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Application of totally automated on-line sample clean up system for extraction and high-performance liquid chromatography separation of peptide leukotrienes*

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Abstract: We have developed a fully automated on-line extraction–purification method for peptide leukotrienes in biological fluids using a column-switching technique. Individual leukotrienes in HPLC-collected fractions are determined by immunoassay techniques. Leukotrienes were extracted from nasal lavage samples and eluted from the solid-phase extraction cartridge into the HPLC column with methanol–ammonium acetate buffer (60:40, v/v) pH 5.4, as a mobile phase. This method provides good recoveries, excellent resolution of leukotrienes and is suitable to be combined with off-line radioimmunoassay (RIA) or enzymoimmunoassay (EIA). The most important losses are observed after evaporation–concentration and lyophilization. We also have compared RIA with EIA determination. Significant differences are found between RIA values and EIA values.

Keywords: *Column liquid chromatography; peptide leukotrienes; C_{18} solid-phase extraction; radioimmunoanalysis; enzymoimmunoanalysis.*

Introduction

Peptide leukotrienes, C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) are lipid mediators involved in inflammatory processes such as asthma and allergic rhinitis [1].

In basal conditions, peptide leukotrienes (p-LTs) are present in extremely low concentrations in nasal secretions [2] and this requires preliminary concentration and purification of these lipoxygenase metabolites before their quantitation by radioimmunoassay (RIA) and enzymoimmunoassay (EIA) techniques [3].

Concentration and purification of p-LTs are generally carried out on C_{18} reversed-phase cartridges but poor recoveries have been reported when compared to other arachidonic acid (AA) metabolites such as the prostanoids or LTB_4 [4]. Subsequent purification of the different peptide leukotrienes is accomplished by the separation of each one of these metabolites by high-performance liquid chromatography (HPLC) [5–7]. In all cases, the limiting step for the determination of p-LTs lies in the required concentration and evaporation of the organic eluent fractions both from the extraction cartridge and from the HPLC column. This step is responsible for important losses prior to quantitation by immunological or gas chromatography–mass spectrometry methods [8].

Commercial automated on-line sample clean up systems are available and we have applied one of these systems for evaluating its suitability in peptide leukotriene extraction and HPLC separation. This paper describes the application of a totally automated on-line sample preparation system which combines a solid-phase extraction and an HPLC separation method which may be combined with RIA or EIA techniques as well as with LC–MS methods for measuring LTC_4 and LTD_4 in nasal washes.

Experimental

Chemicals

Tritiated LTC_4 (157 Ci $mmol^{-1}$) and LTD_4 (157 Ci $mmol^{-1}$) and RIA and EIA commercial kits were purchased from Amersham

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International (Amersham, Bucks, UK); methanol, acetonitrile and EDTA were from Merck (Darmstadt, Germany). LTC_4 and LTD_4 standards were obtained from Cascade Biochem (Berkshire, UK).

Instruments

The automated modular sample handling system (Prospekt, Spark Holland, Emmen, The Netherlands) includes the following modules: a Marathon autosampler with an injection valve; a Prospekt module which contains the solid-phase extraction (SPE) cartridge transport and sealing mechanisms, three programmable six-port valves and also a microprocessor for controlling the modules and a solvent delivery unit (SDU) with one six-port valve and a high pressure pump for the selection and delivery of solvents into the cartridges (see Fig. 1). Valve 1 in the Prospekt module brings the cartridge on-line with the HPLC column when sample preparation is complete; valve 2 dries the cartridge when necessary and valve 3 directs the solvents from the SDU to the sample loop in the Marathon injection valve or flushes them to waste when appropriate (see Fig. 1).

