SYNTHESIS OF $^{2}H_{8}$-LEUKOTRIENE B_4

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SUMMARY

A semi-synthetic method for the preparation of deuterated leukotriene B_4 ($^{2}H_{8}$-LTB_4) from eicosatetraynoic acid (ETYA) is described which involves reduction of ETYA to deuterated arachi - donic acid ($^{2}H_{8}$-AA) with deuterium gas and then biological conversion of this material to $^{2}H_{8}$-LTB_4 by rat peritoneal polymorphonuclear leukocytes (PMNs).

KEYWORDS

Deuterated arachidonic acid, $^{2}H_{8}$-leukotriene B_4.

INTRODUCTION

Leukotriene B_4 (LTB_4), a dihydroxy metabolite of arachidonic acid (AA) is a mediator implicated in inflammatory diseases such as rheumatoid arthritis. It has a variety of pharmacological effects which include aggregation of PMNs, chemotaxis of PMNs and increased venular permeability[1]. LTB_4 not only has effects on

0362-4803/87/030291–06$05.00

Received July 14, 1986

PMNs but is synthesised from membrane bound arachidonate by PMNs. The enzyme responsible for release of membrane bound arachidonate is phospholipase A2 and the released AA is converted to LTB_4 by a group of enzymes called lipoxygenase. Exogenous AA incubated with PMNs is also converted to LTB_4 and many agents such as calcium ionophore A23187, N-formyl-Met-Leu-Phe (FMLP), complement component C5a, and serum treated zymosan (STZ) will stimulate PMNs to produce LTB_4. For the development of a gas chromatography/tandem mass spectrometry (GC/MS/MS) assay for LTB_4 in various inflammatory exudates, we required stable isotope labelled LTB_4 for use as the internal standard. As complete chemical synthesis of $^2H_8$-LTB_4 would be very difficult and inordinately expensive, we have adopted a semi-synthetic approach to the problem. There is currently no reported quantitative GC/MS assay for LTB_4 in biological fluids, and the other methods used for analysis such as HPLC[2], radioimmunoassay[3] and bioassay[4] either lack the required sensitivity, or may lack specificity.

EXPERIMENTAL

Synthesis of Deuterated Arachidonic Acid. To a solution of eicosatetraynoic acid (ETYA) (50 mg) (Roche, Australia) in benzene (10 mL) was added 5% $Pd/CaCO_3$ (10 mg). The resulting suspension was stirred under deuterium gas at room temperature and pressure. Upon consumption of the theoretical amount of deuterium gas the reaction was stopped by venting to the atmosphere. The catalyst was removed by filtration and the solvent removed under a gentle stream of nitrogen to give a yellow semi-solid. TLC of the reaction product indicated the presence of five compounds including material co-chromatographing with ETYA and AA. Mass spectrometry (Finnigan TSQ-46) of a sample of the pentafluorobenzyl (PFB) ester of the crude product

showed that it was composed of $^2H_8$-AA and other less saturated compounds. The crude mixture was purified by high performance liquid chromatography (HPLC). The product was dissolved in methanol and aliquots were injected onto a 250 mm long x 4.2 mm i.d. column packed with 10 μm Spherisorb C18 stationary phase. The flow rate was 1.7 mL/min which gave a column pressure of 1100 psi. The mobile phase was 75% acetonitrile, 25% water and 0.02% acetic acid adjusted to pH 6.8 with ammonium hydroxide, and the detector was set at 200 nm. The fraction corresponding to the arachidonic acid peak was collected, diluted with five times that volume of water, and passed through a SEP PAK C18 extraction cartridge (Waters Associates). The cartridge was then washed with 40 mL of water and the $^2H_8$-arachidonic acid was eluted with methanol. The concentration of arachidonic acid in the resulting solution was determined by re-analysing a small aliquot by HPLC. The extent of incorporation was determined by negative ion chemical ionization mass spectrometry of the pentafluorobenzyl ester of the material. The base peak (m/z 311) corresponds to the anion formed after loss of the PFB group, and the absence of ions in the range m/z 303 to 310 indicated that the purified material was pure $^2H_8$-arachidonic acid. Proton nmr in $CDCl_3$ (Jeol FX-90Q) of the deuterated material showed a very similar spectrum to that of pure arachidonic acid (Sigma), except that the resonances due to the eight olefinic protons were missing (5.42 ppm), and that no splitting of the vinylic protons (2.82 ppm) was observed.

