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Note

Separation of the N-trifluoroacetyl dimethyl esters of leukotriene D and E isomers by semi-preparative high-performance liquid chromatography

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Leukotrienes D (LTD) and E (LTE) are members of a series of 7,9,11,14-eicosatetraenoic acids[1]. Recent evidence has shown that LTD is one of the biologically active components of the human slow reacting substance of anaphylaxis (SRS-A)[2]. SRS-A is one of the chemical mediators, the presence of which gives rise to the clinical symptoms of asthma[3]. To investigate this situation further, a series of pure LTD and the related LTE isomers were required for comparative pharmacological studies.

LTD and LTE can be readily prepared from leukotriene A methyl ester (1b). In the convergent synthesis of 1b three additional major geometric isomers are formed which are isomeric about the 9,10- and 11,12-double bonds[4–7]. The separation of these isomers is described elsewhere[8]. Each geometric isomer of leukotriene A methyl ester exists as a racemic mixture. Individual reaction of 1b with the N-trifluoroacetyl methyl ester of either optically pure L-cysteinylglycine or L-cysteine gives protected LTD[9] (2bii) and LTE[10] (3bii), respectively, and their corresponding isomer pair (2bi and 3bi). A further six isomers of protected LTD and LTE were similarly formed by reaction of 1a, 1c and 1d (Table I). These N-trifluoroacetyl dimethyl ester derivatives were synthesised for ease of chromatographic separation. After chromatographic purification all the derivatised molecules were converted to the corresponding unprotected molecules for comparative pharmacological studies.

The use of semi-preparative high-performance liquid chromatography (HPLC) to separate all of these isomeric molecules is described. Mass spectrometry and ultraviolet (UV) spectroscopy were utilised to characterise the chromatographic peaks.

EXPERIMENTAL

Reagents

The Spherisorb S5W, S5NH, S5CN and S5ODS column packing materials

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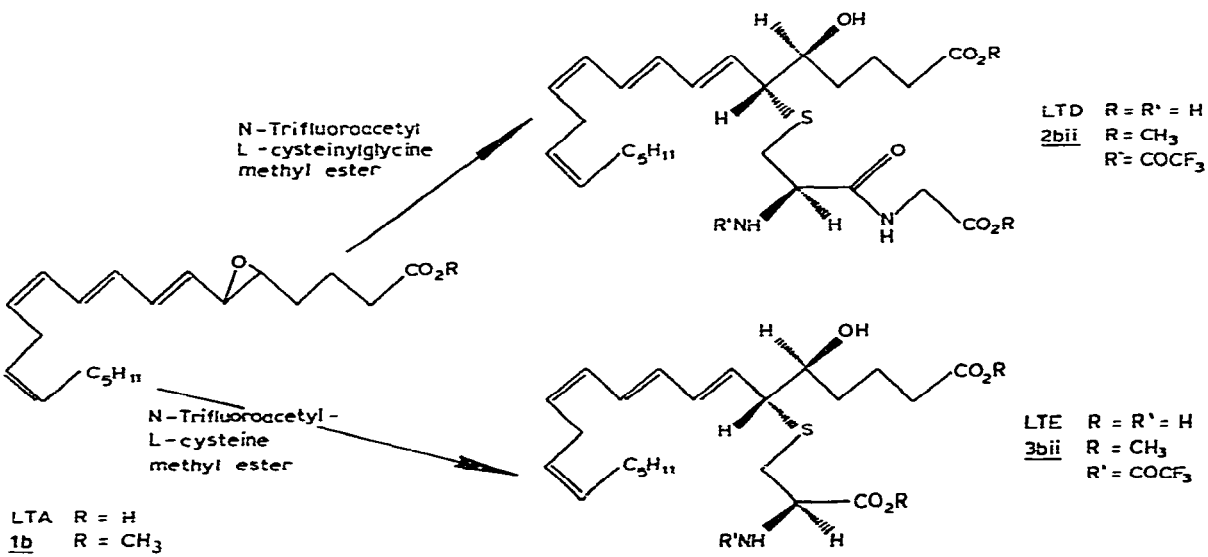
TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF LTD AND LTE ISOMER DERIVATIVES

