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Note

Facile preparation of leukotrienes C_4, D_4 and E_4 containing carbon-13 and nitrogen-15 for quantification of cysteinyl leukotrienes by mass spectrometry

FARIDEH GHOMASHCHI[1], JAMES G. BOLLINGER[1] and MICHAEL H. GELB[1,2,*]

[1]Department of Chemistry, Campus Box 351700, University of Washington, Seattle, WA 98195, USA
[2]Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

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Abstract: With the recent ability to use combined liquid chromatography/electrospray tandem mass spectrometry to analyze for several eicosanoids in biological samples in a single and rapid experiment, heavy isotope-labeled eicosanoids are needed as internal standards in order to quantify eicosanoid analytes. The present study describes a practical preparation of cysteinyl leukotrienes (leukotriene C_4, D_4 and E_4) with three ^{13}C atoms and one ^{15}N atom in the cysteinyl residue. The method involves solid-phase peptide synthesis to make glutathione with heavy isotopes in the cysteinyl residue and reaction of this tripeptide with commercially available leukotriene A_4 methyl ester to give labeled leukotriene C_4 methyl ester, which is hydrolyzed to labeled leukotriene C_4. Labeled leukotriene E_4 is prepared in the same way with the use of labeled cysteine. Labeled leukotriene D_4 is prepared by treatment of labeled leukotriene C_4 with commercially available γ-glutamyl transpeptidase. Copyright © 2007 John Wiley & Sons, Ltd.

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Introduction

The cysteinyl leukotrienes (leukotrienes C_4, D_4 and E_4) are well-known eicosanoids (derived from arachidonic acid) that bind to G protein-coupled receptors and mediate a number of proinflammatory events, especially in the airways.[1] The quantification of cysteinyl leukotrienes is often carried out in studies of inflammation in experimental animals and in humans. This is typically done by some form of immunoassay, such as radioimmunoassay using commercially available kits. However, recent developments using combined liquid chromatography/electrospray ionization tandem mass spectrometry allow a large set of eicosanoids (leukotrienes, prostaglandins and other lipid mediators) to be quantified in a single multiplex analysis.[2,3] Since different molecular species ionize in the mass spectrometer source with different efficiencies, accurate quantification can only be carried out by inclusion of a chemically identical, but isotopically distinguished, internal standard in the sample. Although heavy atom-substituted prostaglandins and leukotriene B_4 are commercially available, there are no commercial sources of labeled cysteinyl leukotrienes. The latter are typically quantified by using a single internal standard, deuterated leukotriene B_4, and determining, by separate analysis, the relative ionization efficiencies of the cysteinyl leukotriene relative to that for leukotriene B_4. However, the addition of heavy atom-substituted internal standard for each molecular species at the earliest step possible to the biological sample is superior since it accounts for any loss of analyte due to absorption to container walls, enzymatic or non-enzymatic degradation of analyte and other factors. For example, cysteinyl leukotrienes are readily degraded by hydrolases present on the surface of inflammatory cells and are known to stick to metal surfaces, such as the injection needle of the autoinjector at the front end of the liquid chromatograph.

The synthesis of cysteinyl leukotrienes containing deuterium in the fatty acid portion has been reported (for example, see Prakash *et al.*[4]), but this requires a

*Correspondence to: Michael H. Gelb, Department of Chemistry, Campus Box 351700, University of Washington, Seattle, WA 98195, USA. E-mail: gelb@chem.washington.edu
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fairly involved synthesis of deuterated leukotriene A_4 methyl ester, which can then be reacted with cysteine, cysteinyl-glycine or glutathione to form deuterated leukotriene E_4, D_4 and C_4, respectively. In this report, we describe the facile synthesis of cysteinyl leukotrienes containing carbon-13 and nitrogen-15 in the cysteine residue. These are readily made by preparing the labeled cysteine-containing materials, which can be reacted with commercially available leukotriene A_4 methyl ester to form the desired labeled cysteinyl leukotrienes.

