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THE PREPARATION OF A ^{11}C-LABELLED 5-LIPOXYGENASE PRODUCT. 5(S)-HYDROXY-6(R)-(N-[1-^{11}C]ACETYL)CYSTEINYL-7,9-TRANS-11,14-CIS-EICOSATETRAENOIC ACID.

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SUMMARY

5(S)-Hydroxy-6(R)-(N-[1-^{11}C]acetyl)cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid (N-[1-^{11}C]acetyl leukotriene E_4) was prepared by the reaction of leukotriene E_4 and [1-^{11}C]acetyl chloride. The product was obtained with 1.3% yield, based on [1-^{11}C]acetyl chloride. The preparation required 50 min from the end of radioisotope production. The chemical and radiochemical purity of an injectable dose of N-[1-^{11}C]acetyl leukotriene E_4 was 95%. The remaining impurities were the 11-trans isomer primarily and the sulfoxides of 5(S)-hydroxy-6(R,S)-(N-[1-^{11}C]acetyl)cysteinyl-7,9-trans-11-(cis,trans)-14-cis-eicosatetraenoic acid. The stereochemistry of the sulfoxides at C-6 and at the C-11 double bond respectively was not identified. The average specific activity of the carrier added preparation was 2 GBq/μmol at the time of application.

Key Words: ^{11}C-Labelling, ^{11}C-acetyl chloride, 5(S)-hydroxy-6(R)-(N-[1-^{11}C]acetyl)-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid, leukotrienes, PET.

INTRODUCTION

Leukotrienes are biologically potent metabolites of arachidonic acid metabolism, produced in the 5-lipoxygenase pathway in some mammalian cells [1-4]. The initial metabolite of the enzymatic oxidation of arachidonic acid at C-5 is 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid, reported as 5(S)-HPETE. Although this molecule is very unstable it is most important, leading to the first member of the leukotriene series which is LTA_4, a 5,6-trans epoxide of the characteristic conjugated trans, trans, cis triene fragment of the leukotrienes. The LTA_4 represents the synthetic precursor to the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4. It was first

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prepared by Corey et al. [5], but various syntheses of these natural compounds and a variety of synthetic derivatives thereof followed, e.g. by Rokach [6-8], which contributed rapidly to the large progress made in the biological evaluation of the leukotrienes as important mediators in disease states in man. Our interest was to the understanding of the metabolic pattern of cysteinyl leukotrienes and their routes of elimination *in vivo* [9-11].

The extremely low concentration of leukotriene metabolites *in vivo* made them difficult to quantify and much effort was spent to correlate metabolite levels, transport and elimination kinetics, and endogenous leukotriene biosynthesis which itself would reflect one of the components of pathogenesis. This work provides for the first time a positron emitting labelled leukotriene metabolite for the non invasive regional mapping of transport kinetics of these important biochemical mediators by positron emission tomography [12].

