Determination of leukotriene B_4 in human plasma by gas chromatography using a mass selective detector and a stable isotope labelled internal standard. Effect of NE-11740 on arachidonic acid metabolism*

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Abstract: A highly selective gas chromatographic method, coupled with selected ion monitoring using a mass selective detector and positive electron ionization, was developed for the determination of leukotriene B_4 (LTB_4) in human plasma. Plasma was separated from whole human blood via centrifugation, proteins precipitated with acetonitrile and LTB_4 recovered (~82.0%) by ethyl acetate extraction. The methyl ester, bis-t-butyldimethylsilyl ether derivative of LTB_4 was formed prior to analysis and determined quantitatively using $[^{18}O]_2$-LTB_4 as an internal standard. The limit of detection ($S/N = 2$) was 425 pg on column (m/z 335/339) using a 1-µl injection. Standard curves were linear over two orders of magnitude with an RSD of <5.0% ($n = 10$). NE-11740, a new anti-inflammatory drug, was shown to inhibit, in a dose-dependent manner ($ED_{50} = 22$ µM) ionophore-stimulated LTB_4 biosynthesis by human whole blood in vitro.

Keywords: Leukotriene B_4; gas chromatography–mass spectrometry (GC–MS); anti-inflammatory drugs; NE-11740 (tebufelone); arachidonic acid.

Introduction

Eicosanoids are a ubiquitous class of naturally occurring C_{20} fatty acids arising from the enzymatic conversion of arachidonic acid (20:4 n–6), which maintain homeostatic control of a variety of physiologic processes [1–3]. Most notable among these natural products are prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), resulting from cyclo-oxygenase (CO) and 5-lipoxygenase (LO) activity, respectively. Both PGE_2 and LTB_4 likely play a rôle in mediating the pain, oedema and tissue destruction associated with inflammatory conditions [4–6].

As such, regulation of arachidonic acid metabolism has long been recognized to be of therapeutic value in the treatment of arthritis, psoriasis and asthma [5, 6]. Traditional non-steroidal anti-inflammatory drugs (NSAIDs, e.g. indomethacin) selectively block PGE_2 formation but do not inhibit LTB_4 biosynthesis. The search for safe NSAIDs possessing dual CO/LO inhibitory activity is an attractive

concept which has been the focus of much research in recent years [5, 7]. NE-11740, {1-[3,5-bis(1,1-dimethylethyl)-4-hydroxy-phenyl]-5-hexyn-1-one}, is a new anti-inflammatory drug (Fig. 1) of the di-t-butylphenol class which blocks both CO and LO activity in a variety of cellular and cell free systems [8].

The high potency, low concentration, structural similarity and limited stability of eicosanoids in biological matrices makes their quantification an analytical challenge [9]. A
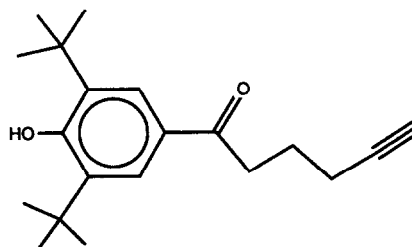

Figure 1
Chemical structure of NE-11740 (tebufelone).

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host of chromatographic [10–12] and immuno-analytical [13, 14] assays have been reported for the measurement of PGE_2 and LTB_4. However, mass spectrometric methods are generally favoured for eicosanoid quantification because of their inherent selectivity [15, 16].

Much effort has been placed against the development of volatile and stable derivatives as well as ionization techniques which provide abundant, high mass ions suitable for GC–MS analysis of eicosanoids [15]. By far, the most common approach has been the use of alkyl-silyl ether/ester derivatization schemes coupled with electron ionization (EI) techniques. Steffenrud and Borgeat [17] reported the favourable EI fragmentation properties of the bis-*t*-butyldimethylsilyl (tBDMS) ether, methyl ester (ME) derivative of LTB_4 relative to a variety of lower molecular weight alkylsilyl ether alternatives. In addition, Murphy [18] further suggested the use of $[^{18}O]_2$-labelled LTB_4 in conjunction with tBDMS/ME derivatization as an internal standard for quantitative purposes. Woollard and Mallet [19] have successfully applied tBDMS/ME derivatization with catalytic hydrogenation to the GC–MS quantification of monohydroxy fatty acids in psoriatic skin.

A facile method for the quantification of LTB_4, as the tBDMS/ME derivative, in human plasma by GC–mass selective detection (MSD) has been developed using positive EI with selected ion monitoring (SIM). The application of this methodology to the determination of the effect that NE-11740 has on ionophore-stimulated LTB_4 biosynthesis by whole human blood *in vitro* has been demonstrated.