Briefly, the HPLC system consisted of two ABI 400 pumps equipped with a 1000s diode array detector incorporating a gradient control board that allows control of the pump (Applied Biosystems, Foster City, CA, USA), a radioactivity detector (Ramona, Issomes, Straubenhardt, Germany) and a 2211 Superrack collector (LKB, Bromma, Sweden). On-line extractions were carried out using C_{18} precolumns (20 mg; particle size 40 μm; length 10 mm; i.d. 2 mm) manufactured by Spark Holland. HPLC separations were performed using an ODS-2 (length 25 cm; i.d. 4.6 mm; particle size 10 μm) from Phase Separations (Deeside, UK) and methanol–ammonium acetate buffer (60:40, v/v) (1 g l^{-1}, adjusted to pH 5.4 with acetic acid) at 1 ml min^{-1}.

Samples

A pool of nasal washes was obtained by instillation of 2 ml of saline into each nostril, while the volunteers extended the neck *ca* 30° from the horizontal and abstained from breathing or swallowing. After 5–10 s, the subjects expelled the nasal washes into propylene tubes. A pool of nasal washes was immediately assayed.

Automated on-line extraction and HPLC separation procedure

Aliquots of nasal lavage pool were automatically extracted on activated C_{18} cartridges and the extracts directly injected into the HPLC column by the Prospekt system (see Fig. 1). The SPE cartridge was activated with methanol (0.75 ml; 30 s at 1.5 ml min^{-1}) and conditioned with acidified water (pH 4.0) with 0.1% EDTA (3 ml; 2 min at 1.5 ml min^{-1}). Samples (0.5 ml) were transferred from the injection loop to the activated cartridge using acidified water (pH 4.0) with 0.1% EDTA (3 ml; 3 min at 1 ml min^{-1}) and after completion of sample loading subsequent washing

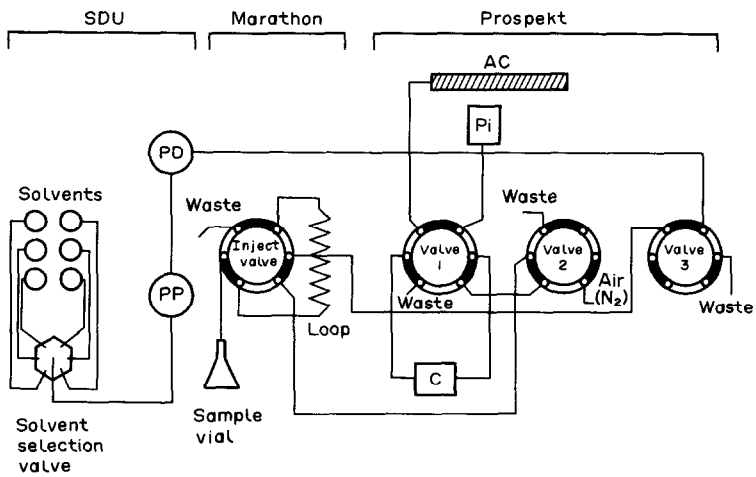

Figure 1
Schematic diagram depicting the Prospekt and solvent delivery unit module valves, the fluid lines layout and the Marathon injection valve. PP = purge pump; PD = pulse damper; AC = analytical column; Pi = HPLC pump; C = cartridge.

of the cartridge was accomplished with the same eluent (0.5 ml; 30 s at 1.5 ml min^{-1}). Following this, leukotrienes were eluted from the cartridge into the analytical column with methanol–ammonium acetate buffer (60:40, v/v) (1 g l^{-1}, adjusted to pH 5.4 with acetic acid), as the mobile phase (6 ml; 6 min at 1 ml min^{-1}) with the high pressure switching valve in the elute position (valve 1). After the initial 6 min, elution was continued with the same mobile phase at 1 ml min^{-1} for 16 min. Once the sample had been transferred to the analytical column, valve 1 remained in the purge position for 6 min in order to purge the lines with methanol (6 ml) and the used cartridge was disposed off and replaced by a new one prior to the next sample clean up cycle.

RIA procedure

After the individually collected HPLC fractions containing the LTC_4 and LTD_4 peaks were taken to dryness by combined centrifugal vacuum evaporation and lyophilization, the dried residues were reconstituted in 500 µl methanol. Aliquots (100 µl) were evaporated to dryness and the resulting residues were directly subjected to RIA. A commercially available LTC_4–LTD_4–LTE_4 radioimmunoassay from Amersham International (Amersham, Bucks, UK) was used in accordance with the supplier's instructions.

