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Leukotriene E_4 Elimination and Metabolism in Normal Human Subjects*

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Radiolabeled leukotriene (LT) E_4 was infused into three healthy subjects in order to assess the production and elimination of sulfidopeptide leukotriene metabolites in urine. Three different radiolabeled tracers were employed, [14,15-^{3}H]LTE_4, [^{35}S]LTE_4, and [^{14}C]LTE_4 in five separate infusion studies. There was a rapid disappearance of radioactivity from the vascular compartment in an apparent two-phase process. The first elimination phase had an apparent half-life of approximately 7 min. Radioactivity quickly appeared in the urine with 10–16% eliminated during the first 2 h following intravenous infusion; 7%, 2–5 h; 4%, 5–8 h; 4%, 8–15 h; and 1.5%, 15–24 h from the [^{14}C]LTE_4 experiments. Unmetabolized LTE_4 was the major radioactive component in the first urine collection, but at later times two more polar compounds predominated. After extensive purification by normal phase-solid phase extraction and reverse-phase high performance liquid chromatography, these compounds were characterized by UV spectroscopy, co-elution with synthetic standards, negative ion electron capture gas chromatography/mass spectrometry, and tandem mass spectrometry. The two major urinary metabolites were structurally determined to be 14-carboxy-hexanor-LTE_3 and the conjugated tetraene, 16-carboxy-Δ^{13}-tetranor-LTE_4. Three other minor metabolites were detectable in the first urine collection only and were characterized by co-elution with synthetic standards as 16-carboxy-tetranor-LTE_3, 18-carboxy-dinor-LTE_4, and 20-carboxy-LTE_4. ω-Oxidation and subsequent β-oxidation from the methyl terminus appeared to be the major metabolic fate for sulfidopeptide leukotrienes in man. The accumulation of the 14-COOH-LTE_3 and 16-COOH-Δ^{13}-LTE_4 may reflect a rate-limiting step in further oxidation of these compounds which places a conjugated triene or conjugated tetraene, respectively, two carbons removed from the CoA ester moiety. Also in the first urine collection there was another minor metabolite identified as *N*-acetyl-LTE_4, however, no subsequent β-oxidation of this metabolite was observed. The major metabolites of LTE_4 might be useful in assessing *in vivo* production of sulfidopeptide leukotrienes in humans.

Sulfidopeptide leukotrienes, leukotriene $C_4/D_4/E_4$, are now known to be the active principles previously referred to as slow reacting substances of anaphylaxis (1). These molecules are derived from arachidonic acid by action of the enzyme 5-lipoxygenase with the intermediate formation of leukotriene A_4 which is conjugated with glutathione (2–4). Sulfidopeptide leukotrienes are synthesized by a variety of cells, for example, mast cells (5), eosinophils (6), and macrophages (7) from arachidonic acid as well as by transcellular biosynthetic mechanisms from LTA_4[1] involving multiple cells such as neutrophils and platelets (8). These molecules possess potent biological activities in that they cause profound bronchoconstriction, vasoconstriction, and increased vascular permeability (9, 10). Substantial evidence has focused attention on the possible role for these eicosanoids in various lung diseases, having been detected in bronchoalveolar lavages from atopic patients following local challenge with specific antigen as well as in bronchial aspirates from patients with acute respiratory distress syndrome (11, 12). However, studies evaluating the biosynthesis of sulfidopeptide leukotrienes at the level of the site of production (*i.e.* within specific regions of the lung) would require the use of invasive techniques. The difficulty in sampling the site for synthesis of these molecules has focused the attention of several investigators on the possibility of identifying metabolites of these molecules in physiological fluids such as plasma or urine which might serve as an index for the production of these molecules much as do metabolites of prostanoids for the synthesis of these active cyclooxygenase products of arachidonic acid.

Detailed information is available concerning the biosynthetic mechanisms leading to the production of sulfidopeptide leukotrienes; however, substantially less is known about the biotransformation of these molecules and subsequent elimination in the human subject. Leukotriene C_4 (LTC_4) is known to be rapidly metabolized by enzymes present in the plasma to the cysteinyl leukotriene E_4 (LTE_4) by way of the cysteinylglycine active metabolite leukotriene D_4 (LTD_4) (13). The subsequent fate of LTE_4 has been the subject of several investigations. In the rat, LTE_4 is converted rapidly into *N*-acetyl-LTE_4 and the structures of several ω- and β-oxidation products of *N*-acetyl-LTE_4 from rat hepatocytes has been elucidated (14). The occurrence of these metabolites in rat bile and in rat urine has also been reported (15). Intravenous infusion of [14,15-^{3}H]LTC_4 into the human subject resulted in the excretion of 12–20% of the radioactivity into urine (16) and infusion of [$^3H_8$]LTC_4 resulted in more than 40% of the radioactivity appearing in the urine (17). In these latter

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[1] The abbreviations used are: LT, leukotriene; RP, reverse-phase; HPLC, high performance liquid chromatography; PFB, pentafluorobenzene; TxB_2, thromboxane; BSTFA, bis(trimethylsilyl)trifluoroacetamide.

studies, LTE_4 was identified as a major metabolite excreted into the urine; however, a substantial amount of radioactivity was associated with compounds more polar and sometimes poorly separated by chromatographic means. The aims of the present investigation were to evaluate the formation of metabolites from LTE_4, infused into human subjects, where a radiolabeled tracer which would not be rapidly converted into tritium-labeled water was employed. The time course for the formation of these metabolites as well as structural characterization of urinary metabolites was a major focus of this study.

EXPERIMENTAL PROCEDURES

Subjects and Study Design—This study was approved by the Human Research Committee of the University of Colorado Health Sciences Center to administer radiolabeled LTE_4 to normal volunteers. Three healthy male volunteers, aged 35–45 years, were investigated. Their weight ranged between 65 and 88 kg. Each subject received an infusion of [^{14}C]LTE_4, and one was infused upon three different occasions with the three different radiolabeled isotopes of LTE_4 employed.