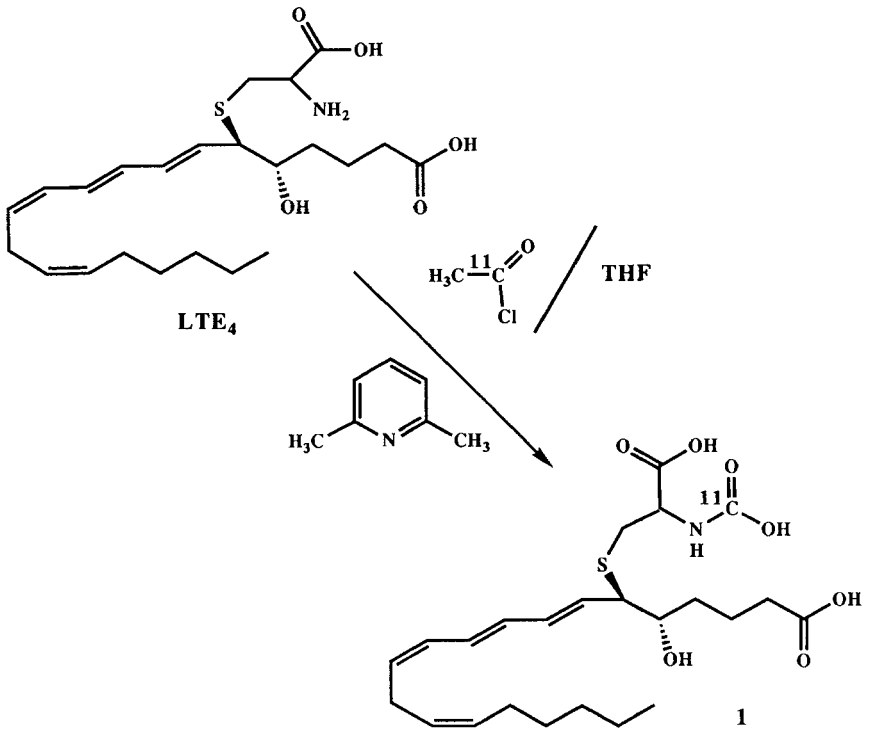


Figure 1: Preparation of 5(S)-hydroxy-6(R)-(N-[1-^{11}C]acetyl)cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid.

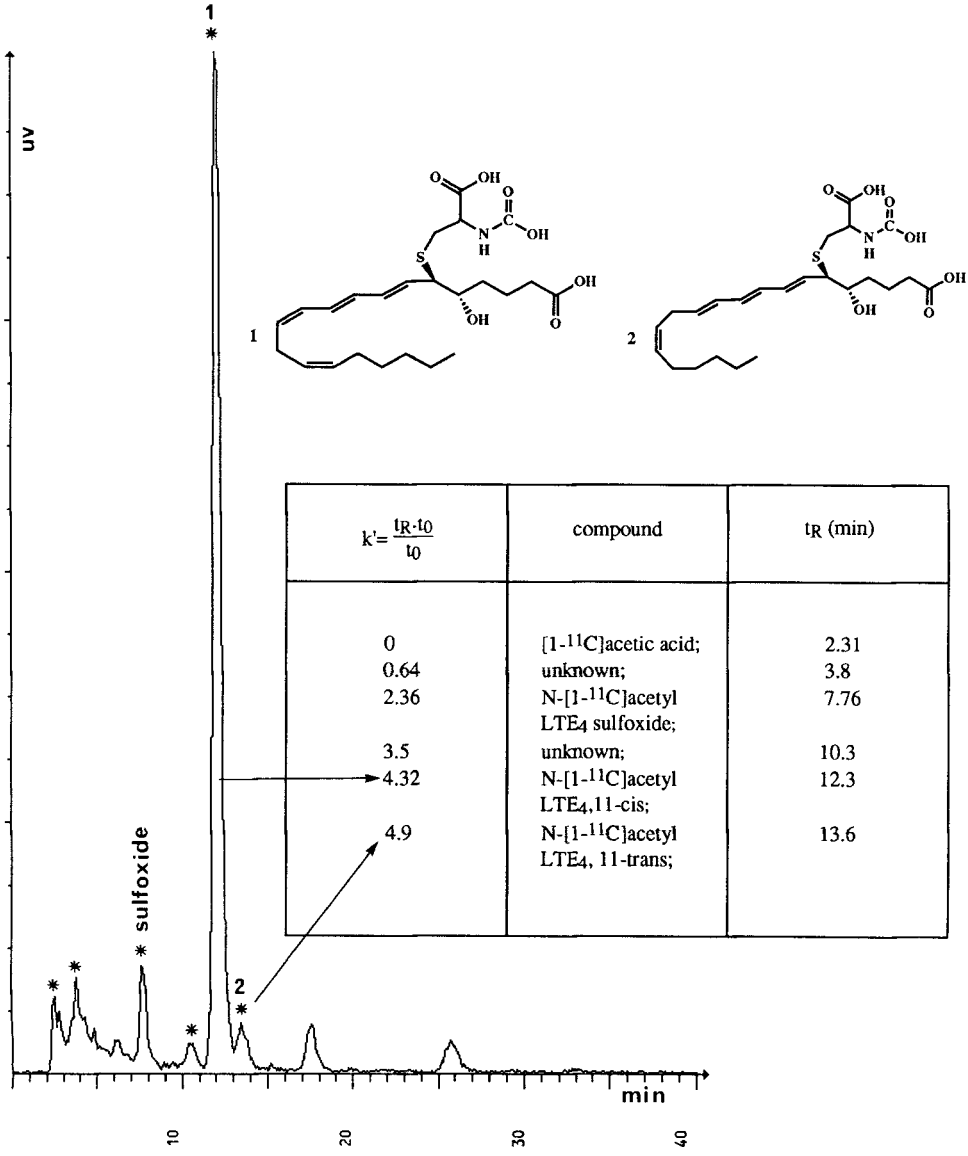
Our attempt has been directed towards 5(S)-hydroxy-6(R)-(N-[1-^{11}C]acetyl)cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid **1**. The metabolism of this compound is restricted to oxidation from the C-20 end of the fatty acid, and deacetylation of the N-acetyl cysteinyl residue (with loss of the label in **1**) does not occur *in vivo* [13]. Therefore we developed a procedure (Fig.1) to prepare **1** from LTE_4 and [1-^{11}C]acetyl chloride. This strategy of N-acetylation was successfully applied by LeBar et al. [14] in the synthesis of a carbon-11 labelled neurohormone and surprisingly proved to be practical with the sensitive LTE_4.

