; 0.05 ng/ml) in urine from asthmatic patients. Immunoreactivity (right axis) that eluted with the retention time of synthetic LTE_4 (retention time denoted by arrow) was assigned to LTE_4. The recovery (mean ± S.D.) of 0.5 ng synthetic LTE_4 in 10-ml aliquots of urine was 74.4 ± 29.3% (n = 5) estimated by the difference between LTE_4 immunoreactivity measured in blanks and samples to which LTE_4 was added. [^{3}H]LTC_4 (left axis), added as an internal standard, was measured by liquid scintillation spectrometry of fractions to calculate recovery.

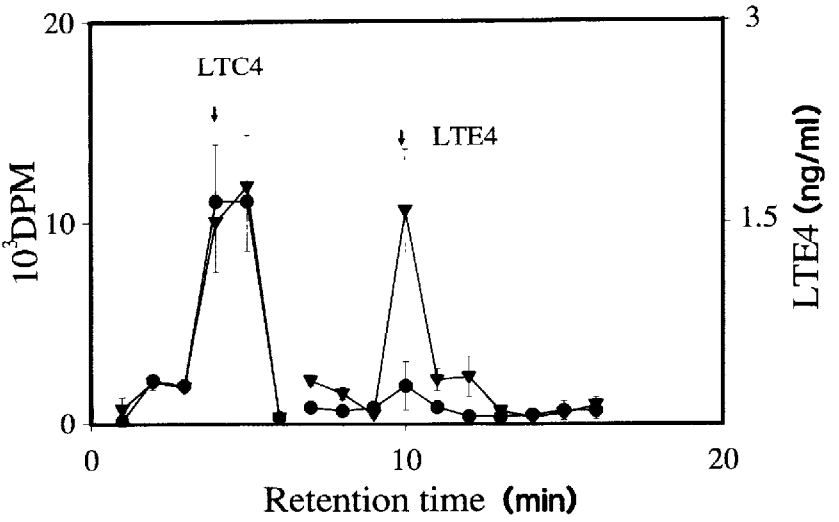

Fig 5 BIO-FAST HPLC of endogenous LTE_4 (●) and endogenous plus synthetic LTE_4 (▼; 0.2 ng/ml) in urine from asthmatic patients. The recovery (mean ± S.D.) of 2.0 ng synthetic LTE_4 was found to be 71.8 ± 31.5% ($n = 7$). See Fig. 4 for further explanation.

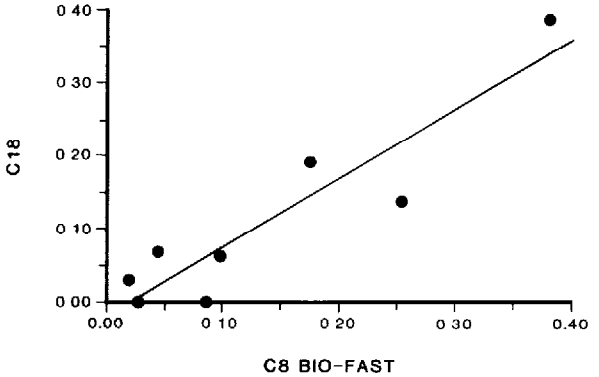

Fig. 6. Cross-validation of the C_{18} [10] and BIO-FAST HPLC methods for LTE_4 extraction and separation. Concentrations of LTE_4 immunoreactivity (ng LTE_4 per ml urine; both axes) were estimated in baseline and post-antigen challenge samples from four asthmatic patients. Each sample was evaluated once by each method. The correlation coefficient is 0.923

A cross-validation of the C_{18} extraction method [10] and the BIO-FAST method was conducted on the baseline and post-antigen challenge samples from four asthmatic patients (see *Biological application* section). Regression analysis of these results is shown in Fig. 6. The coefficient of correlation between the two methods is 0.923.

Biological application

The mean (±S.D.) concentration of LTE_4 immunoreactivity in pre-antigen challenged urine samples from four asthmatic patients was 0.056±0.046 ng/ml, compared with 0.034±0.046 ng/ml for the asthmatic group as a whole. These values were larger than those previously reported for normal subjects (0.017±0.015 ng/ml [10], suggesting that these asthmatics might have an ongoing minor allergic reaction resulting in an elevated baseline production of 5-lipoxygenase products and excretion of LTE_4. Such sub-clinical allergy could account for the discrepancy between their measured baseline respiratory function and the values predicted for equivalent non-asthmatic subjects (92.0±12.2%, mean±S.D. see *Urine samples* section).