EIA procedure

A 100 µl aliquot of the methanol solution was evaporated to dryness and resuspended in EIA buffer. Then, it was transferred to microplate for EIA measurements. LTC_4 and LTD_4 determinations in duplicate were made by means of a EIA kit for LTC_4–LTD_4–LTE_4 kindly provided by Amersham International. Leukotriene measurements were carried out by use of a Labsystems Integrated EIA Management System (iEMS) from Labsystems (Helsinki, Finland).

Recovery calculations

To verify losses at the various steps in the procedure, samples were spiked with 50.000 dpm of tritiated LTC_4 and LTD_4 and radioactivity was then counted after each of the steps of HPLC fraction collection, vacuum evaporation and lyophilization (see Table 1). Also, to check on losses directly arising only from the evaporation–lyophilization process, selected HPLC eluates were spiked with 50.000 dpm of tritiated LTC_4 and LTD_4 and the remaining radioactivity was counted after evaporation and then after lyophilization of the same fractions (see Table 2).

Results and Discussion

Table 1 shows the recoveries obtained for

Table 1
Percentage recoveries obtained for combined extraction and HPLC purification of LTC_4 and LTD_4 from nasal lavage fluid

	Recovery (%)	
	LTC_4	LTD_4
(A) Cartridge extraction + HPLC + fraction collection*	85.3 ± 3.7 (n = 4)	69.0 ± 6.9 (n = 6)
(B) Evaporation/lyophilization†	58.8 ± 13.6 (n = 4)	77.8 ± 8.1 (n = 5)
(C) Extraction + HPLC + evaporation/lyophilization‡	50.0 ± 11.1 (n = 4)	55.5 ± 5.1 (n = 6)

*On-line extraction HPLC purification.
†Recoveries relative to values obtained in (A).
‡Recoveries from (A) + (B) relative to total radiolabel activity added to samples.

Table 2
Percentage recoveries after the vacuum evaporation and lyophilization process

	Recovery (%)	
	LTC_4	LTD_4
(A) Vacuum evaporation of the organic phase component	94.0 ± 1.8 (n = 5)	90.3 ± 1.1 (n = 5)
(B) Vacuum evaporation + lyophilization to dryness	66.6 ± 6.5 (n = 5)	71.0 ± 7.9 (n = 5)

LTC_4 and LTD_4 after direct extraction and purification of nasal lavages using an automated sample extraction and separation system. As indicated in Table 1, an important decrease in recoveries was observed after the evaporation–concentration and lyophilization of the collected HPLC fractions. The collected volume for each fraction was approximately 4 ml which contained 40% ammonium acetate buffer (1 g l^{-1}, adjusted to pH 5.4 with acetic acid) and 60% methanol. The latter was vacuum evaporated in a rotary centrifugal concentrator while the complete elimination of the water phase required overnight lyophilization to dryness. Losses of peptide leukotriene mainly occurred during the lyophilization process, as shown in Table 2. These data confirm that the evaporation–concentration and lyophilization can be limiting steps in the quantitative analyses of these compounds by either spectrometric or immunological techniques. Comparison of data in Tables 1 and 2 indicates that losses of LTD_4 occurred mainly upon HPLC purification whereas LTC_4 is more liable to losses upon evaporation.

Figure 2 shows a typical radiochromatogram obtained from a nasal lavage pool supplemented with tritiated standards of LTC_4, LTD_4 and LTE_4, which was processed by a Prospekt on-line sample clean up system. These data confirm that a complete separation of these peptide leukotrienes was accomplished within 15 min under these isocratic HPLC mobile phase conditions and the lack of additional radiolabel peaks shows that no on-column degradation occurred during the automated extraction and HPLC purification. Overnight pretreatment of the C_{18} cartridges and HPLC column with 0.1% EDTA solution added to the mobile phase was found to be essential for good recoveries, as also discussed by other authors [9]. Samples of nasal washes were then run using the same mobile phase but without EDTA. The lack of EDTA in the HPLC fractions is important in order to avoid the possibility of EDTA interferences in the RIA measurements because it is known that volatility of the chromatographic buffer is a necessary condition when the HPLC is followed by a RIA quantitation step. Along these lines, the use of volatile ammonium acetate in the HPLC eluent makes this method amenable to direct LC–MS, as will be shown elsewhere.

