Urinary Metabolites of Leukotriene B₄ in the Human Subject*

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Leukotriene B₄ (LTB₄) is a potent chemoattractant for neutrophils and is thought to play a role in a variety of inflammatory responses in humans. The metabolism of LTB₄ in vitro is complex with several competing pathways of biotransformation, but metabolism in vivo, especially for normal human subjects, is poorly understood. As part of a Phase I Clinical Trial of human tolerance to LTB₄, four human subjects were injected with 150 nmol/kg LTB₄ with one additional subject as placebo control. The urine of the subjects was collected in two separate pools (0–6 and 7–24 h), and aliquots from these urine collections were analyzed using high performance liquid chromatography, UV spectroscopy, and negative ion electrospray ionization tandem mass spectrometry for metabolites of LTB₄. In the current investigation, 11 different metabolites of LTB₄ were identified in the urine from those subjects injected with LTB₄, and none were present in the urine from the placebo-injected subject. The unconjugated LTB₄ metabolites found in urine were structurally characterized as 18-carboxy-LTB₄, 10,11-dihydroxy-18-carboxy-LTB₄, 20-carboxy-LTB₄, and 10,11-dihydro-20-carboxy-LTB₄. Several glucuronide-conjugated metabolites of LTB₄ were characterized including 17-, 18-, 19-, and 20-hydroxy-LTB₄, 10-hydroxy-4,6,12-octadecatrienoic acid, LTB₄, and 10,11-dihydro-LTB₄. The amount of LTB₄ glucuronide (16.7–29.4 pmol/ml) and 20-carboxy-LTB₄ (18.9–30.6 pmol/ml) present in the urine of subjects injected with LTB₄ was determined using an isotope dilution mass spectrometry assay before and after treatment of the urine samples with β-glucuronidase. The urinary metabolites of LTB₄ identified in this investigation were excreted in low amounts, yet it is possible that one or more of these metabolites could be used to assess LTB₄ biosynthesis following activation of the 5-lipoxygenase pathway in vivo.

Leukotriene B₄ (LTB₄) (5S,12R)-dihydroxy-6,14Z-8,10E-eicosatetraenoic acid) is a biologically active metabolite of arachidonic acid that is chemotactic for the human neutrophil and a potent lipid mediator of inflammation (1). LTB₄ is not stored within a cell but synthesized following activation of certain cells through release of arachidonic acid by the cytosolic phospholipase A₂ (2) and activation of the enzyme 5-lipoxygenase (3). The immediate product of the 5-lipoxygenase pathway is the reactive epoxide intermediate leukotriene A₄ that is transformed by leukotriene A₄ hydrolase into LTB₄ (4). A specific G-protein-coupled receptor for LTB₄ has now been cloned and expressed (5) and is known to be expressed by many cell types including the neutrophil (6). Through this receptor, LTB₄ is a potent stimulus of many functional responses of cells that are involved in immune responses (7) such as neutrophils, monocytes, macrophages, and various lymphocytes. LTB₄ is therefore thought to be an important component of the host defense mechanisms and is currently under investigation as a potent drug for the prophylaxis and/or treatment of infectious diseases.

The inactivation of endogenous LTB₄ takes place through metabolism so that little, if any, escapes into the circulation or appears in urine as an intact molecule. The metabolic transformation of LTB₄ has been studied in various cellular systems including the human neutrophil, which efficiently metabolizes LTB₄ by a specific cytochrome P-450-dependent pathway (CYP4F3) to produce 20-hydroxy-LTB₄ (20-OH-LTB₄) (8). Additionally, other cells carry out this same step of ω-oxidation but do so with different members of this unique family of P-450 enzymes including CYP4F2 and CYP4F4 (8). The initiation of ω-oxidation metabolite, 20-OH-LTB₄, retains significant biological activity (9) but is further metabolized to biologically inactive 20-carboxy-LTB₄ (20-COOH-LTB₄) by both a P-450-dependent pathway (10, 11) as well as an alcohol dehydrogenase-dependent pathway in certain cells (12). Formation of these ω-oxidation products has been observed after the incubation of LTB₄ with human hepatocytes (13). Once ω-oxidation has occurred, 20-COOH-LTB₄ can be further metabolized by β-oxidation into 18-carboxy-LTB₄ (18-COOH-LTB₄) and 16-COOH-LTB₄ (13, 14) which requires formation of the CoA ester at the ω-terminal carboxyl moiety. These chain shortened products, as CoA esters, can undergo additional β-oxidation cycles from the ω-terminus to form substantially less lipophilic metabolites (14).

An additional pathway of LTB₄ metabolism identified in various human cells, including lung macrophages, monocytes, and keratinocytes, occurs via the 12-hydroxyeicosanoid dehydrogenase/Δ¹⁰-reductase pathway (15, 16). This pathway leads to oxidation of the hydroxyl group at C-12 with intermediate formation of the 12-oxo product, which is then reduced at the immediately adjacent 10,11 double bond by a Δ¹⁰-reductase. This leads to a series of 10,11-dihydro metabolites, which can chemical ionization; TMS, trimethylsilyl ether; PFB, perfluorobenzyl ester; 10-HOTrE, 10-hydroxy-4,6,12-octadecatrienoic acid.
be precursors for ω- or β-oxidation. Metabolism by this pathway also results in a substantial reduction in biological activity (17).

LTB₄ can also participate in synthetic metabolic reactions by conjugation with polar molecules at either hydroxy or carboxyl substituents of LTB₄ and oxidized metabolites. The first LTB₄ conjugation metabolite observed was a taurine conjugate of 15- COOH-LTB₂₄ formed in the rat hepatocyte (14). Additionally, when LTB₄ was incubated with human keratinocytes, a glutathione adduct was observed as an intermediate metabolite (16). Finally, glucuronide conjugates of 20-COOH-LTB₄, LTB₆₄, and 10,11-dihydro-LTB₄ have been observed as products following incubation of LTB₄ with human hepatocytes (13).