Materials—Tritiated LTE_4 [14,15-^{3}H]LTE_4 (38 Ci/mmol) was obtained from Du Pont-New England Nuclear; synthetic LTE_4 and LTA_4 were obtained from Cayman Chemical (Ann Arbor, MI). 20-Carboxy-leukotriene E_4 (20-COOH-LTE_4), 18-carboxy-19,20-dinor-leukotriene E_4 (18-COOH-LTE_4), and 16-carboxy-17,18,19,20-tetranor-leukotriene E_3 (16-COOH-LTE_3) were obtained from Oxford Biomedical Research (Oxford, MI). *N*-Acetyl derivatives of LTE_4 and of the different LTE_4 metabolites were synthesized as described below. 14-[^{14}C]Carboxy-15,16,17,18,19,20-hexanor-*N*-acetyl-LTE_3 was biosynthesized using [1-^{14}C]LTE_4 as the starting material and rat hepatocytes as previously described (14). Extraction of urine using Amberlite XAD-8 (Supelco, Bellefonte, PA) was performed in a 200-ml open column, and Supelclean LC-Si cartridges (Supelco) were used for normal phase chromatography. Reverse-phase high performance liquid chromatography (RP-HPLC) was carried out using Ultrasphere ODS (5 μm, 0.46 × 25 cm, Beckman), with a 5-μm ODS, 5-mm precolumn cartridge (Waters Associates, Millipore Corp.) using a binary gradient liquid chromatographic system. Ultraviolet absorbance spectra were recorded every 2.8 s using a Hewlett-Packard 1040 photodiode array detector (Hewlett-Packard, Palo Alto, CA). Radioactivity from HPLC effluents was measured in some experiments using an on-line radioactive monitor (Radiomatic, Tampa, FL) with a Flo-scint II (Packard Radiomatic, Tampa, FL) as scintillation mixture in a ratio of 3:1. All solvents employed were HPLC grade (Fisher), and other reagents were of the highest grade commercially available. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco. Rhodium (5% on alumina powder) and pentafluorobenzyl bromide were obtained from Aldrich.

Preparation of [^{14}C]LTE_4 and [^{35}S]LTE_4—[Cys-^{14}CO]LTC_4 (50 mCi/mmol, obtained from CEA, Saclay, France(18)) was incubated for 3 h at 37 °C with 2 units of γ-glutamyltranspeptidase (Sigma) and 5 units of leucine aminopeptidase (Sigma) in 1 ml of Hanks' balanced salt solution. Four volumes of cold ethanol was added to precipitate proteins, and, following centrifugation, the supernatant was purified by RP-HPLC using methanol/water/acetic acid (70:30:0.02, v/v/v) (system I) as an isocratic mobile phase. Fractions which co-eluted with synthetic LTE_4 were pooled and stored at −70 °C before use.

[^{35}S]LTC_4 was prepared biosynthetically from [^{35}S]cysteine (600 Ci/mmol, Du Pont-New England Nuclear Boston, MA)-labeled human platelets. Human platelets were obtained from 80 ml of platelet-rich plasma and prepared following previously described procedures (19). Resulting platelets were suspended in 5 ml of citrate buffer, pH 6.5, containing PGE_1 (19). Platelets were incubated with 1 mCi of L-[^{35}S]cysteine. After 180 min at room temperature, the cells were washed twice and then resuspended at 0.5 × 10^9/ml in Hanks' balanced salt solution containing 0.5% bovine serum albumin. LTA_4 (10 μM) was then added, and the incubation was carried out for 30 min at 37 °C. At the end of the incubation, γ-glutamyltranspeptidase (2 units) and leucine aminopeptide (5 units) were added for an extra 30 min. Two volumes of ice-cold methanol was then added. After 60 min at −20 °C, the precipitated proteins and cells were pelleted. The supernatant was taken to dryness and reconstituted in 20% methanol in water, and [^{35}S]LTE_4 was isolated by RP-HPLC using system I.

Infusion of Labeled LTE_4—Radiolabeled LTE_4 was taken to dryness under reduced pressure and reconstituted in 10 ml of sterile saline. The solution was analyzed by ultraviolet spectroscopy in order to calculate LTE_4 concentration. An aliquot was taken for measurement of radioactivity content by liquid scintillation counting. Each volunteer was infused in the right hand dorsal digital vein over a 10-min period with LTE_4 filtered through a 0.22-μm Millex sterile filter (Millipore). The actual amount of radiolabeled LTE_4 infused was corrected by subtracting the amount of radioactivity remaining in the infusion set and filter which were subsequently washed with methanol and measured for radioactivity content. During one experiment with [^{35}S]LTE_4, samples of venous blood (2 ml) were taken from the left dorsal digital vein of the volunteer every minute (0–10 min) starting at the end of the infusion period and then at 20 and 40 min following the infusion. These samples were collected in 0.5 ml of heparin, rapidly spun in a Microfuge for 1 min at 12,000 × *g*, and plasma-treated with 4 volumes of cold ethanol. The precipitate was separated, and supernatant was taken for scintillation counting. Urines were collected at 2, 5, 8, 15, and 24 h after infusion, the volume was measured, and the radioactivity content was determined by counting two different aliquots of the urines. Urine samples from 8–15 h were immediately frozen following collection and kept at −20 °C until they were taken for purification. All other samples were processed immediately after collection.