*Isomer**	*Isomer No.*	*Capacity factors, k'*			λ_{max} *(nm)*		
		S5NH	*S5CN*	*S5W*			
5*R*, 6*S*,9-*cis*, 11-*trans*-LTD	2ai	2.5			272.0(sh)	279.5	288.0(sh)
5*S*, 6*R*, 9-*cis*, 11-*trans*-LTD	2aii	3.4			270.5(sh)	278.5	286.5(sh)
5*R*, 6*S*-LTD	2bi	2.8	1.47	10.5	274.0(sh)	282.5	290.5(sh)
LTD (natural 5*S*, 6*R*)	2bii	3.4	1.57	10.5	274.0(sh)	281.5	289.5(sh)
5*R*, 6*S*, 9-*cis*-LTD	2ci	2.6			277.0(sh)	282.0	290.5(sh)
5*S*, 6*R*, 9-*cis*-LTD	2cii	3.4			275.0(sh)	281.5	291.5(sh)
5*R*, 6*S*, 11-*trans*-LTD	2di	2.6			272.0(sh)	279.0	286.5(sh)
5*S*, 6*R*, 11-*trans*-LTD	2dii	3.0			272.0(sh)	278.5	286.5(sh)
5*R*, 6*S*, 9-*cis*,11-*trans*-LTE	3ai	3.8			273.0(sh)	278.5	286.5(sh)
5*S*, 6*R*, 9-*cis*,11-*trans*-LTE	3aii	4.3			272.0(sh)	279.0	286.5(sh)
5*R*, 6*S*-LTE	3bi	3.8	2.8	3.1	277.0(sh)	282.5	292.5(sh)
LTE (natural 5*S*,6*R*)	3bii	4.1	2.9	3.1	276.0(sh)	282.0	291.5(sh)
5*R*, 6*S*, 9-*cis*-LTE	3ci	3.8			277.0(sh)	282.5	290.5(sh)
5*S*, 6*R*, 9-*cis*-LTE	3cii	4.2			277.0(sh)	282.0	289.5(sh)
5*R*, 6*S*, 11-*trans*-LTE	3di	3.4			272.0(sh)	278.5	287.5(sh)
5*S*, 6*R*, 11-*trans*-LTE	3dii	3.6			272.0(sh)	278.0	287.5(sh)

* Order of elution of isomeric pairs assumed to be analogous to that of LTD and 5*R*,6*S* LTD. The stereochemistry of the 7–8 (*trans*) and 14–15 (*cis*) double bonds remains fixed.

were supplied by Phase Separations (Queensferry, Great Britain). HPLC grade hexane, dichloromethane and methanol were purchased from Fisons (Loughborough, Great Britain) and analytical-reagent grade acetic acid from May & Baker (Dagenham, Great Britain).



Instrumentation

A constant-flow Milton Roy Constametric 11G pump coupled to a Cecil Model 212 variable-wavelength UV monitor was utilised for the chromatographic separation. The UV monitor was set at 280 nm or 305 nm for the analytical and semi-preparative work, respectively. The analytical 10-mm path length cell was replaced with one of 1 mm for semi-preparative chromatography. Samples were injected using a Rheodyne variable-volume valve injector (20-μl volume loop for analytical and a 2.0-ml volume loop for semi-preparative separations).

Chromatography

All stainless-steel columns were packed in a vertically upwards mode using a methanol or isopropanol (S5ODS only) slurry of the packing material. Analytical chromatography was performed on 12.5 cm × 5 mm I.D. columns packed with Spherisorb S5W, S5NH, S5CN or S5ODS. For the Spherisorb S5W, S5NH and S5CN columns dichloromethane–methanol (200:1) was used to elute LTD-type molecules and hexane–dichloromethane–methanol (75:25:1) the LTE-type molecules at flow-rates of 1 ml/min. Identical eluting solvents (5 ml/min) were used for the 50 cm × 8 mm I.D. Spherisorb S5NH semi-preparative columns. For the Spherisorb S5ODS column, optimum separations were achieved with either a methanol–water–acetic acid (75:25:0.1) eluent for protected LTDs (2bi and 2bii) or methanol–water–triethylamine (80:20:1) for protected LTEs (3bi and 3bii).