Interest in LTB₄ as an endogenous inflammatory mediator has been somewhat hampered by an inability to assess in vivo production of LTB₄ either in normal individuals or individuals thought to have the leukotriene pathway of arachidonate metabolism activated as a result of pathologic events. One quite successful strategy in assessing in vivo production of prostaglandins and thromboxane A₂ has been the quantitative analysis of corresponding metabolites appearing in urine using sensitive and specific assays for each unique metabolite (18, 19). This approach has also been used to monitor the production of leukotriene C₄ in human subjects through measurement of leukotriene E₄ excreted into urine (20). LTB₄ has been observed in urine only in individuals with a deficiency of fatty aldehyde dehydrogenase (Sjögren-Larsson Syndrome) and Zellweger patients who lack peroxisomes and have limited LTB₄ metabolism (11). The production of LTB₄ either in normal individuals or individuals with a deficiency of fatty aldehyde dehydrogenase (Sjögren-Larsson Syndrome) and Zellweger patients who lack peroxisomes and have limited LTB₄ metabolism (11).

In the course of investigations into LTB₄ as a potential agent useful in the prophylaxis or treatment of infections, synthetic LTB₄ was administered into healthy human volunteers in studies of safety, tolerability, pharmacokinetics, and pharmacodynamics. These experiments afforded the possibility to examine the urine for excreted LTB₄ and LTB₄ metabolites when known quantities of LTB₄ were administered intravenously to human subjects. Detailed study of the urine was therefore undertaken to investigate whether or not previously identified metabolites of LTB₄ described above were present in human subjects.

**EXPERIMENTAL PROCEDURES**

**Materials**—The free acid of LTB₄ for the injection into human subjects was obtained from Cascade Biochem, Ltd. (Reading, UK), in solution at a concentration of 2 mg/ml in ethanol/water (90:10, v/v). The purity of this LTB₄ (96%) was assessed by reversed phase HPLC with UV monitoring at 235 and 270 nm. The major contaminants were 5- and 12-epi-LTB₄ (2%), 14-trans-LTB₄ (1%, tentative identification), and the δ-lactone of LTB₄ (0.5%). Very small amounts of 6-trans- and 6-trans-12-epi-LTB₄ were present (0.1% each). Leukotriene B₄, 20-COOH-LTB₄, and 6,7,14,15-H₄-LTB₄ (d₄-LTB₄) were used as analytical standards for quantitative analysis and mass spectrometry were purchased from Cayman Chemical (Ann Arbor, MI). Type VII-A β-glucuronidase from Escherichia coli and bis(trimethylsilyl)trifluoroacetamide were purchased from Sigma. Pentafluorobenzyl bromide and N,N-diisopropylethylamine were purchased from Aldrich. All solvents were high performance liquid chromatographic (HPLC) grade and were obtained from Fisher.

**Preparation of LTB₄ for Injection**—In order to prepare LTB₄ for injection into human subjects, ethanolic LTB₄ was converted into the sodium salt by the addition of 1 eq of aqueous sodium hydroxide. This ethanolic solution of neutralized LTB₄ was evaporated to dryness under reduced pressure at 40°C. The clear yellowish oily residue was immediately redissolved in sodium phosphate (30 mM) buffered 0.7% sodium chloride containing 0.01% EDTA to a final concentration of 0.7 mg/ml LTB₄. The pH of the solution was adjusted to 7.5 ± 0.1, and the solution was sterilized by filtration through a 0.22-μm microporous membrane filter. The sterile solution of LTB₄ was then lyophilized and treated in 10-ml clear glass vials, which were sealed and stored at −80°C. Solutions of LTB₄ for injection were analyzed prior to use to assess sterility, endotoxin content, concentration, and impurity levels. The purity of LTB₄ for injection was also ~96% with a profile of impurities similar to that of the starting material with the exception that the δ-lactone of LTB₄ was not present, but an impurity corresponding to the ethyl ester of LTB₄ (~0.5%) was now detectable.

**Injection of LTB₄**—Subjects enrolled in this study were members of the community at large (Quebec, Canada) who were screened according to inclusion and exclusion criteria. In addition, individuals were tested for the presence of alcohol as well as human immunodeficiency virus and hepatitis B and C. Subjects abstained from alcohol for 2 days prior to inclusion in the study. Subjects also provided written informed consent and agreed to abide with the study restrictions, including collection of urine samples. Study subjects excluded were those with any clinically significant abnormalities as assessed from an electrocardiogram, clinical laboratory tests, or had abused alcohol or used illicit drugs. Other exclusion criteria included the use of other lipophilic or highly immunodeficiency virus or an active infection caused by herpes simplex virus, or a virus of the orthomyxovirus or rhinovirus families, and any clinically significant surgery or illness within the previous month. None of the subjects had used antiviral, antibiotic, or corticosteroids within 14 days preceding the study or had used an investigational drug within 30 days preceding this study.

After overnight fasting for 10 h, subjects were injected with LTB₄ as a bolus intravenous dose of 50 μg/kg (150 nmol/kg) or an identical placebo injection of sterile saline. Both the LTB₄ and the placebo were administered in a total volume of ~5 ml as a bolus injection completed within ~1 min. A total of four subjects received LTB₄, and one subject received the placebo injection.

The subjects fasted for at least 1 h after drug administration, after which time they were served a controlled breakfast, and standard meals at ~4, 9, and 13 h post-injection. Water was permitted ad libitum. Subjects remained lying down for 1 h after LTB₄ injection, and vigorous physical activity was prohibited at all times during the study. Subjects were asked to empty their bladder prior to administration, and urine was then collected at two interval pools of 0–6 and 7–24 h post-injection. The urine samples were collected in polypropylene containers and kept in an ice water bath; at the end of both collection periods, the urine samples were quickly frozen and stored at ~80°C until analyzed. Throughout the study, subjects were monitored for adverse events, and a qualified medical investigator was on-site during injection of LTB₄, and until 4 h after injection.