Purification of Metabolites from Urine—Each urine sample was acidified to pH 4 with formic acid and then applied to an exaustively washed XAD 8 column (20). The column was then washed twice with 200 ml of water, and the radioactivity eluted with 200 ml of methanol. Radioactivity not retained by the column, but eluted in the water washes, was determined. The methanolic eluate was dried under reduced pressure (0.1 torr) and reconstituted in 15% methanol/water for injection on a RP-HPLC using a linear gradient from 16 mM ammonium formate made pH 4.8 with HCl to methanol/water/acetic acid (90:10:0.1, v/v/v) over 55 min (system II). The effluent of a separate aliquot (5–20%) was analyzed with an on-line radioactive monitor for determination of the metabolic profile of each timed urine sample. The remaining urine sample was purified by an identical RP-HPLC system and 1-min fractions were collected. Designation of metabolites refers to retention time on this HPLC separation step (H*xx*; H = human; *xx* = retention time in minutes). Those fractions which contained significant amounts of radioactivity were then taken to dryness, reconstituted in 4 ml of methanol/ethyl acetate (50:50, v/v), and applied to a normal phase cartridge prewashed with 4 ml of CH_2Cl_2, 4 ml of methanol, and 4 ml of ethyl acetate. The cartridge was then washed with 4 ml of methanol/ethyl acetate (7:3, v/v) and eluted with 4 ml of methanol/water/formic acid (95:5:0.1, v/v/v). This latter fraction was dried under reduced pressure, reconstituted in 15% methanol and injected onto RP-HPLC using a methanol/water/acetic acid mixture adjusted to pH 6 with ammonium hydroxide as an isocratic eluting system. The exact composition was 15:85:0.02 (v/v/v) for H31 and 25:75:0.02 (v/v/v) for H36. H60 was purified using system I, and H40, H45, H50, and H58 were not subjected to this purification step. The amount of radioactivity present in each fraction was determined from a small aliquot taken from 1-min fractions. Final purification (system III) and co-elution with biological and synthetic standards were performed with RP-HPLC using a linear gradient from 3.2 mM ammonium formate made at pH 4 with HCl (solvent A) to a mixture of solvent A in acetonitrile (solvent B). For the metabolites H31, H36, H40, and H45, solvent B was 1:1 (solvent A/acetonitrile, v/v), and the gradient lasted 55 min. For metabolite H50, solvent B was 6:4 (v/v) and the gradient was over 30 min; for H58 and H60, solvent B was 60:40:0.02 formic acid (v/v/v), and the gradient was over a 25-min period.

Acetylation Conditions—The acetylation of metabolite H31 and other standards was carried out in 150 μl of ethanol following the addition of 80 mM sodium bicarbonate, 150 μl of triethanolamine (80 mM water), and 10 μl of acetic anhydride (10% in ethanol). The reaction was allowed to proceed for 30 min at 4 °C, then stopped with 4 ml of water. The reaction mixture was acidified with formic acid and applied to a Sep-Pak cartridge (Waters Associates) conditioned with 5 ml of methanol and 5 ml of water. After washing with additional 5 ml of water following application of the sample, the *N*-acetylated compounds were eluted with 4 ml of methanol. All derivatives were stored at −70 °C.

Mass Spectrometry—Aliquots of metabolites obtained from the final HPLC purification were subjected to catalytic desulfurization using 5% rhodium on alumina. Briefly, the samples were dissolved in 1 ml of 50% methanol/water and 5–10 mg of Rh/Al_2O_3, which had been prewashed with methanol, was added. Hydrogen gas was slowly

bubbled through the solution for 30 min followed by treatment with 50 μl of 1 N NaOH. The sample was centrifuged to a separate catalyst, and the supernatant was acidified with a drop of formic acid and extracted with 2 volumes of ethyl acetate. The organic layer was taken to dryness under a stream of dry nitrogen and treated with 10% *N,N*-diisopropylethylamine and 10% pentafluorobenzylbromide in acetonitrile for 20 min at room temperature to yield the pentafluorobenzyl ester. Any hydroxyl groups present in the metabolite remaining following the hydrogenation step were converted into the trimethylsilyl ether derivative by reaction with BSTFA (1:1 in acetonitrile) for 15 min at 60 °C. Products were analyzed by gas chromatography-mass spectrometry (Finnigan SSQ70, San Jose, CA). Capillary gas chromatography was carried out using a splitless injector at 280 °C, and the column temperature programmed from 200–300 °C at 15 °C/min. The capillary column employed was a DB-1 column, (10 m × 0.25 mm inside diameter, 0.2-μm film thickness; J&W Scientific, Rancho Cordova, CA). The mass spectrometer was operated in a negative ion electron capture (chemical ionization) mode using methane as moderating gas at 0.5 torr. A mixture of C-19 through C-27 fatty acids derivatized to pentafluorobenzyl ester was used to determine the carbon number equivalent for each reduced and derivatized metabolite. The number of carbons of the various fatty acyl groups of these standard PFB derivatives was plotted against the measured retention time using the above described conditions. The resulting linear regression ($r = 1.0$) was used to calculate the carbon number equivalent for each metabolite.

Tandem mass spectrometry was carried out on a Finnigan TSQ-70B mass spectrometer (Finnigan Corp., San Jose, CA). The GC and source conditions were the same used with the mass spectrometric experiments described above with argon as collision gas in the second quadrupole field region with an energy of 30 eV (laboratory frame of reference).

RESULTS

No adverse effects were observed in any of the volunteers during the infusion of LTE_4. The amount of LTE_4 infused into each subject (Table I) ranged from 2.4 to 45 μg and the radioactivity from 0.25 to 4.0 μCi. The amount of radioactivity recovered in the urines appeared to be related to the type of radiolabeled tracer LTE_4 employed and ranged during the first 24 h after infusion between 30 and 44% when [^{14}C]- or [^{35}S]LTE_4 was employed, but was only 9.9% when [14,15-^{3}H] LTE_4 was used (Table II). While there was a 6-fold difference in the absolute quantity of LTE_4 injected in those experiments involving [^{14}C]LTE_4, there was only a slight difference in the total percentage of radioactivity recovered in the urine during a 24-h period. This suggested that there was no relationship between the amount of LTE_4 infused and the percent excretion of LTE_4 metabolites at the doses employed.

As seen in Fig. 1, the disappearance of [^{35}S]LTE_4 from the vascular compartment was rapid during the first 10 min following infusion of one individual. Using the early time points of the curve of disappearance of radiolabel from plasma, the half-life of LTE_4 in plasma was estimated to be 6 to 7 min. This suggested that an efficient mechanism for the uptake of LTE_4 from blood and removal from the venous compartment exists. The time course for appearance of radioactive metabolites in three subjects is shown in Table IV and for one subject infused with [^{35}S]LTE_4 in Table V. For all individuals, the LTE_4 was most abundant in the first urine collection (2 h) dropping to one-tenth of that value by the second urine collection (3 h). After 5 h following infusion, no radiolabeled LTE_4 was detectible. There also appeared to be a second elimination phase of substantially longer duration perhaps related to the appearance of metabolites in plasma and their subsequent removal by excretion from the kidney into the urine.