Results and discussion

The preparation of isotopically substituted leukotriene C_4 with heavy isotope in the cysteinyl portion requires that labeled glutathione be prepared. This was carried out using standard FMOC-based solid-phase peptide synthesis. Labeled and protected cysteine ([1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]*N*-FMOC-Cys(*S*-trityl from Cambridge Isotopes, Inc.)) was used because it is common to all of the cysteinyl leukotrienes. During this process, the cysteine residue is protected with a trityl group on its sulfhydryl. The cleavage of the peptide from the resin and side-chain deprotection is best carried out in the presence of scavengers, such as thioanisole and 1,2-dithioethane, to prevent capture of the trityl cation by the cysteine sulfhydryl and other peptide nucleophiles. It is important that 1,2-dithioethane be completely removed since it can react with leukotriene A_4 methyl ester during the subsequent step. This was accomplished by trapping the glutathione on a strong cation exchange column (Dowex 50 in the H^+ form) and washing with water to elute the 1,2-dithioethane in the void volume. The glutathione is then eluted by washing with aqueous NH_4OH. The glutathione at this point still contains protecting group fragments and is purified by high-performance liquid chromatography (HPLC) on a reverse-phase (C18) column. Glutathione elutes in the void volume, and the more hydrophobic protecting group fragments are retained on the column. HPLC purification is preferred over the use of a solid-phase extraction cartridge since some of the impurities elute just after the void volume peak. The reaction of labeled glutathione with commercially available leukotriene A_4 methyl ester provides labeled leukotriene C_4 methyl ester, which is converted to labeled leukotriene C_4 by hydrolysis under alkaline conditions (Figure 1).

Using the same strategy, leukotriene D_4 synthesis requires labeled cysteinyl-glycine, but this was not the preferred procedure because of three reasons: (1) we found that labeled leukotriene C_4 could be completely converted to labeled leukotriene D_4 by incubation with commercially available transpeptidase in the presence of a mixture of amino acids as glutamyl acceptors (Figure 1). It is more efficient and faster to carry out a single solid-phase peptide synthesis of glutathione rather than having to cleave two portions of resin and carry out two peptide purifications to also obtain labeled cysteinyl-glycine; (2) yields for the solid-phase synthesis of cysteinyl-glycine were very low (<5%), the major product being the dipeptide-derived diketopiperazine. Diketopiperazine formation occurs on occasion during solid-phase synthesis of dipeptides, probably as a result of intramolecular attack of the dipeptide amino terminus onto the carbonyl carbon of the ester involving the C-terminal amino acid and the resin-bound leaving group. This cyclization can occur either during removal of the FMOC group from the *N*-terminal amino acid or during the subsequent step of side-chain deprotection combined with cleavage of the peptide from the resin under acidic conditions; (3) we obtained very low yields of leukotriene D_4 methyl ester (<1–2%) for the reaction of leukotriene A_4 methyl ester with cysteinyl-glycine. The major products were leukotriene B_4 methyl ester (resulting from hydrolysis of leukotriene A_4 methyl ester) and the disulfide derived from cysteinyl-glycine. It seems that disulfide formation from this dipeptide occurs more readily than disulfide formation from glutathione or from cysteine during the synthesis of leukotriene C_4 methyl ester and leukotriene E_4 methyl ester, respectively.

Leukotriene E_4 was prepared via reaction of leukotriene A_4 methyl ester with commercially available labeled cysteine followed by alkaline hydrolysis of the methyl ester. The labeled cysteine could also be obtained by deprotection of the same protected cysteine that was used in the solid-phase synthesis of the labeled glutathione. However, the use of commercially available cysteine is more practical as it avoids the steps needed to remove protecting group fragments from the cysteine that form during deprotection of *N*-FMOC-cysteine(*S*-trityl).