**Urinary Extraction**—The two urine sample pools from each of the five subjects (representing 0–6 and 7–24 h time periods after injection of LTB₄ or placebo) were thawed and kept at 4°C. The volume of urine in each sample was measured, then transferred into polypropylene 50-ml conical tubes, and centrifuged at 4°C for 10 min at 750 × g. The urine samples were then acidified to pH 3.7–3.8 using formic acid and subjected to solid phase extraction using 1-g cartridges (Waters, Milford, MA). The cartridges were first conditioned with 2 volumes of methanol (40 ml) and then equilibrated with 2 volumes of water. The acidified urine samples were then loaded onto the cartridges and washed with 2 volumes of water. Solvent flow through the extraction cartridges was 20 ± 5 ml/min. Lipophilic metabolites were eluted with 1 volume of methanol directly into 20-ml glass ampoules. A maximum of 500 ml of urine was loaded on each 20-ml cartridge, and when the urine sample volume exceeded 500 ml, the extraction cartridge was reconditioned, and the remaining volume of the urine sample was extracted using the same reconditioned solid phase extraction cartridge. Two extraction blanks (0.9% saline acidified with formic acid) were also prepared. The ampoules were flame-sealed and stored at ~80°C until analysis. The urine sample volumes varied from 160 to 400 ml for the 0–6h samples and 625–1050 ml for the 7–24-h collections.

Aliquots of the methanol eluate, typically 5%, were taken for subsequent extraction, purification, and treatment with β-glucuronidase. For some studies, solid phase extraction aliquots from the 0–6-h collection of each subject in the study were evaporated to dryness under vacuum and then reconstituted in 5 ml of 100 mM phosphate buffer, pH 7. The pH of the mixture was adjusted to 3.8 using formic acid, and the leukotrienes present in the solid phase-extracted urine were extracted as described above. After vortexing, each sample was centrifuged at 135 × g for 5 min to separate the two layers. This liquid-liquid extraction procedure was repeated 3 additional times, and the combined organic layers from each extraction were pooled and taken to dryness under vacuum.

**Normal-phase HPLC and RP-HPLC**—The 1:1 hexane/ethyl acetate extract was further purified in a normal phase chromatography using an Ultrasphere 5-μm silica 250 × 4.6 mm column (Phenomenex, Torrance, CA). The normal phase solvents used were 90:10:0.1 hexane/isopropyl alcohol/acetic acid (solvent A) and 90:10:0.3 isopropyl alcohol, 20 mM ammonium acetate, acetic acid (solvent B). The initial mobile phase was...
10% solvent B at a flow rate of 1 ml/min. This initial mobile phase was held for 3 min and then a linear gradient was started to 30% solvent B at 13 min. This was followed by a second linear gradient to 40% solvent B at 25 min and finally a third linear gradient to 85% solvent B at 43 min. The column effluent was monitored using UV detection at 270 and 235 nm, and one fraction was collected each minute for 43 min.

Due to the complexity of the urinary matrix, it was necessary to perform reversed phase chromatography of the normal phase fractions of interest in order to increase the extent of purification. Normal phase fractions of interest were pooled, dried down under vacuum, and analyzed by reversed phase HPLC using a Synergi 10-μm Hydro-RP 250 × 4.6 mm column (Phenomenex, Torrance, CA). The reversed phase solvents used were 0.3 mm acetic acid adjusted to pH 5.7 with ammonium hydroxide (solvent A) and 85:35 acetonitrile/methanol (v/v) (solvent B). The initial mobile phase was 15% solvent B at a flow rate of 1 ml/min. This initial mobile phase was held for 3 min, and then a linear gradient was started to 70% solvent B at 43 min. This was followed by a second linear gradient to 100% solvent B at 55 min. The column effluent was monitored using UV detection at 270 and 235 nm, and one fraction was collected each minute for 55 min.

The final analysis was carried out using reversed phase fractions of interest, which had been dried under vacuum, and then subjected to a second reversed phase HPLC separation (subsequently termed the analytical RP-HPLC) using on-line RP-HPLC with electrospray mass spectrometry (LC/MS), which employed a 150 × 1.0-mm Ultremex C18 column (Phenomenex, Torrance, CA) at a flow rate of 50 μl/min. The same reversed phase solvents were used that are described above. The initial mobile phase was 15% solvent B, which was held for 3 min, and then a linear gradient was started to 60% solvent B in 30 min. This was followed by a second linear gradient to 100% solvent B at 45 min. The column effluent was monitored using UV detection at 270 and 235 nm, and one fraction was collected each minute for 55 min.

**Table 1**

| Metabolite | UV chromophore | Reversed phase retention time(s) | MRM transitions monitored(s) |
|------------|----------------|---------------------------------|-----------------------------|
| 10-HO-LTB4 glucuronide | - | - | - |
| 10,11-Dihydro-LTB4 glucuronide | - | - | - |
| LTB4 glucuronide | - | - | - |
| 17-, 18-, 19- and 20-OH-LTB4 glucuronide | - | - | - |
| 10,11-Dihydro-20-COOH-LTB4 | - | - | - |
| 20-COOH-LTB4 | - | - | - |
| 10,11-Dihydro-18-COOH-LTB4 | - | - | - |
| 18-COOH-LTB4 | - | - | - |

- Reversed phase retention time using the reversed phase solvent system.
- Identification of α-oxidized metabolite elution order required GC/MS analysis and detection as the pentafluorobenzyl ester, trimethylsilyl ether derivatives (Fig. 3B).
- Ion transitions reported previously (13, 26) for each indicated m/z pair in a tandem quadrupole mass spectrometer.
- The reversed phase HPLC retention time and MRM transitions monitored are for the aglycone formed after hydrolysis by β-glucuronidase.
- Chromophore identified as characteristic for a conjugated triene with evidence of vibronic shoulders (± 10 nm) on either side of the reported λmax.
- Chromophore identified as characteristic for a conjugated diene at the reported λmax.