Metabolite Purification—The extraction of acidified urine using Amberlite XAD-8 resulted in a substantial amount of radioactivity being retained by the column which could be subsequently eluted with methanol. The relative amount of extractable radioactivity decreased with time of urine collection, ranging from 63 to 80% of the radioactivity in urine during the 0–2-h collection period to 26 to 42% in those urines collected between 15 and 24 h after infusion (Table III). This suggested that more polar metabolites not retained by the XAD-8 column were being formed and eliminated in urine as time following the infusion period increased.

Analysis of the methanol eluate from XAD-8 of the first urine collections (0–2 h) always revealed a major radioactive peak at the retention time of synthetic LTE_4 (H60); however, several other peaks were also observed (H58, H50, H46, H40, H36, and H31) in substantially less amounts (Fig. 2*A*). XAD-8-retained material from the second urine collection (2–5 h)

TABLE I

Radiolabeled tracers employed in human metabolism studies

Volunteer	Weight	LTE_4 tracer	Amount	Injected
	kg		*μCi*	*μg*
N. V.	80	^{14}C	0.25	2.5
J. M.	65	^{14}C	1.5	15
R. M.	88	^{14}C	0.36	3.6
		^{35}S	4	45
		^{3}H	2.1	2.4

TABLE II

Time course for appearance of radioactivity in collected urines

Values as percent of the total radioactivity infused.

	^{3}H	^{14}C[a]	^{35}S
0–2	7.3	13.4	13.6
2–5	1.8	7.5	12
5–8	0.8	4.3	5.7
8–15	ND[b]	4.3	7
15–24	ND	1.5	6
Total	9.9	30.0	44.3

[a] Average of three subjects.
[b] ND, not detectable.

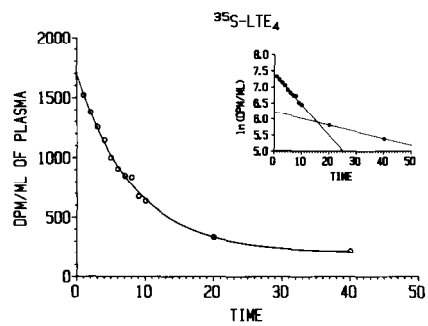

FIG. 1. **Disappearance of radiolabel from plasma after intravenous infusion with 4 μCi of [^{35}S]LTE_4 into one normal volunteer (R. C. M.).** *Inset,* log transformation of plasma radioactivity and linear regression of values between 1 and 10 min.

TABLE III

Distribution of urinary radioactivity from [^{14}C]LTE_4 following XAD-8 extraction

Results are averages of three subjects.

	H_2O		MeOH	
	Amount in urine	Total injected	Amount in urine	Total injected
	%		%	
0–2	26.2	3.8	73.8	9.6
2–5	40.1	3.1	59.9	4.4
5–8	47.8	2.1	59.2	2.2
8–15	60	2.8	40	1.5
15–24	67.4	1	32.6	0.5
Total		12.8		18.2

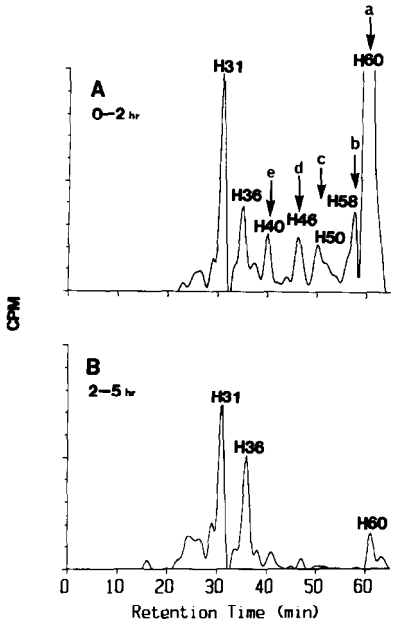

FIG. 2. **Reverse-phase HPLC (system I) separation of methanol eluate from the XAD-8 extraction.** *A*, sample from the first urine collection, 0 to 2 h after the infusion with [^{35}S]LTE$_4$. The *lettered labels* indicate the expected retention times for synthetic standards separated by this RP-HPLC system as follows: *a*, LTE$_4$; *b*, *N*-acetyl-LTE$_4$; *c*, 20-COOH-LTE$_4$; *d*, 18-COOH-LTE$_4$; and *e*, 16-COOH-LTE$_3$. *B*, sample from the second urine collection, 2 to 5 h after the infusion with [^{35}S]LTE$_4$. Radioactive metabolites designated as per HPLC retention time.

TABLE IV

Appearance of human metabolites of LTE$_4$ in timed urine samples following infusion with [^{14}C]LTE$_4$ into three separate human subjects

Values are means ± S.E.; n = 3.