Materials and Methods

Reagents and materials

NE-11740 [>99.8% pure by liquid chromatography (LC)] was obtained from Norwich Eaton Pharmaceuticals, a subsidiary of the Procter & Gamble Co. Calcium ionophore A23187 was purchased from Sigma Chemical Co. LTB_4 was purchased from Cayman Chemical Company (98% pure). Eicosanoid-free plasma and $[^3H]$-LTB_4 (32.8 Ci mmol^{-1}) were obtained from New England Nuclear Products.

N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoracetamide, MTBSTFA, was purchased from Pierce Chemical Co.; *t*-butyldimethylchlorosilane–imidazole–DMF reagent was purchased

from Alltech Associates. All reagents were derivatization grade. Acetonitrile was silylation grade and was also purchased from Pierce Chemical Co. Ethereal diazomethane was produced by addition of alkali to Diazald (Aldrich Chemical Co.) in a distillation apparatus manufactured at our facility and made of Clear-Seal joints. The procedure provided with the Diazald Kit was followed precisely, with the exception that all reagent volumes were reduced by a factor of 10.

Preparation of stable isotope labelled internal standards

Stable isotope labelled (SIL) LTB_4 was produced in this laboratory by incorporating two $[^{18}O]_2$-atoms into the acid moeity using a procedure similar to that initially described by Murphy and Clay [20].

Briefly, the procedure consisted of removing the $[^{16}O]$-water from 100 μl of esterase (Porcine Liver, Sigma Chemical Co.; 280 units) by evaporation in a vortex evaporator. The enzyme was reconstituted with the same volume of $[^{18}O]_2$-water (Cambridge Chemical Co.; 98% purity). An appropriate mass of LTB_4 was dissolved in ethanol (Gold Shield; 200 Proof), and the volume was reduced under a nitrogen stream to approximately 10–20 μl. The eicosanoid solution was added slowly to the esterase solution, and allowed to incubate at 37°C for 1 h. After removal from the controlled-temperature water bath, 2 ml of ethanol was added and the mixture was centrifuged. The liquid fraction was decanted and saved. One millilitre of 0.1 M sodium formate buffer (pH 3.2, Certified ACS, Fisher Scientific) was added to the solid fraction. The resulting aqueous portion was extracted with three 2.0-ml portions of ethyl ether (J.T. Baker Inc.) which were combined with the ethanol fraction, and taken to dryness. Recovery of LTB_4 was monitored by UV spectroscopy (λ_{max} = 272 nm). Recoveries normally ranged from 60–65%. Successful incorporation of $[^{18}]O$ was confirmed by full scan GC–MS, and the purity of the $[^{18}O]_2$-LTB_4 isotopimer, as measured by SIM (*m/z* 335.3/339.3), always exceeded 97%.

Ionophore stimulation

Fresh, heparinized whole blood (60 ml) was collected by venous puncture from volunteers who had not been exposed to NSAIDs for at least 6 weeks prior to the study. Blood was

then aliquoted (4 ml) into polypropylene tubes and placed in a water bath (37°C) for 3 min. Following preincubation, NE-11740 was added to a final concentration of 0, 1, 5, 10, 25 or 50 μM. Then calcium ionophore (A23187), dissolved in 10 μl DMSO, was added to each tube (50 μM final concentration) to stimulate LTB₄ biosynthesis. The mixture was shaken vigorously and stirred continously for 25 min in the water bath. Plasma was separated from whole blood via centrifugation (3000 rpm × 15 min) and weighed accurately. Two volumes of acetonitrile were slowly added to each plasma sample to precipitate serum proteins. The sample was centrifuged (3000 rpm × 5 min), the supernatant decanted, and finally, stored frozen (−80°C) until assay.

Preparation of samples and standards

Typically, the entire portion of deproteinated plasma was taken for GC–MS analysis. An appropriate mass of [¹⁸O]₂-labelled LTB₄ internal standard was added to each sample. The typical amount ranged from 200–500 ng and was determined on the basis of the anticipated analyte concentration. A 5-fold volume of distilled water was added to each sample, and the pH was adjusted to 3.0–3.5 using 0.1 N HCl. The sample was extracted with two 2.0-ml volumes of ethyl acetate (J. T. Baker Inc.), which were combined, and taken to dryness under nitrogen.

Five LTB₄ calibration standards were prepared with each set of samples. SIL internal standard, in an amount identical to that added to each sample, was also added to each

calibration standard yielding an amount ratio (mass LTB₄/mass [¹⁸O]₂-LTB₄) in the ~0.1–~10.0 range.