**Hydrolysis of Glucuronides**—An aliquot of methanol eluate (5%) from the 0–6-h pool of each subject injected with LTB4 or the placebo was dried down under vacuum and brought back up in 4.5 ml of 100 mM phosphate buffer, pH 7. This solution was adjusted to pH 7, and 5,000 units of β-glucuronidase in 0.5 ml of 100 mM phosphate buffer (pH 7) was added (26). This mixture was incubated in a 37 °C shaking water bath for 16 h for the quantitation experiments. For the time course experiments, the incubation times ranged from 1 to 24 h. After treatment, the liquid-liquid extraction procedure described in the initial metabolite extraction section was carried out followed by normal and reversed phase HPLC as described.

**Quantitation of LTB4, Glucuronide and 20-COOH-LTB4**—The quantity of LTB4 glucuronide and 20-COOH-LTB4 present in the urine of subjects injected with LTB4 and the placebo was determined using a stable isotope dilution LC/MS/MS protocol, essentially as described previously (27). The amount of LTB4 glucuronide present in urine was determined by adding 150 pmol of LTB4 as internal standard to an aliquot of the solid phase extracted urine in 5 ml of 100 mM phosphate buffer, pH 7. Hydrolysis of glucuronides was then carried out using the procedure described above followed by extraction and HPLC separation. The MRM transitions monitored were m/z 335 → 195 for LTB4 and m/z 339 → 197 for d5-LTB4. A standard curve generated used various amounts of authentic LTB4, mixed with 150 pmol of d5-LTB4, which was linear over the range 9–900 pmol LTB4. Additionally, the amount of 20-COOH-LTB4 was also calculated in the urine samples by using d5-LTB4 as the internal standard. A standard curve was constructed using known amounts of 20-COOH-LTB4 mixed with d5-LTB4 (150 pmol). The MRM transition monitored for 20-COOH-LTB4 was m/z 365 → 195.
RESULTS

The administration of LTB4 to four healthy male subjects at the dose of 150 nmol/kg was well tolerated with no report of severe adverse effects. A full report of the safety and tolerability of the intravenous administration of LTB4 to healthy human subjects as well as pharmacokinetic and pharmacodynamic data will be published separately.

The urine of subjects treated with LTB4, as well as the one placebo-treated subject, was collected into two separate pools. The first pool was 0–6 h after administration of LTB4 or placebo, and the second collection was 7–24 h after the procedure. Due to the complexity of the urine matrix, a total of five purification steps were performed in order to prepare the sample for mass spectrometric analysis. The first step in purification involved a solid phase extraction using a reversed phase protocol to remove lipophilic components from the urine matrix. The pH of the urine was adjusted to pH 3.8 prior to solid phase extraction in order to maximize recovery of LTB4 metabolites (28). The next purification step was a liquid-liquid extraction carried out on separate aliquots of the methanol eluate using a hexane/ethyl acetate extraction procedure that had been shown to result in excellent recovery of LTB4 as well as several of the target metabolites (data not shown). The solvent-extracted components were then subjected to normal phase HPLC using a silica column with gradient elution maximized for the separation of LTB4, 20-OH-LTB4, and 20-COOH-LTB4 (data not shown). The retention times of these leukotrienes were 7.6, 14.9, and 16.8 min, respectively, and these specific fractions (±1 min) were collected. The normal phase fractions of interest were introduced onto a 4.6-mm reversed phase HPLC column where the retention time of LTB4, 20-OH-LTB4, and 20-COOH-LTB4 standards were 36.1, 21.8, and 17.3 min, respectively. Finally, the reversed phase fractions that eluted at the expected retention times (±1 min) were introduced onto a 1-mm analytical RP-HPLC column and analyzed by on-line mass spectrometry (LC/MS). The retention times for standards and known UV absorption characteristics of leukotrienes were used as a guide to screening additional fractions than those noted for metabolites of LTB4. For example, fractions that eluted from the normal phase column after 16.8 min were analyzed for metabolites more polar than 20-COOH-LTB4. Another experimental strategy used to verify the identity of LTB4 metabolites was to monitor two MRM transitions for each known LTB4 metabolite. This strategy was critical to identify unambiguously the metabolites listed below. The MRM transitions used in this study were obtained from a previous study of the collision-induced dissociation mass spectra derived from the corresponding metabolites (13, 29).

Collisional activation of intact LTB4 glucuronide-conjugated metabolites had been shown previously to result in a neutral loss of 176 daltons to yield the aglycone (parent) anion as well as a glucuronic acid anion at m/z 193 (13). These ions and the neutral loss event did not provide structural details concerning the aglycone portion of the metabolite but did provide relevant
information as to the existence of glucuronides eluting from the HPLC column. Because glucuronide conjugates of 20-COOH-LTB\textsubscript{4}, LTB\textsubscript{4}, and 10,11-dihydro-LTB\textsubscript{4} had been previously observed following incubation of LTB\textsubscript{4} with human hepatocytes, a constant neutral loss scan was carried out for the LC/MS analysis of the solid phase extracted urine. However, a complex mixture of components was clearly evident in the placebo control as well as in the urine from the individual receiving the LTB\textsubscript{4} injection. Despite the solid phase extraction of this sample, it was clear that the use of a single ion transition, in this case a constant neutral loss of 176 daltons, as well as a precursor mass specific for LTB\textsubscript{4} were not sufficiently unique criteria to identify specific glucuronides present in the complex urine mixture. The most likely reason for this was that multiple LTB\textsubscript{4} glucuronide conjugates (see below) were present and each one at sufficiently low levels and that naturally occurring glucuronide conjugates from unrelated molecules produced signals that were greater in magnitude compared with any single LTB\textsubscript{4} glucuronide. Thus it was necessary to carry out the second stage of reversed phase HPLC as well as hydrolyze the glucuronides prior to analysis.