All three cysteinyl leukotrienes were purified by HPLC on a C18 reverse-phase column. This is an important step so that leukotriene B_4 is removed. Since leukotriene B_4 is not labeled with stable isotopes, it must be removed from the labeled cysteine leukotrienes to avoid contamination of biological samples (in cases where sample-derived leukotriene B_4 is being quantified). Leukotriene B_4 methyl ester forms in small amounts by hydrolysis of leukotriene A_4 methyl ester during the reaction of the latter with the thiol component.

All three labeled cysteinyl leukotrienes gave the expected peak for the $(M\text{-}H^+)^-$ ion in negative-ion electrospray mass spectrometry (see Experimental). For all three cysteinyl leukotrienes, the peak at $(M\text{-}H^+)^-$

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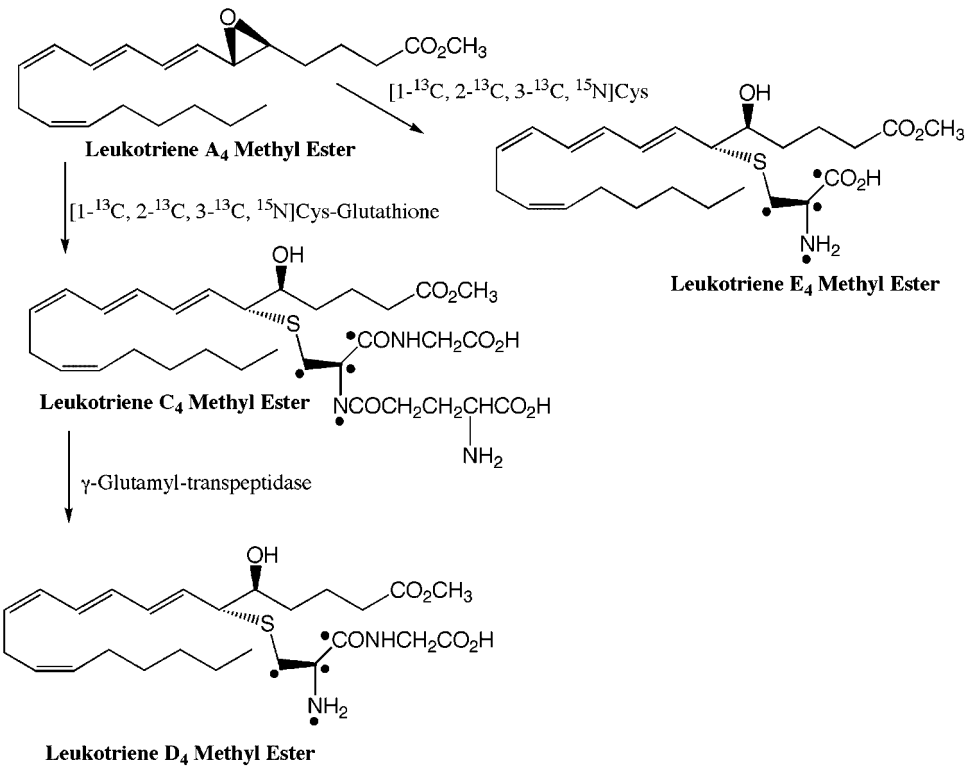

Figure 1 Synthesis of labeled cysteinyl leukotriene methyl esters. The black dots indicate the location of the ^{13}C and ^{15}N isotopes. Saponification leads to the labeled cysteinyl leukotrienes (not shown).

plus 1 was 17% as intense as the $(M\text{-}H^+)^-$, and no peaks were observed at $(M\text{-}H^+)^-$ plus 2 indicating no cross-contamination of the labeled cysteine leukotrienes into the mass channel for the non-labeled species. Thus, these labeled cysteinyl leukotrienes are appropriate for use as internal standards to quantify the amount of non-labeled cysteinyl leukotrienes in biological samples (see Henderrson *et al.*,[3] for an example). Major fragment ions for all three labeled cysteinyl leukotrienes include loss of H_2O and the lipid fragment due to cleavage of the Cβ–S bond of the cysteinyl residue.