	[^{14}C]LTE$_4$ injected		
	H60	H36	H31
		%	
0–2 h	6.7 ± 0.7	1.0 ± 0.9	0.7 ± 0.5
2–5 h	0.5 ± 0.2	2.1 ± 0.6	1.4 ± 0.3
5–8 h		1.3 ± 0.1	0.7 ± 0.1
8–15 h		0.6 ± 0.5	0.4 ± 0.3
Total in urine	7.2 ± 0.8	5.0 ± 1.3	3.2 ± 0.5

typically revealed little radioactivity at the retention time of LTE$_4$, with two separated peaks accounting for the majority of the radioactivity in these samples (Fig. 2*B*). These peaks (H31 and H36) were clearly detectable in all subsequent urine samples up to 15 h after infusion and together accounted for more than 8% of the total infused radioactivity (Table IV). Other metabolites were virtually undetectable following the 5-h time period of urine collection. The time course for appearance of radioactive metabolites from [^{14}C]LTE$_4$ averaged for three subjects is shown in Table IV and for one subject infused with [^{35}S]LTE$_4$ in Table V; 5 h after infusion, no radiolabeled LTE$_4$ was detectable in any subject. The major metabolites had a different and prolonged elimination time course typified in Table V with maximal excretion between 2 and 5 h following infusion. Only in one subject (J.M.) of the five infusion studies was there some alteration in the time course shown in Table V. This individual had a prolonged urinary excretion period of radioactive metabolites that was fairly constant during each of the first three collection periods (8 h). There were no major differences in the appearance of these more polar human metabolites of LTE$_4$ when ^{14}C- or ^{35}S-labeled LTE$_4$ was employed as tracer. However, these

TABLE V

Recovery of [^{35}S]LTE$_4$ and radiolabeled metabolites in partially purified urine from one human subject (R. M.)

Values are means ± S.E.; n = 3.

	Injected LTE$_4$		
	H60	H36	H31
		%	
0–2 h	7.3 (3300 ng)	0.6 (280 ng)	1.3 (590 ng)
2–5 h	0.8 (360 ng)	1.4 (610 ng)	2.1 (940 ng)
5–8 h		0.6 (280 ng)	0.7 (310 ng)
8–15 h		0.4 (180 ng)	0.5 (230 ng)
Total	8.1 (3660 ng)	3.0 (1350 ng)	4.6 (2070 ng)

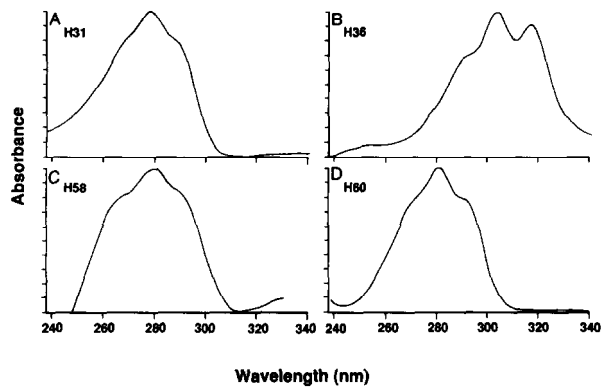

FIG. 3. **UV absorption spectra of human LTE$_4$ metabolites.** *A*, metabolite H31; *B*, metabolite H36; *C*, metabolite H58; *D*, metabolite H60.

metabolites were totally absent in all urine samples collected from the volunteer using [14,15-^{3}H]LTE$_4$ as tracer. Correspondingly, the amount of radioactivity present in the urine samples from the tritium tracer experiment almost coincided with the amount of LTE$_4$ present in urine, the small differences represented by H40, H46, H50, and H58 was detectable in the first urine collection. These results are in agreement with those previously published in two other human experiment studies (16, 17). Thus, it would appear that these major metabolites of LTE$_4$ involved metabolism which led to exchange of the protons on carbon 14 and carbon 15 to remove this tracer from the metabolite structure. Full ultraviolet spectra were obtained for four of the human urinary metabolites as these molecules eluted from the HPLC (system III) and are shown in Fig. 3.

Metabolite H60—Urinary metabolite designated H60 eluted at the retention time of synthetic LTE$_4$ as mentioned above. It had a UV spectrum consistent with the injected LTE$_4$ (Fig. 3*D*). Furthermore, this metabolite co-eluted with authentic LTE$_4$ in two separate HPLC systems involving different mobile phases (system I and system III). FAB mass spectrometry revealed an ion at m/z 438 $(M - H)^-$ which, by tandem mass spectrometry and collision-induced dissociation, decomposed to ions at m/z 115, 235, 333, and 351 identical with that observed for synthetic LTE$_4$. Therefore, metabolite H60 was identified as unmetabolized LTE$_4$. Concerning the two different RP-HPLC systems used, it is important to note that the acetonitrile/water (system III) mobile phase was substantially different from the methanol/water (system I) mobile phase, eluting *N*-acetyl derivatives later rather than prior to the corresponding nonacetylated compound.

Metabolite H36—The urinary metabolite H36 had a unique ultraviolet absorption spectrum with maximum absorbance at 306 nm with shoulders at 295 and 318 (Fig. 3*B*). A similar UV absorption spectrum was seen for rat metabolite E which corresponded to 16-COOH-Δ^{13}-*N*-acetyl-LTE$_4$. Reduction of

H36 using rhodium on alumina followed by derivatization as the pentafluorobenzyl ester trimethylsilyl ether resulted in a derivative which could be analyzed by electron capture GC/MS. The retention time, expressed as carbon number equivalent, of the major component obtained following reduction and derivatization was 25.45 (Table VI). The mass spectrum (negative ion electron capture) of this metabolite (Fig. 4*B*) showed abundant ion at *m/z* 553, corresponding to the carboxylate anion. The retention time and mass spectral behavior was identical with that obtained from 1,16-bis(pentafluorobenzyl)-5-trimethylsiloxyhexadecanedioate obtained from reduction of synthetic 16-COOH-LTE_3 (15). Due to losses suffered during each purification step, the amount of this metabolite obtained from human urine for mass spectral studies was less than 40 ng, and the less abundant ions in the authentic mass spectrum of this derivative could not be seen above the instrument noise level as shown in Fig. 4*B*. However, the RP-HPLC retention time of metabolite H36 in three different HPLC systems does not correspond to the retention time of the synthetic 16-COOH-LTE_3, but the UV data indicated that a thioether sulfur atom was still allylic to a conjugated tetraene as the chromophore. These observations were consistent with identification of metabolite H36 as 16-carboxy-17,18,19,20-tetranor-Δ^{13}-LTE_4. The exact stereochemistry of this metabolite has not been investigated due to the limited quantities available for further study. The geometry of double bond at C13-14 in particular is not known; however, it is assumed that the stereochemistry of the remaining portion of the molecule has not been altered from that found in LTE_4.