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18-COOH-LTB\textsubscript{4} and 10,11-dihydro-18-COOH-LTB\textsubscript{4}—The pooled normal phase fractions eluting between 19 and 20 min were injected onto a 4.6-mm reversed phase HPLC column and a component eluted that produced a signal at 12 min with an UV absorption spectrum consistent with that of a conjugated triene (data not shown). Subsequent LC/MS/MS analysis of the components eluting between 11 and 13 min from the 4.6-mm reversed phase column was then carried out for this region where components somewhat less lipophilic than 20-COOH-LTB\textsubscript{4} eluted. The specific transitions $m/z$ 337 $\to$ 141 and 337 $\to$ 195 were investigated since they correspond to 18-COOH-LTB\textsubscript{4}, a known metabolite in cell studies (13). Both transitions were clearly present in the unhydrolyzed urine (not treated with /H\textsubscript{9252}-glucuronidase) at a retention time of 13.1 min (Fig. 1, a and b), which had a retention time shorter than that expected for 20-COOH-LTB\textsubscript{4} (17.1 min). The unique UV absorption spectrum as well as the coelution of each MRM transition confirmed the presence of 18-COOH-LTB\textsubscript{4} in the aliquot of urine taken from the subject injected with LTB\textsubscript{4}. This experiment was repeated for all the subjects (data not shown), but the placebo-treated individual did not generate a component eluting at the appropriate retention time. Additionally, the

![Image of reversed phase HPLC and mass spectrometric analysis by multiple reaction monitoring (LC/MS/MS) of an aliquot (1 ml) of urine from an LTB\textsubscript{4}-treated subject after prior purification by solid phase extraction, solvent extraction, normal phase fractions 17–18, and reversed phase fractions 17–18. Elution of 20-COOH-LTB\textsubscript{4} was detected by the ion transitions $m/z$ 365 $\to$ 169 (a) and $m/z$ 365 $\to$ 195 (b), and 10,11-dihydro-20-COOH-LTB\textsubscript{4} was detected by the ion transitions $m/z$ 367 $\to$ 169 (c) and $m/z$ 367 $\to$ 115 (d). The inset to a is the UV spectrum of the component eluting at 17.05 min. The inset to c is the collisional activation of $m/z$ 367 with resultant product ion mass spectrum. The observed product ions and corresponding relative abundance agreed with the previous report characterizing this metabolite (13).]
MRM transitions \( m/z \ 339 \rightarrow 115 \) and \( m/z \ 339 \rightarrow 141 \) were monitored to determine whether or not 10,11-dihydro-18-COOH-LTB4 was present in these HPLC fractions. Both of these MRM transitions were present in the unhydrolyzed urine (Fig. 1, c and d) at a slightly more lipophilic retention time (13.6 min) compared with 18-COOH-LTB4, which suggested that 10,11-dihydro-18-COOH-LTB4 was present in the urine of subjects injected with LTB4.

20-COOH-LTB4 and 10,11-Dihydro-20-COOH-LTB4—The normal phase and reversed phase retention times of 20-COOH-LTB4 were determined to be 16.8 and 17.1 min, respectively. Therefore, the pooled normal phase fractions 15–16, and reversed phase fractions 21–23 were subjected to RP-HPLC on a 4.6-mm column, and a conjugated triene chromophore was observed for a component eluting at 17.3 min (Fig. 2a, inset). The RP-HPLC fractions 17–18 were pooled and analyzed by LC/MS/MS. The expected ion transitions for the collisional activation of 20-COOH-LTB4 \( m/z \ 365 \rightarrow 195 \) and \( m/z \ 365 \rightarrow 169 \) (29) were monitored in an MRM assay. When unhydrolyzed urine was subjected to the prior normal-phase and RP-chromatographic separation, LC/MS/MS analysis revealed the elution of a component indicated by these specific ion transitions at the suggested retention time and with the appropriate relative abundant ratios (Fig. 2, a and b). In addition to monitoring the MRM transitions for 20-COOH-LTB4, the MRM transitions at \( m/z \ 367 \rightarrow 115 \) and \( m/z \ 367 \rightarrow 169 \) were also monitored in
order to assess whether or not 10,11-dihydro-20-COOH-LTB₄ was present in these HPLC fractions. Both of these MRM transitions were significant in the unhydrolyzed urine at a slightly more lipophilic retention time (17.6 min, Fig. 2, c and d), which suggested that indeed 10,11-dihydro-20-COOH-LTB₄ was present in urine samples of subjects injected with LTB₄. The collisional activation of \( m/z \) 367 yielded a product ion mass spectrum (Fig. 2c, inset) that was virtually identical to that published previously (13) for 10,11-dihydro-20-COOH-LTB₄. The abundant product ions observed at \( m/z \) 115 and 169 were consistent with cleavage adjacent to the hydroxyl substituents with charge retention on the corresponding moiety as reported previously (29).