Experimental

Synthesis of [1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]Cys-Glutathione

All steps with peptide synthesis resin were carried out at room temperature in a 5-ml glass vial with a Teflon septum-lined screw cap. To wash the resin, the vial was centrifuged at ~800 rpm for ~30 s in a clinical centrifuge, and most of the supernatant was removed with a Pasteur pipet, being careful not to take resin beads. DMF is anhydrous grade (Aldrich). FMOC-Gly-Wang Resin (Novabiochem Inc., 0.78 mmol/g) (0.5 g) was washed 4 times with 10 ml portions of DMF. Piperidine (20% by vol.) in DMF (10 ml) was added to the resin, and the vial was swirled briefly. After 1 min, the liquid was removed, and the resin was treated with a second portion of piperidine (20%) in DMF for 30 min with mixing on a rotating wheel. The resin was washed 4 times with 10 ml portions of DMF and then dried in a vacuum desiccator.

Anhydrous HOBt (Novabiochem Inc., 0.27 g, 2 mmol) and TBTU (Novabiochem Inc., 0.64 g, 2 mmol) were dissolved in 5 ml DMF by stirring. The solution was stored in a capped vial for up to 2 weeks at room temperature. Gly-Wang resin (72 mg, 56 μmol Gly) was placed in vial. The vial was charged with 50 mg of [1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]*N*-FMOC-Cys(S-trityl) (Cambridge Isotopes, Inc.), followed by 0.21 ml of HOBt/TBTU/DMF solution, followed by 29 μl of diisopropylethylamine (Aldrich) and finally followed by 0.4 ml DMF. The capped vial was mixed on a rotating wheel overnight (~12 h) at room temperature. The resin was washed 6 times with 4 ml portions of DMF. The resin was treated with 20% of piperidine in DMF as above. The resin was washed 6 times with 4 ml portions of DMF and then 3 times with 4 ml portions of ether. The resin was dried in a vacuum desiccator.

A vial containing dried Cys-Gly-Wang resin (37 mg, 29 μmol peptide) (see above) was charged with

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N-FMOC-Glu, with its α-COOH group protected as its *t*-butyl ester (Bachem, Inc.) (49 mg, 116 μmol), 0.29 ml of HOBt/TBTU/DMF solution (116 μmol) (see above) and 40 μl of diisopropylethylamine (232 μmol). The capped vial was mixed overnight at room temperature on a rotating wheel. The resin was washed with DMF, treated with piperidine/DMF and washed with DMF and ether as above. The resin was dried briefly in a vacuum desiccator and then treated with 1 ml of trifluoroacetic acid/thioanisole/1,2-ethanedithiol/water (85/5/5/5 by vol.) for 1 h at room temperature. The solution was filtered through a glass wool-plugged Pasteur pipet. Most of the liquid was removed with a stream of N_2 at room temperature (in a fume hood, stench). To the residue was added 1 ml water (not all solid dissolves), and the mixture was applied to a column of AG 50W-X8 strong cation-exchange resin (BioRad, 1.5 × 1.5 cm bed volume). The resin was previously washed with ~10 ml of 1 N NaOH, then with water until the eluant was at neutral pH (pH paper), then with ~10 ml of 1 N HCl, and finally with water until the eluant was at neutral pH. After loading the crude peptide, the column was washed with five portions of water (6 ml each). The column was washed with five portions of 6 N aqueous NH_4OH (6 ml each), collecting each wash into a separate glass tube. The six fractions were brought to dryness in a Speed-Vac concentrator (Savant Instruments, Inc.). Negative-ion electrospray ionization mass spectrometry analysis on a Bruker Esquire ion trap instrument showed that the glutathione peptide was present in fractions 1 and 2 (mainly as the reduced form, i.e. non-disulfide). The residue in fractions 1 and 2 was dissolved in 0.7 ml of water, and the solution was submitted to HPLC on a C18 column (Vydac 218TP1010, 1 × 25 cm) that was previously equilibrated with water containing 0.05% trifluoroacetic acid. The eluant was monitored with a UV detector set to 214 nm. The desired peptide eluted in the void volume, with the first impurity UV peak appearing just after the void volume peak. The void volume fraction was brought to dryness in a Speed-Vac.