DISCUSSION

The N-acetylated LTE_4 was prepared previously to study the influence of polar substituents of leukotrienes on their biological activity [15]. This method conveniently proceeded through the reaction of excess acetic anhydride in methanol/water at 273 K. Introduction of the [1-^{11}C]acetyl moiety into the cysteinyl residue by that procedure would not be practical from the radiochemical point of view. Attempts using free [1-^{11}C]acetic acid together with suitable peptide coupling agents, e.g. the benzotriazolyl-N-oxy-tris(dimethyl amino)phosphonium hexafluorophosphate, BOP [16], interestingly yielded some labelled material, but the reaction conditions seemed extremely difficult to control with respect to leukotriene chemistry. As a result of these experiments we finally applied an approach using [1-^{11}C]acetyl chloride [17] without any moderation of its reactivity during the labelling procedure. From this reaction sufficient amounts of ^{11}C-labelled N-acetyl-LTE_4 (**1**) were obtained allowing animal studies [12].

The positive identification of 5(S)-hydroxy-6(R)-(N-[1-^{11}C]acetyl)-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid (**1**) was made by HPLC using carrier added ^{11}C preparations, ^{3}H-labelled LTE_4, the N-[1-^{14}C]acetyl-LTE_4, and defined chemical transformations of the product combined with UV spectroscopy.

The crude reaction mixture contained various labelled compounds as indicated within the peak identification table of the chromatogram in Fig.2. Not all of them were identified. Among the strong polar components of the mixture eluting within the first 7 min of chromatography only residual [1-^{11}C]acetic acid produced from unreacted [1-^{11}C]acetyl chloride was characterized.



$k' = \frac{t_R \cdot t_0}{t_0}$	compound	t_R (min)
0	[1-^{11}C]acetic acid;	2.31
0.64	unknown;	3.8
2.36	N-[1-^{11}C]acetyl LTE$_4$ sulfoxide;	7.76
3.5	unknown;	10.3
4.32	N-[1-^{11}C]acetyl LTE$_4$,11-cis;	12.3
4.9	N-[1-^{11}C]acetyl LTE$_4$, 11-trans;	13.6

Figure 2: Chromatography of the crude reaction mixture. Radioactivity was traced simultaneously with the 282 nm UV absorption (radioactive peaks are identified by an asterisk) and displayed into one window using the ADC board of a Canberra FLOW-ONE A250 radioactivity detection system connected to a Waters 490E multiwavelength detector. For the chromatographic conditions see experimental. Only the radioactive components are identified in the table.