Standard and internal standard were each dissolved in ethanol prior to mixing. The ethanol was removed under a nitrogen stream and the calibration standard and sample residues derivatized as described below.
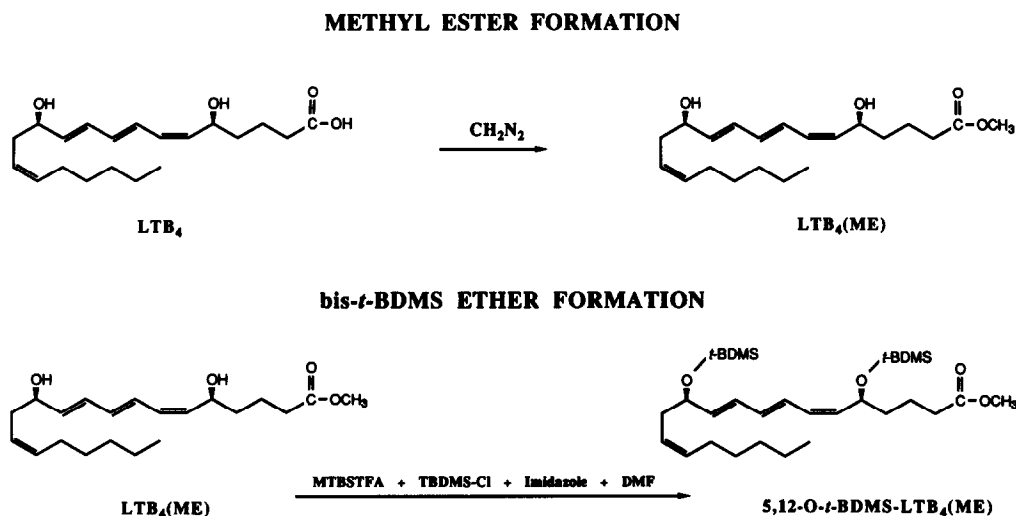
Derivatization procedure

The derivatization sequence is outlined in Scheme 1. Exactly 100 μl of ethereal diazomethane was added to each sample or standard residue, and allowed to stand for 2–3 min. The ethereal mixture was then taken to dryness under a nitrogen stream. Fifty microlitres of acetonitrile, 50 μl of MTBSTFA and 50 μl of TBDMS-Cl–Imidazole–DMF reagent, were added to the methylated eicosanoid sample. Samples were placed in a heated reaction block for 1 h at 60°C. After removal from the reaction block, samples were taken to dryness in a vortex evaporator. The soluble portion of the derivatized residue was reconstituted in an appropriate volume of hexane (usually 50 μl or less), and chromatographed.

Experimental conditions

All analyses were performed using a Hewlett Packard 5790 gas chromatograph. The detector was a 5970A Hewlett Packard Mass Selective Detector (MSD) equipped with a 59970B Series 200 controller and Revision 3.1.2 MSD operating system software. A direct capillary-ion source interface was employed.

The analytes were separated on a 30M DB-5

METHYL ESTER FORMATION

LTB₄ → (CH₂N₂) → LTB₄(ME)

bis-t-BDMS ETHER FORMATION

LTB₄(ME) → (MTBSTFA + TBDMS-Cl + Imidazole + DMF) → 5,12-O-t-BDMS-LTB₄(ME)

Scheme 1
TB₄ derivatization sequence.

narrow bore (0.25-μm film) fused silica capillary column (J&W Scientific) using splitless injection and an injection volume of 1 μl. Since the sample was reconstituted in 50 μl or less of solvent, conical shaped, glass vial inserts (National Scientific Co.) were used. Helium was employed as the carrier gas at a constant pressure of 8 psi. Oven temperature was ramped from 280 to 320°C, at 4.0°C min^{-1} following an initial hold at 280°C for 2 min. Injection port and interface temperatures were maintained at 300°C. LTB$_4$ and [^{18}O]$_2$-LTB$_4$ were detected by monitoring ions m/z 335.3 and 339.3, respectively. Fragmentation was achieved via positive EI (70 eV). The dwell time was 100 ms for each ion.