[1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]Cys-Leukotriene C_4

The labeled glutathione was dissolved in 1 ml of water. The concentration of free SH was determined by standard Ellman assay with 5,5′-dithio-bis(2-nitrobenzoic acid) using a 2 μl aliquot of the glutathione solution and standard cysteine solution for calibration. An aliquot of the stock solution of labeled glutathione (0.5 μmol) was transferred to a small glass vial and the solution was brought to dryness in a Speed-Vac. To the vial, 20 μl of methanol/water (6/1, v/v) was added. The pH was adjusted to 9–10 by the addition of 0.5 μl aliquots of triethylamine with spotting of a small solution aliquot onto moistened pH paper. Into a second vial, 50 μg of leukotriene A_4 methyl ester (solution in hexane/1% triethylamine, Cayman Chemicals) was added. The solvent was removed with a stream of N_2. To this vial, the solution of glutathione was immediately added. The capped vial was kept at room temperature for 4 h in the dark. Then 0.5 ml of 0.1 M K_2CO_3 in water was added, and the capped vial was kept at room temperature for 3 h in the dark. The mixture was brought to pH ~5 (pH paper) with small aliquots of 96% formic acid. The solution was applied to a C18 solid-phase extraction cartridge (50 mg solid phase, Waters Inc., which was pre-conditioned by washing with 1 ml of methanol/0.1% formic acid and then with two portions of water/0.1% formic acid, 1 ml each). After application of the reaction mixture and centrifugation, the cartridge was washed with two portions of water/0.1% formic acid (1 ml each) (the solution was first delivered to the empty reaction vial and then transferred to the cartridge), and the product was eluted into a clean receiving tube using two 1 ml portions of methanol/0.1% formic acid (again rinsing the empty reaction vial prior to transfer to the cartridge). The eluant was concentrated to dryness in a Speed-Vac. The residue was dissolved in 100 μl of methanol and submitted to purification by HPLC (two injections of 50 μl each). The column (Zorbax SB-C18, 2.1 × 100 mm, 3.5 μ, Agilent Inc.) was equilibrated with 20% solvent B (acetonitrile with 0.1% formic acid) and 80% solvent A (water, Milli-Q, Millipore, Inc.). The solvent program was 0–10 min, 20% B, 10–15 min 38% B, 15–25 min 65% B, 25–35 min 100% B. The absorbance was monitored at 280 nm. The product eluted at ~22 min. The product solution was concentrated to dryness in a Speed-Vac, and the residue was dissolved in ethanol (prior to use, the ethanol was subjected to several cycles of vacuum degassing, N_2 purging). The solution was stored in aliquots in flame-sealed ampules or Teflon-septum capped glass vials under N_2 at −80°C. The yield is 15–20%. The structure was confirmed based on HPLC retention time identical to leukotriene C_4 authentic standard (Cayman Chemicals). Negative-ion electrospray ionization mass spectrometry gave the expected value for $(M\text{-}H^+)^- = 628.5$ and a collisional-induced dissociation spectrum identical to that of the authentic standard.

The concentration of labeled leukotriene C_4 was measured by spiking a known volume of stock solution with a known amount of non-labeled leukotriene C_4 (Caychem Chemicals, Inc.) and measuring the relative parent ion signals by negative-ion electrospray mass spectrometry.