Samples obtained in the first 3 h after inhaled antigen in these subjects showed large peaks of endogenous LTE_4 immunoreactivity that eluted with the same RP-HPLC retention time as synthetic LTE_4 (Fig. 7). Antigen provocation in these subjects caused a considerable acute bronchoconstriction (to 64.5±20.8% of baseline FEV_1; mean±S.D.; see Experimental section) and resulted in significantly elevated urinary LTE_4 concentrations (0.213±0.162 ng/ml; mean±S.D.; Fig. 8). This further confirms the role of leukotrienes in bronchial asthma suggested by recent studies [24,25].

CONCLUSIONS

Leukotriene metabolites in urine from asthmatic patients can be rapidly and reproducibly extracted and separated by BIO-FAST HPLC using a C_8 solid-

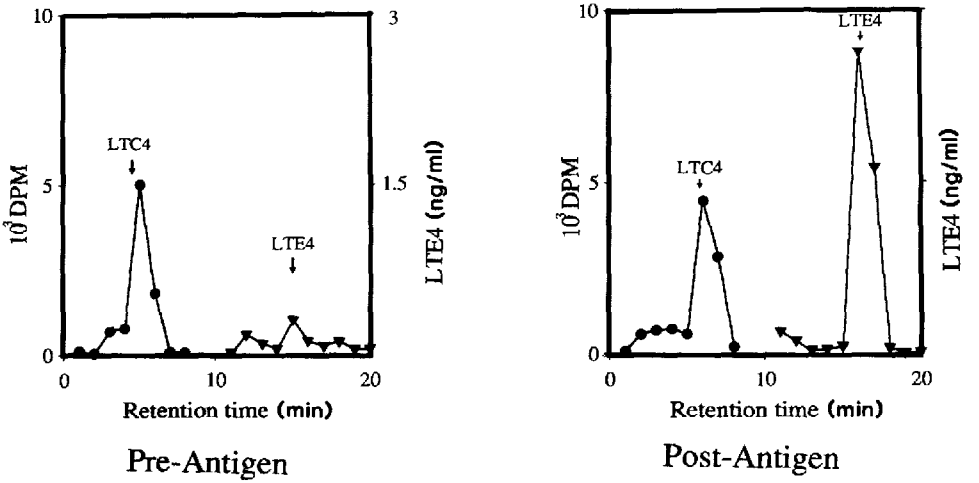

Fig. 7. Detection of increased co-eluting LTE_4 immunoreactivity in urine from asthmatic subjects with allergen-induced bronchoconstriction. Left panel: prior to antigen inhalation, only a small peak of immunoreactivity (right) co-eluted with synthetic LTE_4. [^{3}H]LTC_4 (left peak) was added to estimate recovery. Right panel: urine collected after antigen provocation in the same subjects showed a twenty-fold increase in co-eluting immunoreactivity, whilst recovery of [^{3}H]LTC_4 was comparable.

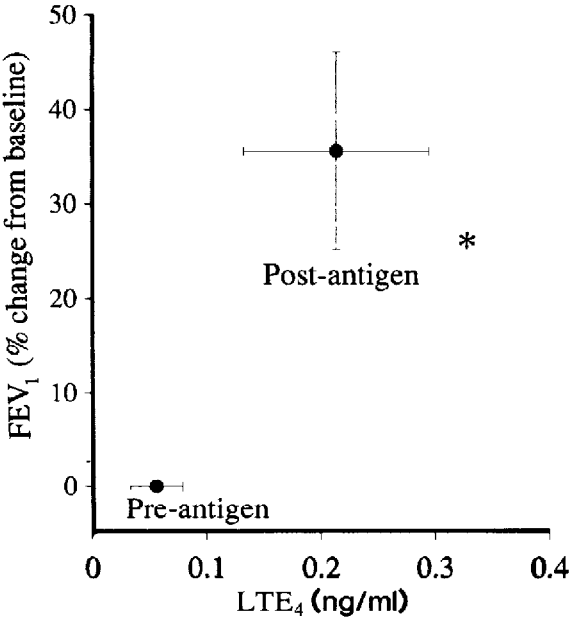

Fig. 8. Urinary LTE_4 concentrations in asthmatics measured by BIO-FAST HPLC and RIA. Antigen inhalation by four subjects caused an acute bronchoconstriction ($38.0 \pm 25.4\%$ fall in FEV_1; mean ± S.D.; left axis) and resulted in a significantly increased concentration of LTE_4 in the urine ($p < 0.005$, paired t-test).

phase extraction cartridge and C_{18} analytical column. Exogenous LTE_4 added at concentrations comparable to those observed in both unchallenged allergic asthmatics, and in subjects suffering from bronchoconstriction as a result of inhaled antigen, was measurable by RIA after BIO-FAST HPLC and demonstrated excellent recovery and reproducibility. Work is currently in progress towards developing the fully automated or true BIO-FAST HPLC assay. Analysis of human urinary leukotrienes by FAST-HPLC and RIA may thus prove to be a powerful tool for elucidating the role of leukotrienes in bronchial asthma and other inflammatory diseases.

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