Mass spectrometry

The required HPLC eluate was manually collected and reduced in volume before transference to a sample tube where it was blown to dryness. All spectra were obtained using an LKB 9000S mass spectrometer. Samples (3 μg) were analysed by direct insertion probe at 90°C using an ion accelerating voltage of 3.5 kV, an electron voltage of 20 eV and a source temperature of 270°C.

Ultraviolet spectroscopy

The UV absorption spectra were recorded in cyclohexane on a Pye-Unicam SP8-100 spectrophotometer, calibrated at 279.4 nm with a Holmium filter.

RESULTS AND DISCUSSION

Both μBondapak C_{18} (refs. 6, 10, 11) and Nucleosil C_{18} (ref. 12) analytical HPLC columns have been used to purify microgramme quantities of LTD and to check the co-elution of samples from biological and synthetic sources. Compound 2bii has also been chromatographed[6] on a μBondapak C_{18}column. Minimal attention has yet been paid to the HPLC of LTE and 3bii although both compounds have been chromatographed[13] using reversed-phase HPLC. The performance of these analytical columns or their suitability for scaling up to semi-preparative requirements was not discussed.

It was anticipated that for both the comparative pharmacological studies and complete spectral characterisation of all isomers, milligramme rather than microgramme quantities of material would be required. Consequently, semi-preparative rather than analytical columns were utilised for the chromatographic separation.

Preparative HPLC usually involves a compromise between sample capacity, column resolution and separation time[14]. As the chromatographic separation between isomeric peaks was expected to be small, it was considered important to carry out the analytical work on high efficiency columns, which can be scaled up for semi-preparative operations without loss in column performance. In practice it would not be possible to sacrifice the separation of the diastereoisomers for increased sample throughput. Consequently, to compare their separation characteristics, four analytical columns, 12.5 cm in length, were packed with 5-μm spherical packing material.

Analytical chromatography

It was anticipated that the presence of the carboxylic acid and amino-group functionalities in the parent eicosatetraenoic acids would diminish differential chromatographic characteristics observed between the optical-isomer pairs. Hence separations were developed on the fully derivatised (2ai–2dii and 3ai–3dii) rather than underivatised molecules. The initial HPLC separation was developed for isomers 2bi and the natural isomer 2bii. This latter isomer was the most important and the most readily available. It was assumed that conditions selected for one optical-isomer pair could be utilised for the separation of the remaining isomeric pairs. A similar approach was used for isomeric pairs 3ai–3dii.

The separations achieved for the four pairs of optical isomers of each type are shown in Table I. For 2bi and 2bii optimum separations were obtained on a Spherisorb S5NH column. When using a dichloromethane–methanol eluent an α value of 1.2 was obtained. No separation was observed on a Spherisorb S5W column using the same solvent system even though surprisingly long retention times were observed. Partial separations were obtained on the less retentive Spherisorb S5CN (α = 1.07) and S5ODS phases (α = 1.1). As similar separations were achieved for the optical isomers of 2a, 2c or 2d, the Spherisorb S5NH column was used for scaling up to semi-preparative separations. Although the optical-isomer pairs were well resolved, the geometric isomers (2ai–2di) are not completely separated, confirming the need to chromatograph 1a–1d at the previous synthetic stage. Similar observations can be made for the alternative geometric isomers (2aii–2dii).

Optical isomers 3bi and 3bii were more difficult to separate than those in the corresponding LTD-type series. Using a less polar eluent the optimum separation was again achieved using a Spherisorb S5NH column but with a reduction in α value to only 1.1. The Spherisorb S5ODS column gave a separation of 3bi and 3bii showing an α value of 1.09. The reduction in polarity and increase in size of the 6-substituent would therefore seems to be advantageous in separating the LTD-type isomers compared with those of LTE type.