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[1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]Cys-Leukotriene D_4

The conversion of leukotriene C_4 to leukotriene D_4 was carried out enzymatically, essentially as described in Anderson *et al.*[5] The enzyme is γ-glutamyl transpeptidase (Sigma Cat. G-9270, 11 units/mg powder). The enzyme stock is prepared by dissolving 0.8 mg of powder in 1 ml of purified water (Milli-Q). The reaction buffer contains 40 mM sodium phosphate, pH 7.4, 0.5 mM L-alanine, 0.12 mM L-arginine, 0.25 mM L-asparagine, 0.02 mM L-aspartic acid, 0.04 mM L-cystine, 0.04 mM L-glutamate, 0.4 mM L-glutamine, 0.08 mM L-histidine, 0.06 mM L-isoleucine, 0.15 mM L-leucine, 0.2 mM L-lysine, 0.04 mM L-methionine, 0.13 mM L-phenylalanine, 0.19 mM L-serine, 0.2 mM L-threonine, 0.05 mM L-tryptophan, 0.08 mM L-tyrosine, 0.2 mM L-valine, and 0.4 mM L-glycine. Glutamine is added immediately before the addition of enzyme. The reaction mixtures contain 5 μg of [1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]Cys-Leukotriene C_4, 214 μl of reaction buffer and 11 μl of enzyme stock solution. The mixture is incubated at 37°C for 30 min and then stored at −20°C prior to purification. The sample is centrifuged at $\sim 12\,000 \times g$ for 1 min to remove any solid (none observed) prior to injection of half of the mixture onto a reverse-phase C18 HPLC column (Zorbax SB-C18, 2.1 × 100 mm, 3.5 μm, Agilent Cat. 861753-902) previously equilibrated with 80% solvent A (water, Milli-Q)/20% solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 1.5 ml/min. The absorbance at 280 nm is monitored. The solvent program is: 0–10 min, 20% B; 10–15 min, 20–38% B; 15–25 min, 38–65% B; 25–35 min, 65–100% B. Leukotriene D_4 elutes at about 28 min. The yield is 70%. The reaction was also carried out on a 2-fold larger scale (10 μg of labeled leukotriene C_4) with purification on a semi-prep C18 HPLC column (Vydac 218TP1010, 0–10 min, 20% B; 10–15 min, 20–38% B; 15–35 min, 38–65% B; 35–55 min, 65–100% B at a flow rate of 1.5 ml/min) and with a yield of 80%. The structure was confirmed based on HPLC retention time identical to leukotriene D_4 authentic standard (Cayman Chemicals). Negative-ion electrospray ionization mass spectrometry gave the expected value for $(M\text{-}H^+)^- = 499.3$ and a collisional-induced dissociation spectrum identical to that of the authentic standard.

[1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]Cys-Leukotriene E_4

This was prepared, stored and quantified as for leukotriene C_4 using labeled cysteine ([1-^{13}C, 2-^{13}C, 3-^{13}C, ^{15}N]Cys, Cambridge Isotopes, Inc.). The yield is 40–50%. The structure was confirmed based on HPLC retention time identical to leukotriene E_4 authentic standard (Cayman Chemicals). Negative-ion electrospray ionization mass spectrometry gave the expected value for $(M\text{-}H^+)^- = 442.2$ and a collisional-induced dissociation spectrum identical to that of the authentic standard.

Conclusions

Using commercially available reagents, heavy isotope-labeled cysteinyl leukotrienes (leukotriene C_4, D_4 and E_4) are readily prepared in amounts sufficient for thousands of liquid chromatography/electrospray ionization mass spectrometry quantitative analyses of leukotrienes in biological samples. The use of chemically identically but isotopically differentiated cysteinyl leukotrienes as internal standards provides the most accurate way to quantify these eicosanoids. The use of internal standards corrects for any loss of analytes due to sample processing or adsorption to surfaces of the liquid chromatography/mass spectrometry instrument.

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