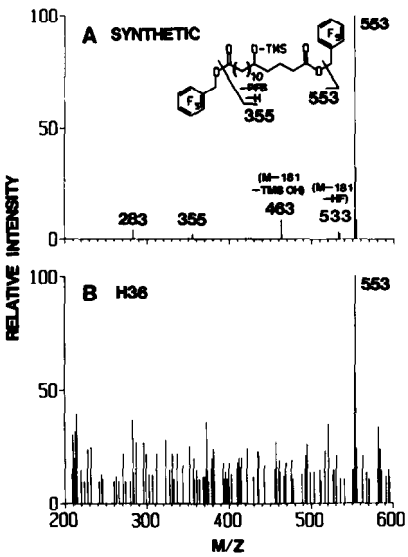

FIG. 4. **Negative ion electron capture mass spectrum of the PFB, trimethylsilyl derivatives obtained from 5-hydroxy-1,16-hexadecanedioic acid obtained by catalytic desulfurization starting from 500 ng of synthetic 16-COOH-LTE_3 (*A*) and metabolite H36 after catalytic desulfurization starting from a maximum of 20 ng of HPLC-purified human urinary metabolite (*B*).**

TABLE VI

Gas chromatographic equivalent

	Carbon number values for standards and metabolites[a]
5-Hydroxy-1,16-hexadecanedioic acid	25.45
Metabolite H36 (reduced)	25.45
Metabolite H31 (reduced)	23.45

[a] As the pentafluorobenzyl ester, trimethylsilyl ethers; capillary column, DB-1 (10 m × 0.25 mm inside diameter, 0.2-μm film thickness).

Metabolite H31—The least lipophilic major metabolite had a UV spectrum typical of a sulfidopeptide leukotriene containing a thioether bond allylic to the conjugated triene with UV maximum at 277 nm and shoulders at 267 and 290. Desulfurization of this metabolite using rhodium on alumina and subsequent conversion to the pentafluorobenzyl ester trimethylsilyl ether derivative yielded a compound analyzed by electron capture gas chromatography/mass spectrometry. The retention time of this derivative (expressed as carbon number equivalents) was 23.45 (Table VI), exactly 2 carbon eq lower than the retention time of standard 1,16-

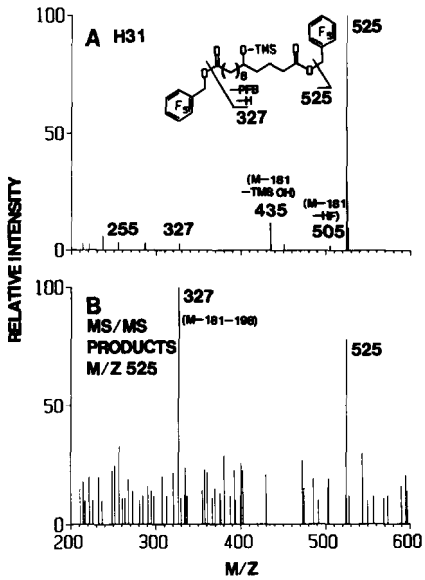

FIG. 5. **Negative ion electron capture mass spectrum of the PFB, trimethylsilyl derivative obtained from metabolite H31 after catalytic desulfurization starting from 100 ng of HPLC-purified human urinary metabolite (*A*) and collisional activated decomposition of *m/z* 525 obtained in spectrum 5A by tandem MS/MS (*B*).**

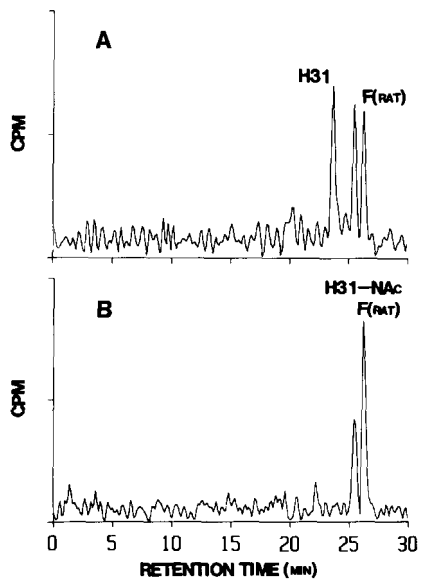

FIG. 6. **Reverse-phase HPLC (system III) separation of metabolite H31 and metabolite F (14-carboxy-15,16,17,18,19,20-hexanor-*N*-acetyl-LTE_3 from rat hepatocytes incubated with [^{14}C]LTE_4) (*A*) and metabolite H31 (after *N*-acetylation carried out as described under "Experimental Procedures") and metabolite F.**

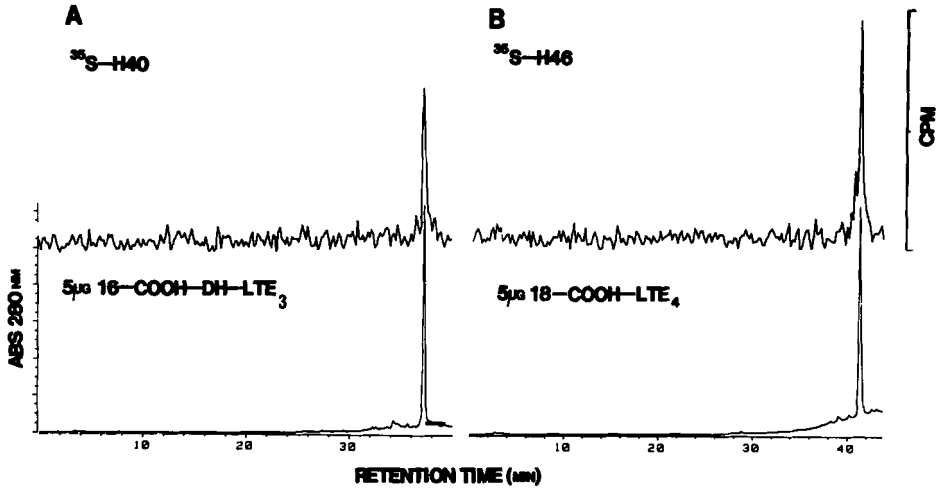

FIG. 7. **Comparison of retention behavior of metabolite H40 and 5 μg of synthetic 16-carboxy-17,18,19,20-tetranor-LTE$_3$ (*A*) and metabolite H46 and 5 μg of synthetic 18-carboxy-19,20-dinor-LTE$_4$ under certain conditions of reverse phase-HPLC (system IV) (*B*).**