Reaction following of LTE formation

It was observed during the development of the separation for 3bi and 3bii that samples were contaminated with the corresponding 11-*trans* LTE isomers (3di and 3dii). As 3di and 3dii are only slightly less retained than 3bi, its purification was made more difficult by the presence of these *trans*-isomers. Monitoring the formation of 3bi and 3bii from the appropriate isomer of LTA methyl ester indicated that the reaction was essentially complete in 20 min. Isomers 3di and 3dii were only formed when the reaction products were allowed to stand in methanol–triethylamine. Hence, by ensur-

ing the chromatographic separation was carried out soon after completion of the reaction, minimal formation of interfering 3di and 3dii was observed.

Semi-preparative chromatography

Using a 50 cm × 8 mm I.D. Spherisorb S5NH column with over 40,000 theoretical plates, up to 3 mg of a mixture of 2bi and 2bii dissolved in 1 ml of eluent were separated per injection. The attempted separation of larger amounts of material caused loss in column resolution to an unacceptable level. The relatively low sample capacity of this column (0.2 mg/g packing material) reflects[9] the difficulty of the separation. Surprisingly, volume overload effects were also observed when the isomers were dissolved in greater than 1 ml of eluent. This rate of separation allowed the collection of several milligrams of 2bi and 2bii, although less of the other three isomer pairs was collected. A chromatogram showing the elution positions of 2bi and 2bii and a spiked mixture of all eight optical isomers of protected LTD is shown in Fig. 1a and b, respectively.