Glucuronide Conjugates of 20-, 19-, 18-, and 17-OH-LTB₄—
The normal phase retention time of 20-OH-LTB₄ standard was 14.9 min, and the reversed phase retention time was 21.8 min using a 4.6-mm column. The pooled normal phase fractions 15–16 were separated on the 4.6-mm RP column, and fractions 21–23 of the β-glucuronidase-treated and untreated urine aliquots were analyzed by LC/MS/MS. Any metabolites of LTB₄ that were hydroxylated near the methyl terminus were detected using the MRM transition \( m/z \) 351 → 195 (29). This transition was clearly observed in urine that had been treated with β-glucuronidase (Fig. 3a) but was not observed in the urine not treated with β-glucuronidase. Furthermore, four separate HPLC components eluted within a 3-min time window at 21.4, 22.4, 22.9, and 23.6 min in the β-glucuronidase-treated urine. In order to establish the identity of the four components that share the MRM transition \( m/z \) 351 → 195, reversed phase fractions 21–23 were derivatized for GC/MS analysis as the pentafluorobenzyl ester (PFB) and trimethylsilyl ether (TMS) derivative. The NCI of these derivatives had the most abundant ion at \( m/z \) 567 (Fig. 3b), which corresponded to a trihydroxy-TMS-derivatized carboxylate anion. Negative ion chemical ionization was used to determine the retention times (9.98, 10.10, 10.17, and 10.42 min) for the \( \omega \)-hydroxylated LTB₄ compounds of interest. After the retention times were established, EI was used to provide detailed structural information regarding the hydroxyl group position from bond cleavage that occurs adjacent to the trimethylsilyl ether positions. The EI mass spectrum of the derivatized metabolite with a retention time of 9.98 min (Fig. 3c) indicated that there are hydroxyl groups at C5 from the \( m/z \) 369 fragment (TMS-O=CH-(CH₂)₅CO₂PFB) and C12 from the \( m/z \) 549 (TMS-O=CH-(CH₂)₅CH(TMSO)CO₂PFB) (24). Additionally the ions at \( m/z \) 73 (TMS⁺) and \( m/z \) 181 (C₇H₇F₅⁻) were from the derivative groups. Finally, the major fragment ion at \( m/z \) 145 (TMS-O=CH(CH₂)₅CH₃) was indicative of a 17-hydroxylated metabolite due to a-cleavage of the C16–C17 bond adjacent to the trimethylsilyl ether (25). From the EI spectrum shown in Fig. 3c, it was determined that the LTB₄ metabolite with a retention time of 9.98 min was 17-OH-LTB₄. The EI spectra of the hydroxylated LTB₄ metabolites with retention times of 10.10 and 10.18 min (Fig. 3b) were obtained and showed major
α-cleavage ions at \( m/z \) 131 (TMS-O\(^+\) = CHCH\(_2\)CH\(_3\)) and \( m/z \) 117 (TMS-O\(^-\) = CHCH\(_3\)), respectively, as expected for \( ω\)-1- and \( ω\)-2-hydroxylated metabolites (25). In addition the relative abundance of other ions in the electron ionization mass spectra (data not shown) was identical to spectra published previously (30, 31) of the \( ω\)-1- and \( ω\)-2-hydroxylated metabolites of LTB\(_4\). These data were consistent with the identification of the metabolites with retention times of 10.10 and 10.18 min as 18-OH-LTB\(_4\) and 19-OH-LTB\(_4\). Finally, the EI spectrum of the derivatized LTB\(_4\) metabolite with a retention time of 10.42 min, identical to the gas chromatographic retention time of the 20-OH-LTB\(_4\) standard, was identical to the EI mass spectrum of 20-OH-LTB\(_4\) (24). The GC/MS data indicated that glucuronide conjugates of 20-, 19-, 18-, and 17-OH-LTB\(_4\) were present in urine of subjects injected with LTB\(_4\) and that the most abundant \( ω\)-oxidized metabolite was 17-OH-LTB\(_4\).

**Glucuronide Conjugates of LTB\(_4\) and 10,11-Dihydro-LTB\(_4\)**

The normal phase and reversed phase retention times of LTB\(_4\) were determined to be 7.6 and 36.1 min, respectively. Therefore, pooled normal phase fractions 7-9 were subjected to RP-HPLC. Only the β-glucuronidase-treated urine revealed a conjugated triene chromophore with a \( λ_{max} \) of 270 nm at 35.8 min (Fig. 4, inset). The pooled reversed phase fractions 35-37 of the β-glucuronidase-treated and untreated samples were then subjected to LC/MS/MS analysis. In order to ascertain if LTB\(_4\) was present in the urine, the MRM transitions \( m/z \) 335 \( \rightarrow \) 195 and \( m/z \) 335 \( \rightarrow \) 129 were monitored, and neither of these transitions were present in the unhydrolyzed urine (Fig. 5, b and c). However, the MRM transitions for LTB\(_4\) were detected at 36.9 min when the β-glucuronidase-treated urine was analyzed (Fig. 4, a and b). In addition to monitoring the MRM transitions for 10,11-dihydro-LTB\(_4\) (Fig. 5, a) and LTB\(_4\) (Fig. 5, b and c), the internal standard \( d_4\)-LTB\(_4\) was detected by the ion transition \( m/z \) 339 \( \rightarrow \) 197 (d).
transitions for LTB₄, the MRM transitions m/z 337 → 115 and m/z 337 → 225 for 10,11-dihydro-LTB₄ were also monitored in the unhydrolyzed and β-glucuronidase-treated urine. The most abundant of the MRM transitions for 10,11-dihydro-LTB₄ was not present in the unhydrolyzed urine (Fig. 5a); however, both of these MRM transitions were present at the correct relative intensity ratio at the slightly more lipophilic retention time compared with LTB₄ of 37.9 min (Fig. 6, a and b). This mass spectrometric data indicated that glucuronide conjugates of LTB₄ as well as 10,11-dihydro-LTB₄ were present in urine of the subjects injected with LTB₄.

Glucuronide Conjugate of 10-Hydroxy-4,6,12-octadecatrienoic Acid (10-HOTrE)—Normal phase fractions eluting between 4 and 6 min were also subjected to RP-HPLC on a 4.6-mm column, and reversed phase fractions eluting between 39 and 41 min were subject to LC/MS/MS. The specific MRM transitions for 10-HOTrE that were monitored during this LC/MS/MS assay were m/z 293 → 137 and 293 → 153 (29). Neither of these transitions were present in the specific fractions of the unhydrolyzed urine (Fig. 5a); however, both of these MRM transitions were present at the correct relative intensity ratio at the slightly more lipophilic retention time compared with LTB₄ of 37.9 min (Fig. 6, a and b). This mass spectrometric data indicated that glucuronide conjugates of LTB₄ as well as 10,11-dihydro-LTB₄ were present in urine of the subjects injected with LTB₄.