Synthesis of Deuterated Leukotriene B_4 Male wistar rats (300g) were injected intraperitoneally with 20 mL of a 3% peptone solution in 0.9% saline and the PMNs were harvested 5 hours later by peritoneal lavage. The cells were collected into calcium and magnesium free Hank's buffer solution, and were isolated by centrifugation and purified by further washing and centri-

fugation. The cells were suspended in the buffer solution to give a concentration of cells of 1 x 10^7 per mL. To six tubes containing 1 mL of the cell suspension were added calcium chloride, magnesium sulphate, FMLP and $^2H_8$-AA to give final concentrations of 2 mM, 8 mM, 0.1 mM and 0.1 mM respectively. The cells were incubated at 37°C for 20 minutes, at which time the suspension was centrifuged and the supernatant passed through a SEP-PAK C18 cartridge as described above. The cartridge was washed with 20 mL water, and dried by passing nitrogen gas through it at 60 psi. The cartridge was then washed with 1 mL of hexane and 1 mL of dichloromethane, and the product eluted with 2 mL of methanol which was evaported to dryness under nitrogen. The residue was dissolved in 100 µL of methanol, and the LTB_4 was separated from the other incubation products by HPLC. The fractions corresponding to LTB_4 were collected and extracted as described above. The HPLC system was similar to that used for the purification of AA except that the detector was set at 269.5 nm and the mobile phase was composed of 63% methanol, 37% water and 0.02% acetic acid adjusted to pH 6.8 with ammonium hydroxide solution. The concentration of LTB_4 in the SEP-PAK C18 eluate was determined by HPLC and the relative amounts of $^2H_8$- and ^1H-LTB_4 were determined by negative ion chemical ionization GC/MS of the pentafluorobenzyl ester t-butyldimethylsilyl (TBDMS) ether derivative (Figure 1). The ion traces at m/z 571 and 563 correspond to the anions formed after loss of the PFB group from $^2H_8$- and ^1H-LTB_4 respectively.

DISCUSSION

Biosynthesis of deuterated LTB_4 by PMNs following stimulation with FMLP and added $^2H_8$-arachidonic acid results in the formation of deuterium labelled LTB_4 with only 3.5% contamination with ^1H-LTB_4 (Figure 1). It is interesting to note that the bonded phase capillary GC column used is capable of

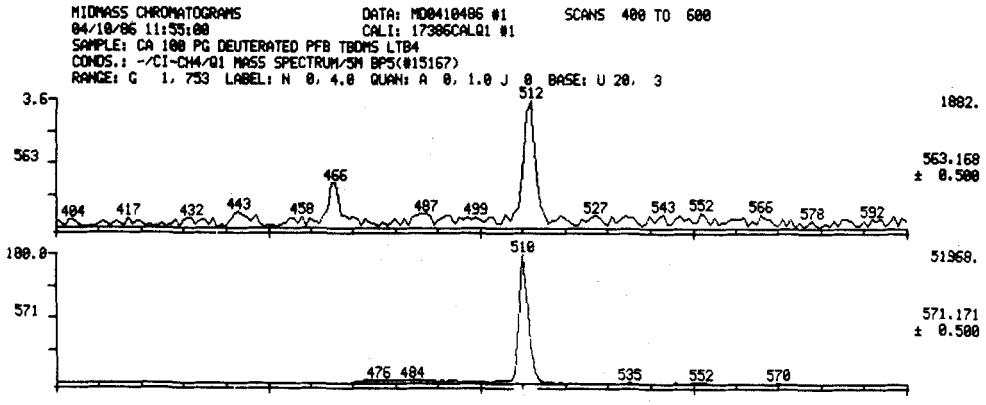

Figure 1. Ion traces for m/z 563 and 571 from deuterated LTB_4

slight separation of $^2H_8$- and ^1H-LTB_4; the maximum of the $^2H_8$-LTB_4 peak occurs at scan number 510, while that of ^1H-LTB_4 occurs at scan number 512 (Figure 1).

The deuterated LTB_4 synthesised by stimulation of PMNs with FMLP is suitable for use as an internal standard for the quantitative analysis of LTB_4. The small amount of ^1H-LTB_4 added to unknown samples can be compensated for during standard curve construction. Table 1 shows the standard curve data obtained after injection of 2 uL aliquots of solutions containing 10-400 pg/µL of PFB-TBDMS-LTB_4 and 100 pg/µL of $^2H_8$-LTB_4 onto a bonded phase capillary GC column with the mass spectrometer operating in chemical ionization negative ion, collision activated decomposition mode, monitoring the daughter ions of m/z 563 and 571 at m/z 299 and 305 respectively.

Table 1. Standard curve data obtained after injection of 10-400pg of LTB_4 and 100 pg of $^2H_8$-LTB_4 onto the column.

LTB_4 (pg/ µL)	Peak height ratio ($LTB_4/^2H_8$-LTB_4)
10	0.06
40	0.21
100	0.66
200	1.32
400	2.62

$y = 0.00662\ x - 0.0187 \quad r = 0.9997$

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ACKNOWLEDGEMENTS: M. Forrest is a Merck Sharp and Dohme (Aust) research fellow and C. McGee was supported by a grant from Pfizer Pty Ltd.