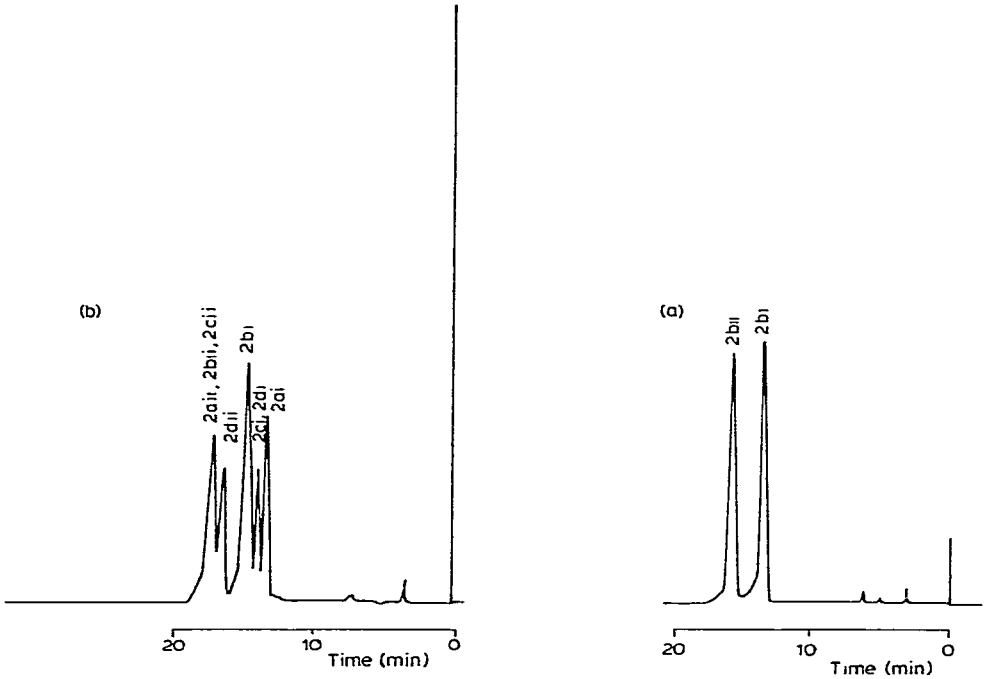


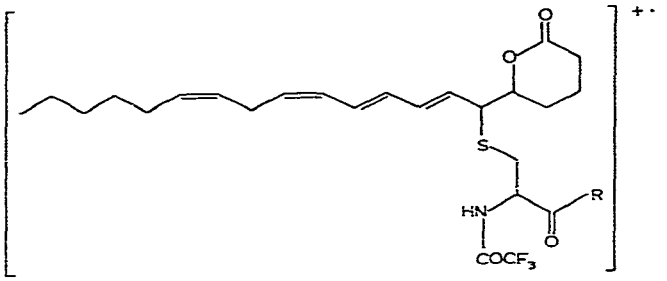
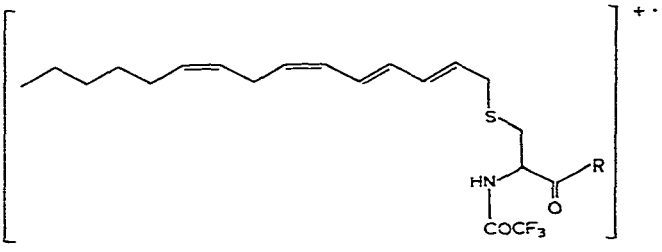
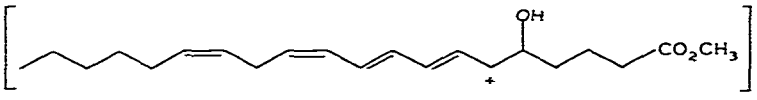
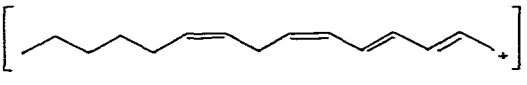
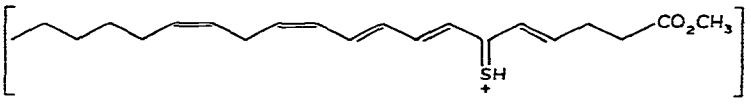
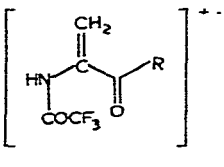
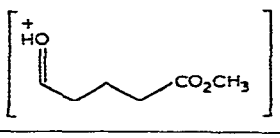
Fig. 1. (a) Chromatogram of derivatised leukotriene D isomers 2bi and 2bii. (b) Chromatogram of a mixture of all eight isomers of derivatised leukotriene D.

The preparative separation of the LTE-type isomers was, as expected from the analytical work, more difficult than that for the corresponding LTD-type isomers. Although "heart cutting" of peaks was employed it was necessary to re-inject a sample up to three times to obtain the required purity. Over a milligramme of 3bi and 3bii was collected but less of the other six isomers.

TABLE II

CHARACTERISTIC IONS OBSERVED IN THE MASS SPECTRUM OF LTD- AND LTE-TYPE DERIVATIVES

LTD type: R = $NHCO_2CH_3$; LTE type: R = OCH_3.

m/e values		*Ion structure*
LTD-type derivative	*LTE-type derivative*	
620	563	$[M]^{+\cdot}$
588	531	
490	433	
333	333	
203	203	
347	347	
254	197	
131	131	

Purity and chromatographic yield

As the presence of small amounts of one isomer in another could markedly affect the biological activity of the major component, strict control of isomer purity was essential. The purity of the eight protected LTD isomers as measured by analytical HPLC and assuming equi-molar UV response was >99.5% and that for the LTE series >99%. The recovery of a sample of protected LTD following re-injection of 2bii on the semi-preparative Spherisorb S5NH column was almost quantitative. No stability problems were thus met during the time elapse of the chromatographic separation although a considerable amount of material was lost when samples were stored at ambient temperatures or taken to dryness. The isomer appears to adhere to the glassware. Hence, once isolated, it was important to store the isomers in solution at –60°C to minimise loss.

Mass spectrometry

The spectra of each isomer of protected LTD and LTE showed a characteristic range of ions although significant differences were observed in the relative abundance of these ions. It is anticipated one will be able to correlate these variations in ion abundance with stereochemical assignments within the molecules. A weak molecular ion and the loss of water and methanol is observed for all isomers. Fragment ions characteristic of the eicosatetraenoate and peptide portions of the isomers are observed (see Table II).

Ultraviolet spectroscopy

The UV absorption maxima in cyclohexane of eight isomers of both derivatised LTD and LTE are listed in Table I. The geometric-isomer pairs showed characteristic absorption maxima although, as expected, the optical isomers with the same double-bond geometry were very similar.

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