Results and Discussion

Assay development

The EI spectra for derivatized LTB$_4$ and [^{18}O]$_2$-LTB$_4$ appear in Fig. 2. Generally, tBDMS ether derivatives produced abundant M$^+$-57 ions under EI conditions due to the loss of *t*-butyl radical [15]. In the case of LTB$_4$ ([^{18}O]$_2$-LTB$_4$), the ions corresponding to *t*-butyl loss at m/z 521 (525) were observed, but not in sufficient abundance to be useful for trace quantitation. The most prominent ions, which also retained the [^{18}O]$_2$-label, were observed at m/z 467 (471), 335 (339) and 245 (249), respectively. Cleavage alpha to the 12-O-tBDMS functionality gives rise to m/z 467 (M$^+$-111) with subsequent loss of tBDMS-OH (M$^+$-111-132) yielding m/z 335 [18]. In addition, fragmentation adjacent to the 5-O-tBDMS group produces the ion at m/z 245 (M$^+$-333). The greater abundance and favourable (higher) nominal mass associated with the 335 (339) ion pair were best suited for quantitative purposes.
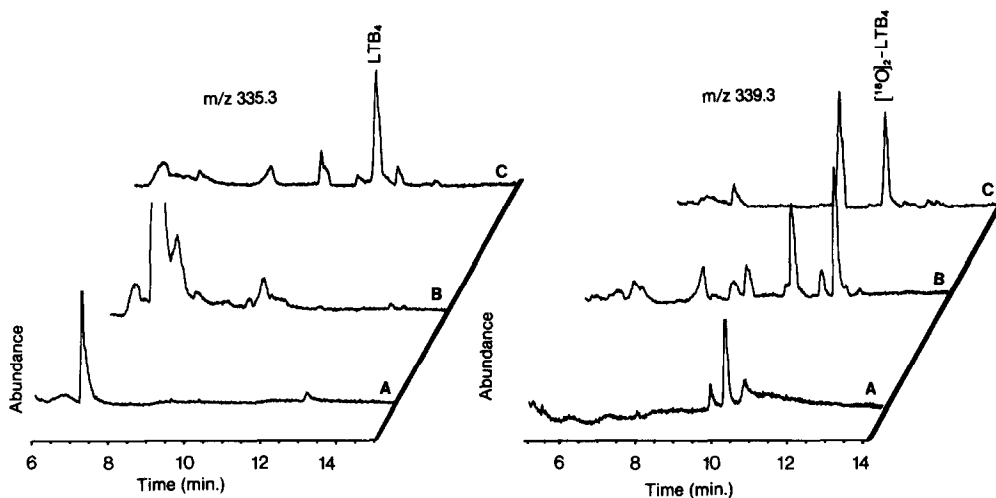
Selected ion chromatograms (m/z 335.3 and 339.3) for a typical human plasma sample spiked with LTB$_4$/[^{18}O]$_2$-LTB$_4$ and carried through the derivatization protocol are shown in Fig. 3. Under the assay conditions LTB$_4$ (t_R = 11.3 min) was well resolved (R_s > 2.0) from all potentially interfering plasma components.

Calibration and assay validation

A typical isotope dilution standard curve was prepared by derivatizing the standards directly. Standards covered the range of amount ratios
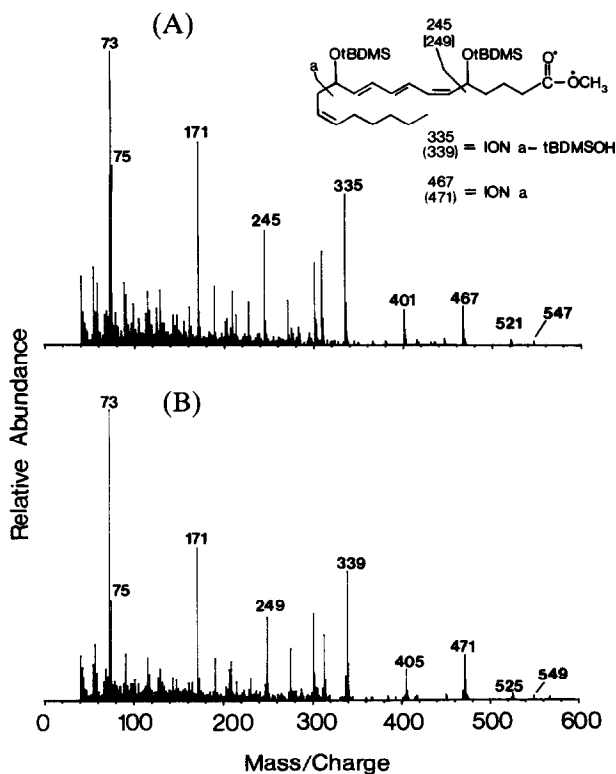
Figure 2
EI spectra of bis-tBDMS ether, methyl ester derivatives of (A) LTB$_4$ and (B) [^{18}O]$_2$-LTB$_4$.