Quantitation of urinary metabolites of LTB₄ following injection into four separate human subjects (150 nmol/kg) using d₄-LTB₄ as a quantitative internal standard

| Subject | LTB₄ glucuronide (pmol/ml urine) | 20-COOH-LTB₄ (pmol/ml urine) |
|---------|--------------------------------|-------------------------------|
| 1       | 29.4                           | 22.9                          |
| 2       | 28.6                           | 18.9                          |
| 3       | 19.3                           | 30.6                          |
| 4       | 16.7                           | 21.3                          |
| Placebo | 0                              | 0                             |

*LTB₄ was analyzed after treatment of urine with β-glucuronidase.*

achieved by incubating the solid phase extracted urine with β-glucuronidase for 16 h at 37 °C. After hydrolysis and purification using RP-HPLC, the MRM transitions for LTB₄ and the internal standard d₄-LTB₄ were monitored during LC/MS/MS analysis. A time course for the hydrolysis of urine was also carried out (Fig. 8) on an aliquot of the solid phase extracted urine to follow the yield of released LTB₄. The yield of free LTB₄ was found to maximize between 15 and 24 h of treatment, and therefore a hydrolysis time of 16 h was routinely used in subsequent studies. The quantity of 20-COOH-LTB₄ was also determined in each urine sample using the d₄-LTB₄ as an analog internal standard. The standard curve for this latter assay was linear over the range of 9–900 pmol with an excellent linearity and correlation coefficient (data not shown).
of numerous glucuronide conjugate positional isomers, perhaps greater than five, was possible when considering acyl glucuronides (32). Additionally, glucuronide conjugates of LTB₄ metabolites derived from the pathways described above were also possible that could result in an exceedingly complex mixture of metabolites, making identification of prominent metabolites of LTB₄ excreted into the urine difficult.

Previous studies of LTB₄ metabolism in intact animals had involved the use of tritium-labeled LTB₄ to assist in isolation and purification of metabolites. When rats were injected with radiolabeled LTB₄, both bile and urine were found to contain metabolites. The bile contained 20–25% of the injected radioactivity, whereas the urine contained less than 10% of the injected dose (33). Reversed phase HPLC analysis of the bile and urine revealed that most metabolites were less lipophilic than LTB₄; however, structural identification of these metabolites was not reported. In another study, the in vivo metabolism of LTB₄ in the monkey was reported (34) after infusion with radiolabeled LTB₄ (8 μmol/kg). In this investigation, 25% of the injected radioactivity was recovered in urine after 24 h. More than 70% of the infused radioactivity was reported as volatile metabolites, and no intact LTB₄ was detected in urine. Purification of nonvolatile radiolabeled metabolites was carried out, and one metabolite was found to coelute with 20-hydroxy-LTB₄. The structures of the additional urinary metabolites were not reported, although several metabolites were separated.

In the present study where LTB₄ (150 nmol/kg) was injected into human subjects in a Phase I Clinical Trial, an alternative strategy was employed to identify LTB₄ metabolites based upon the previous known metabolites of LTB₄ using sensitive and specific LC/MS/MS assays. Because the retention times and relative intensities of ion transitions were known for several LTB₄ metabolites, it was possible to take advantage of the chromatographic behavior of metabolites under both reversed and normal phase conditions. For example, it was possible to obtain data consistent with the existence of 20-COOH-LTB₄, a component present in the urine of individuals treated with LTB₄, but not present in the urine of the untreated control subject. The criteria for metabolite identification was based upon HPLC retention times as well as the relative abundance of several specific ion transitions. The relative intensity of mass 365→195 was 0.70 (Fig. 2, a and b), which was similar to that published previously (29). If a single ion transition was used, multiple components could often be detected (Figs. 1d and 7a). This was mostly the result of the presence of unrelated compounds in urine that generated signals with the same ion transition channel, most likely due to the large number of molecules present in urine at levels much higher than those observed for the true LTB₄ metabolites. Therefore, criteria were established where two MRM transitions for each of the identified metabolites had to coincide at the correct retention time and, if possible, UV absorption spectra in order to report the presence of a specific metabolite. By using this strategy, it was also possible to detect the presence of 18-COOH-LTB₄, 10,11-di-hydroxy-18-COOH-LTB₄, and 10,11-dihydroxy-20-COOH-LTB₄ excreted into the urine in each of the subjects, even though authentic standards were not available to unambiguously establish the ratio of MRM transitions. In the case of 18-COOH-LTB₄ and 10,11-di-hydroxy-18-COOH-LTB₄, additional information was employed in that the compound with the correct MRM transitions eluted as a less lipophilic component compared with 20-COOH-LTB₄. Additionally, 10,11-di-hydroxy-20-COOH-LTB₄ had the correct MRM transitions and eluted at a slightly more lipophilic retention time than 20-COOH-LTB₄. No data could be obtained consistent

![Graph](image-url)
with the presence of 16-COOH-LTB4 as urinary metabolite of LTB4. It was very clear, however, that neither 20-OH-LTB4 nor LTB4 itself were present as excreted products in urine of subjects treated with LTB4. The fact that LTB4 was not present in urine of these subjects suggested that LTB4 was rapidly metabolized following injection and was consistent with previous primate studies where ~50 times more LTB4 was injected (34).

One of the major pathways of LTB4 metabolism in human subjects in this study was glucuronic acid conjugation, which is a common means that the intact organism can enhance the excretion of lipophilic compounds (35, 36). In order to establish unambiguously that glucuronide conjugates of LTB4 were present in the urine of subjects injected with LTB4, a strategy that employed analysis of identical aliquots of β-glucuronidase-treated and untreated samples was analyzed by LC/MS/MS. A further complication of multiple glucuronides for any single LTB4 metabolite reduced the overall sensitivity of the analysis of each individual metabolite as an intact glucuronide. Because β-glucuronidase can cleave ether-linked as well as 1-O-acetylated conjugates, it was employed as the most applicable method to remove the glucuronic acid conjugate. Studies employing acid or base catalyzed hydrolysis led to extensive degradation of LTB4 metabolites (data not shown). The LC/MS/MS data clearly indicated the existence of several excreted glucuronide metabolites of the injected LTB4. These included 20-, 19-, 18-, and 17-OH-LTB4, 10-HOTrE, 10,11-LTB4, and even intact LTB4 glucuronides. None of these components were present in the nonhydrolyzed urine samples nor were they present in the untreated placebo urine. The identity of each of these metabolites (after hydrolysis) could be clearly ascertained using the criteria described above.