bis(pentafluorobenzyl)-5-trimethylsilyloxyhexadecanedioate obtained from reduction of synthetic 16-COOH-LTE$_3$. The mass spectrum of this metabolite is shown in Fig. 5*A* and presents abundant ions at *m/z* 525 (M − 181), corresponding to the carboxylate anion of a monohydroxytetradecandioic acid derivative. The ions at *m/z* 435, loss of trimethylsilanol from M − 181, further support the presence of a monohydroxy group derivatized as the trimethylsilyl ether. Collision-induced decomposition of the ion at *m/z* 525 in a tandem quadrupole mass spectrometer yielded a unique ion at *m/z* 327 corresponding to the loss of pentafluorobenzyl alcohol indicating the presence of a second PFB-derivatized carboxyl (Fig. 5*B*). This M − 198 ion is the unique ion also observed in the CAD spectra of authentic, derivatized 5-hydroxyhexadecandioic acid. As shown in Fig. 6, co-injection of metabolite H31 with rat metabolite F (14-carboxy-*N*-acetyl-LTE$_3$) resulted in clearly separable components between the rat hepatocyte metabolite and the human urinary metabolite, even though the rat metabolite F consisted of two components in this HPLC system. These two components were most likely the 11-*trans*-14-COOH-*N*-acetyl-LTE$_3$ and 14-COOH-*N*-acetyl-LTE$_3$ derivatives, respectively, as indicated by the shift to lower wavelengths of the UV spectrum of the former compound. When the human metabolite H31 was acetylated using conditions to acetylate only free amino groups, the *N*-acetylated human metabolite then co-eluted with the rat metabolite 14-carboxyl-*N*-acetyl-LTE$_3$. Thus, metabolite H31 was consistent with the structure 14-carboxy-15,16,17,18,19,20-hexanor-LTE$_3$. The exact stereochemistry of this metabolite has not been investigated, but it is assumed to be unaltered from that found in LTE$_4$.

Metabolite H58 and Minor Metabolites—The ultraviolet spectrum of metabolite H58 revealed the typical sulfidopeptide leukotriene spectrum with absorption maximum at 280 nm and shoulders at 270 and 292. Furthermore, this metabolite co-eluted with standard *N*-acetyl-LTE$_4$ in two HPLC systems involving methanol/water and acetonitrile/water as mobile phases (system I and system III).

Insufficient material was available to obtain ultraviolet spectra for metabolites H40, H46, or H50. However, each of these metabolites as measured by radioactivity co-eluted with synthetic standards as shown in Fig. 7. These metabolites corresponded to 16-COOH-LTE$_3$ (H40), 18-COOH-LTE$_4$ (H45), and 20-COOH-LTE$_4$ (H50) (data not shown).

DISCUSSION

Following discovery of the sulfidopeptide leukotrienes over a decade ago, there has been a considerable amount of interest concerning the biological role that these potent metabolites of arachidonic acid may play in human physiology and pathophysiology. A primary role for these molecules as lipid mediators requires mechanisms for inactivation and elimination from the organism. LTC$_4$ is known to be rapidly converted into LTD$_4$ and LTE$_4$ by various peptidases present in cells as well as tissues including blood. Furthermore, all sulfidopeptide leukotrienes were shown to have energy-dependent uptake mechanisms in the liver (21). Previous human studies using tritium-labeled LTE$_4$ revealed rapid elimination from the blood and appearance of significant radioactivity in urine. Of interest is the observation reported in this study of the importance of the position and type of radiolabeled tracer employed in determination of the exact amount of radiolabeled metabolites appearing in urine. Sulfur 35-labeled LTE$_4$ was recovered in the highest amounts in urine as opposed to LTE$_4$ labeled with two tritium atoms attached to the 14,15-double bond that resulted in only 10% recovery of radioactivity in the urine. These data suggest that a major metabolite pathway for LTE$_4$ results in removal of protons at carbons 14 and 15 of the original arachidonic acid backbone.

The results from this study also support the observation that LTE$_4$ is rapidly removed from blood and eliminated via urine as an intact molecule within the first 2 h (16, 17, 22). Subsequent to that, however, LTE$_4$ is a minor component and other metabolites of LTE$_4$ predominate. When using ^{14}C- and ^{35}S-labeled LTE$_4$, it became evident that these more polar and less lipophilic metabolites are excreted in amounts that exceed the elimination of unmetabolized LTE$_4$. This would suggest that they may play an important role in assessing *in vivo* sulfidopeptide leukotriene biosynthesis.

In studies with isolated rat hepatocytes, LTE$_4$ was previously shown to be initially metabolized into *N*-acetyl-LTE$_4$ which was a substrate for ω-oxidation followed by a series of sequential β-oxidation transformations (14). Major metabolites formed include the 16-COOH-*N*-acetyl-LTE$_3$ which resulted following the action of 2,4-dienoyl-CoA reductase (23) on the 18-COOH-*N*-acetyl-LTE$_4$ CoA ester intermediate in the β-oxidation cycle. Just as β-oxidation is recognized to be a major route of metabolism of prostaglandins from the carboxyl terminus (24), the major route of metabolism of the sulfidopeptide leukotrienes involves β-oxidation from the methyl terminus following an initial ω-oxidation. Structures of the human metabolites identified by ultraviolet spectroscopy and mass spectrometry are shown in Fig. 8. These structures suggest that a major pathway for LTE$_4$ metabolism in humans involves an initial ω-oxidation to 20-hydroxy-LTE$_4$