Figure 3
Typical selected ion chromatograms (m/z 335.3 and 339.3) for human plasma (A) alone, (B) spiked with $[^{18}O]_2$-LTB$_4$ (360 ng) internal standard, or (C) both LTB$_4$ (920 ng) and $[^{18}O]_2$-LTB$_4$ (360 ng).

(LTB$_4$/$[^{18}O]_2$-LTB$_4$) from 0.1 to 10. When data were fitted using a linear-regression algorithm the slope was 0.109 and the intercept 0.22, with a correlation coefficient (r^2) or 0.998. The use of a SIL internal standard whose molecular weight is 4 units greater than LTB$_4$ minimized natural abundance effects (curve bending) common at high analyte concentrations with M + 1 and M + 2 SIL internal standards. The assay precision as determined by the RSD ($n =$ 10) was <5% at all points on the standard curve, and as little as 425 pg LTB$_4$ could be detected (m/z 335.3/339.3) ($S/N =$ 2) using this methodology.

Similar results were obtained when standards and SIL LTB$_4$ were spiked into leukotriene-free plasma (charcoal stripped, New England Nuclear). Hence, all subsequent standard curves were prepared by derivatizing LTB$_4$ standard solutions directly. In addition, loss of $[^{18}O]_2$-label due to isotopic back-exchange was not observed nor expected since the internal standard was added to plasma following the precipitation of serum protein. However, to determine if back-exchange due to esterase activity could occur, a standard curve was prepared by spiking both analyte and SIL internal standard into plasma 30 min prior to protein removal. As in the previous experiment, no significant change in the curve was observed, indicating that the $[^{18}O]_2$-LTB$_4$ was stable to short term exposure to plasma.

The use of a SIL internal standard for quantitative purposes precluded the need to correct for analyte loss subsequent to sample handling and pretreatment. Nevertheless, recovery of LTB$_4$ following ethyl acetate extraction from both stimulated and unstimulated whole blood was determined using $[^3H]$-LTB$_4$ (2 and 20 ng ml^{-1}). Scintillation counting revealed that 82.5 and 83.2% of the activity was recovered in the organic phase for the unstimulated and stimulated blood samples, respectively. Only 2.0–2.5% of the activity remained in the aqueous portion of each sample, indicating that ~15% of the activity was associated with the packed cells.

Determination of LTB$_4$ in human plasma

In normal subjects, circulating levels of LTB$_4$ are believed to be in the pg ml^{-1} range. Stimuli such as calcium ionophore (A23187) can be used to induce LTB$_4$ biosynthesis by leukocytes, thereby mimicking the inflammatory condition [21, 22]. A23187 treated (50 μM) whole blood produced significant levels (~120 ng ml^{-1}) of LTB$_4$ versus unstimulated controls (<10 ng ml^{-1}). NE-11740 inhibited ionophore-induced LTB$_4$ production in vitro and the effect was dose-dependent (Fig. 4).

Complete inhibition of LTB$_4$ biosynthesis was not observed at the highest dose (50 μM) tested. The ED_{50} of the drug was determined to be 22 μM by probit analysis. The LO inhibitory activity of NE-11740 may in part contribute to the compound's anti-inflammatory potency.
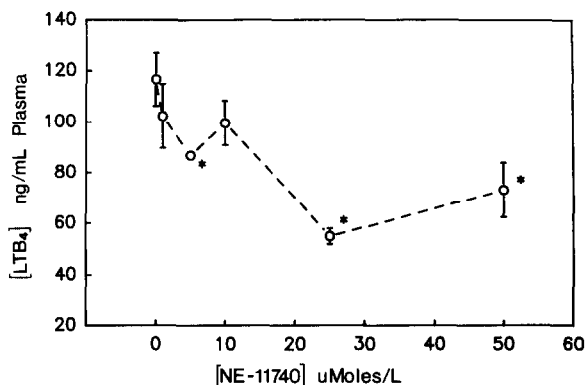
Figure 4
Effect of NE-11740 on ionophore-induced LTB$_4$ biosynthesis by human whole blood *in vitro*. *Significantly different from control at $\alpha < 0.05$. ED_{50} was calculated to be 22 μM by probit analysis assuming controlled yields.

Conclusion

A highly selective GC–MSD assay for the determination of LTB$_4$ in human plasma using SIM and positive EI has been developed. This facile method can be used to evaluate the LO inhibitory activity of compounds of pharmacologic interest. NE-11740, a new anti-inflammatory drug, effectively blocked A23187-induced LTB$_4$ biosynthesis *in vitro*. This activity may offer an improved approach to the treatment of a variety of inflammatory conditions.

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