Of some interest was the unexpected observation of 17-OH-LTB4, 18-OH-LTB4, and 19-OH-LTB4 glucuronide. Powell and Gravelle (30) found that rat polymorphonuclear leukocytes could form both 19-OH-LTB4 and 18-OH-LTB4 upon incubation with arachidonic acid and stimulation by A23187. Additionally, rat liver microsomes were found to convert LTB4 into 20-OH-LTB4 along with small amounts of 19-OH-LTB4 (31). Recently, recombinant CYP4F5 and CYP4F6 were found to convert LTB4 into 17-OH-, 18-OH-, and 20-OH-LTB4 during in vitro incubation (37). These P450 isozymes were found expressed in hepatocytes of rats (37). During the LC/MS/MS assay, detection of four separate components with the ion transition of m/z 351 → 195 was observed after treatment with β-glucuronidase (Fig. 3a). In order to determine the position of hydroxylation of the four components, reversed phase fractions 21–23 were pooled and analyzed by GC/MS as the PFB ester and TMS ether derivative. The four trihydroxy-TMS derivatized carboxylate anions (m/z 567) are shown in the NCI-GC/MS chromatogram in Fig. 3b at retention times of 9.98, 10.10, 10.18, and 10.42 min. Electron ionization was then used to provide structural information concerning the hydroxyl group position from cleavage that occurs adjacent to the TMS ether position. The EI mass spectra at retention times of 9.98, 10.10, and 10.18 min showed a major a-cleavage ion at m/z 145 (Fig. 3c), m/z 131, and m/z 117, respectively. From these data it was concluded that 17-OH-, 18-OH-, and 19-OH-LTB4 were present as glucuronide conjugates in the urine of subjects injected with LTB4. Additionally, the metabolite with a retention time of 10.42 min was identified as 20-OH-LTB4. The main ω-hydroxylated LTB4 metabolite found in the β-glucuronidase-treated urine of subjects injected with LTB4 was found to be 17-OH-LTB4, whereas 20-OH-LTB4 was a minor peak. This unexpected finding might reflect a situation where 20-OH-LTB4 can undergo β-oxidation rather than forming a glucuronide (Fig. 9). Thus, the glucuronidation pathway was a more predominant fate for ω-1-, ω-2-, and ω-3-hydroxylation products of LTB4 because subsequent β-oxidation was not possible.

The most abundant metabolites identified were LTB4 glucuronide and 20-COOH-LTB4. Therefore, quantitative assays based on stable isotope dilution or analog dilution using stable isotope-labeled LTB4 was employed to measure specifically these two metabolites of LTB4. The quantity of the LTB4 glucuronide (after hydrolysis) was found to range between 15 and 30 pmol/ml of urine. The enzymatic hydrolysis experiment was performed five separate times for one subject, and the error was determined to be ±1.7 pmol/ml of urine. The amount of

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**Fig. 9. Proposed pathway for metabolism of LTB4 in the human subject with urinary metabolites identified in this study indicated.** Required intermediate metabolites not observed are indicated in brackets. UDP-glucuronosyltransferase (UGT)-dependent pathways resulting in glucuronide metabolites and cytochrome P-450 (CYP4F) pathways are indicated.
Metabolism of LTB₄

20-COOH-LTB₄ was also determined in these urine samples, and it ranged from 18 to 31 pmol/ml of urine. This quantitation was found to have an analytical error of ±0.86 pmol/ml in five replicate analyses. From these results, it appeared that approximately the same amount of 20-COOH-LTB₄ and LTB₄ glucuronide were excreted into the urine of each subject. It is not known if a single glucuronide isomer predominated for LTB₄ glucuronide; however, based on previous studies it was likely that a number of glucuronides existed. The total amount of 20-COOH-LTB₄ and LTB₄ glucuronide represented only 0.2% recovery of the injected LTB₄ for each of the subjects. This rather low recovery was expected based on the previously described studies of LTB₄ metabolism as well as the metabolism of prostanooids into individual metabolites (18–20).

It is important to note that the metabolic products of LTB₄ in vivo reported above were only found in the urine sample from a 0- to 6-h period after injection of LTB₄. The 7–24-h urine sample was also analyzed, and none of these metabolites were observed. This was consistent with the time course described previously (33, 34) for appearance of LTB₄ metabolites in urine. In various animal experiments. These studies indicated that LTB₄ underwent degradation via multiple pathways leading to extensive chain shortening typical of fatty acids as well as glucuronidation (Fig. 9). Nevertheless, some caution must be exercised in extrapolation of the observations reported here to the metabolic fate of LTB₄ synthesized from endogenous arachidonic acid in vivo. This study involved administration of high doses of LTB₄ that could experience a different metabolic fate in quantitative terms from LTB₄ synthesized within a cell.

In summary, the metabolism of LTB₄ in the human subjects is quite complex with multiple pathways responsible for the degradation of LTB₄ prior to elimination into urine. Some of the most abundant products were those derived from conjugation with glucuronic acid of either intact LTB₄ or other highly lipophilic metabolites. In this investigation, 11 different metabolites of LTB₄ were identified in urine subjects injected with 150 nmol/kg LTB₄. Four unconjugated metabolites were excreted into urine and identified by mass spectrometry as 18-COOH-LTB₄, 10,11-dihydro-18-COOH-LTB₄, 20-COOH-LTB₄, and 10–11-dihydro-20-COOH-LTB₄. The glucuronide conjugates of 20-, 19-, 18-, and 17-OH-LTB₄, 10-HOTrE, 10,11-dihydro-LTB₄, and intact LTB₄ itself were also observed. None of these metabolites exceeded 1% of the injected dose; however, it was possible to detect the glucuronide conjugates by mass spectrometry after hydrolysis to the aglycone metabolite. It is possible that these LTB₄ metabolites may be relevant targets to assess the in vivo production of LTB₄, but additional experiments will be required to define which of these observed metabolites from the exogenously administered LTB₄ best reflects endogenous LTB₄ production.

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