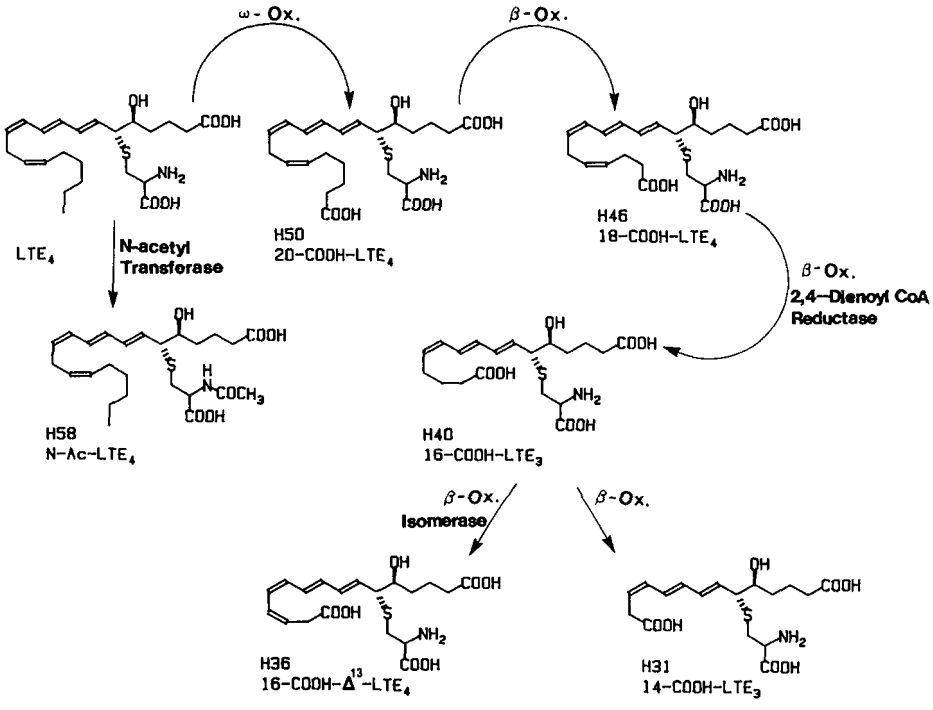

FIG. 8. **Proposed pathway for LTE_4 metabolism in human subjects.** The exact stereochemistry of the new structurally characterized metabolites has not been established (see text).

followed by oxidation to 20-carboxy-LTE_4. A small amount of *N*-acetyl-LTE_4 was observed in the urine of all volunteers; however, none of the subsequent ω/β-oxidized metabolites involve the *N*-acetyl group. This is consistent with the proposed pathway for the metabolism of LTE_4 in monkeys in which the 20-hydroxy- and 20-COOH-LTE_4 metabolites were observed. In human subjects, continued metabolism via β-oxidation of 20-carboxyl-LTE_4 leads to the accumulation of two metabolites, the 16-COOH-Δ^{13}-LTE_4 (H36) and the 14-COOH-LTE_3 (H31). Minor amounts of the 16-COOH-LTE_3 were also observed suggesting the importance of 2,4-dienoyl-CoA reductase in human metabolism of LTE_4. While not indicated in Fig. 8, which shows the identified structures, these reactions proceed as the CoA esters of the carboxyl group starting at the C20 terminus.

The accumulation of 14-carboxy-LTE_3 suggests a limiting step in further β-oxidation of this metabolite which places the conjugated triene now two carbons removed from the CoA ester moiety in the β-oxidation complex. Further steps of β-oxidation would involve some stage of reduction of the triene in order to continue the cleavage of two carbon fragments through a thiolase-mediated reaction. The biochemical steps involved in such reactions are poorly understood at the present time. A similar situation exists for the further metabolism of the 16-carboxyl-tetraene metabolite which would now place a conjugated tetraene two carbons removed from the CoA ester. Interestingly, the formation of the tetraene metabolite was not observed when [^{3}H]LTE_4 was used as the radiolabeled tracer in this study or in previous human studies (16, 17). As noted in the metabolism of LTE_4 in the isolated rat hepatocyte (14), the specific activity of the tetraene metabolite (metabolite E) was substantially reduced over the starting material suggesting that exchange of the protons on carbons 14 and 15 occurred during the mechanism of formation of this tetraene metabolite in the rat. As previously suggested, this might involve oxidation of the 16-COOH-LTE_3 metabolite (as the CoA ester) into the Δ-2,3 unsaturated intermediate, for example, catabolized by peroxisomal acyl-CoA oxidase (25) followed by the action of *cis,trans*-3,2-enoyl-CoA isomerase working reversibly. This mechanism would account for exchange of these exact protons with water and thereby loss of the tritium label from [14,15^{3}H]LTE_4 in human metabolism studies.

The appearance of radiolabeled LTE_4 metabolites in the three subjects investigated was remarkably similar in terms of the abundance of each of the reported major metabolites. However, it should be noted that all three subjects were healthy males of similar age. It is not known whether any of these subjects were slow or fast acetylators (26) and whether acetylation phenotype plays any significant role in the appearance of *N*-acetyl-LTE_4 and related metabolites.

The absolute amount of the LTE_4 metabolites eliminated in urine is similar to that observed for the major metabolites of prostaglandins which are routinely measured as an index of prostanoid production *in vivo* (27). For example, the major metabolites of thromboxane, either 2,3-dinor-TxB_2 or 11-dihydro-TxB_2 represent 6–7% each of the fractional conversion of exogenously administered low doses of TxB_2 (28). The H31 and H36 metabolites of LTE_4 reported in this study appear in urine in similar concentrations to these prostanoid metabolites.

Nevertheless, measurement of the β-oxidation metabolites of LTE_4 would eliminate potential problems associated with kidney LTE_4 production and elimination. For example, measurement of TxB_2 and prostaglandin E_2 in urine reflects kidney production of these metabolites rather than whole body production of these prostaglandins. We suggest that measurement of the major LTE_4 metabolites structurally characterized here might better reflect total body production of sulfidopeptide leukotrienes.

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