Finally, aliquots the same HPLC extracts were subjected to RIA and EIA, respectively. Data shown in Fig. 3, reveal that RIA values

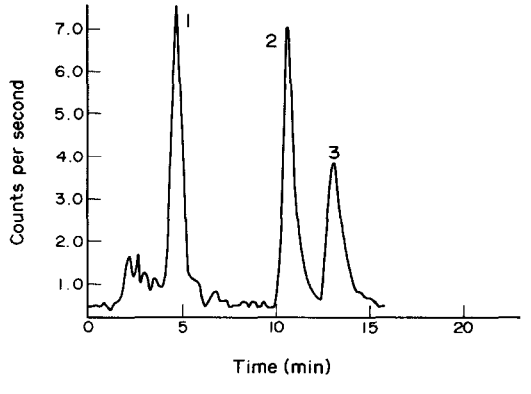

Figure 2
Typical radiochromatogram of nasal lavage spiked with tritiated peptide leukotriene LTC_4 (1), LTD_4 (2) and LTE_4 (3) standards. Analyses were performed with an ODS-column (length 15 cm; i.d. 0.4 cm; particle size 5 μm) isocratically eluted with a mixture of 60% methanol and 40% H_2O–ammonium acetate, pH 5.4, as mobile phase. Flow rate was 1 ml min^{-1}.

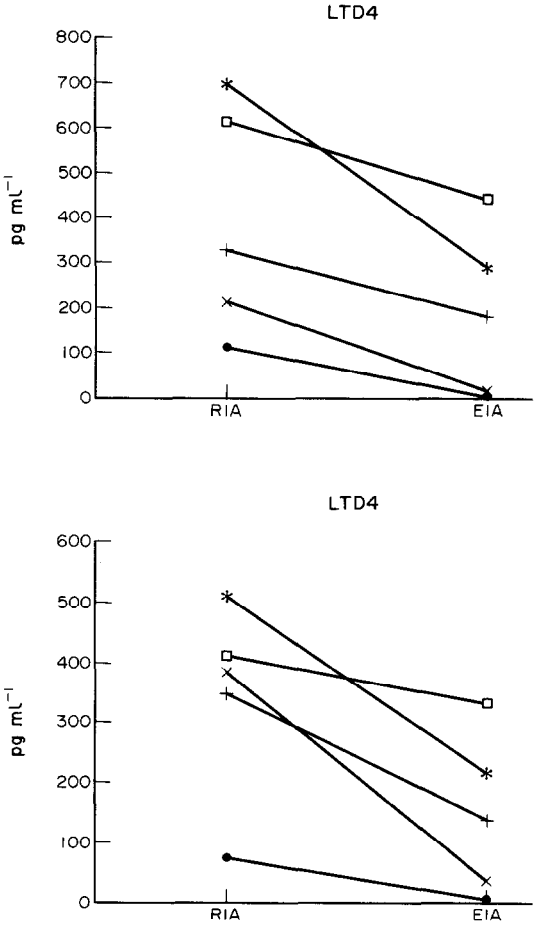

Figure 3
Comparison between RIA and EIA values from the same nasal lavage aliquots, taken from five different individual samples.

were always higher than EIA values. These significant differences seem to be associated with the immunological procedures applied and further studies are under way in order to validate both immunological assays by gas chromatography and mass spectrometric techniques.

In conclusion, peptide leukotrienes can be extracted and purified from nasal lavages using an automated on-line method compatible with off-line immunological techniques.

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