The [1-^{11}C]acetic acid eluted with the dead volume and gave the t_0 value for determining the distribution factor k'. Typical leukotriene UV absorption spectra were observed at retention times of 3.8 min, 7.8 min, 10.3 min, 12.3 min, and 13.6 min. All of these products contributed to the radioactivity of the mixture, thus all of them should contain the [1-^{11}C]acetyl residue together with an intact triene structure fragment. The elution peaks at 17.8 min and at 26 min which are also shown, were not radioactive, but had the characteristic UV absorbance of leukotrienes. They were assigned to remaining unreacted LTE_4 (26 min, k' = 10.26) and its sulfoxide (17.8 min, k' = 6.7).

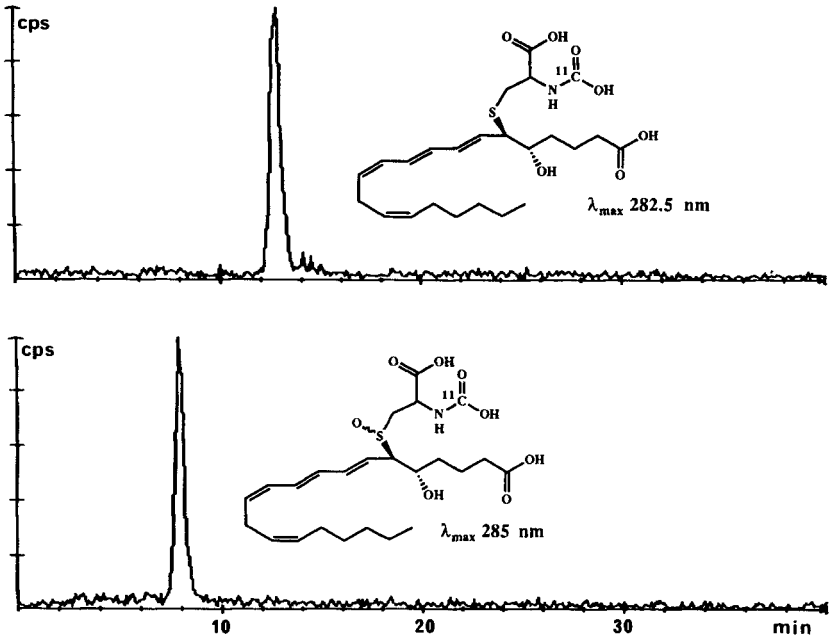


Figure 3: Chromatography of the periodate oxidation of **1.** **(a)** Isolated N-[1-^{11}C]acetyl-LTE_4 obtained after separation from the crude reaction mixture. **(b)** Sulfoxide of N-[1-^{11}C]acetyl-LTE_4 obtained after the periodate oxidation. Only the radioactive traces are shown in both chromatograms. An identical chromatographic pattern was obtained during UV detection, after loading both samples with $2x10^{-8}$ moles of non radioactive reference material. Good UV absorption spectra could be obtained from more concentrated elution peaks using a Merck L-3000 diode array detector. The corresponding λ_{max} are shown for both compounds.

The target compound **1** was identified by careful comparison with independently prepared ^{3}H- and ^{14}C- labelled reference material, which confirmed the chemical identity of **1** as 5(S)-hydroxy-6(R)-N-acetyl-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid. Final support for **1** was given by oxidation of its thioether functionality [18] using excess sodium periodate (about 25 equivalents, 338 K, 20 min) directly in the corresponding HPLC fraction (11 to 14 ml). The oxidation product was identical with the expected sulfoxide of N-[1-^{11}C]acetyl-LTE_4. It eluted at about 8 min, whereas **1** had disappeared quantitatively. The UV absorption maximum of the sulfoxide was shifted to lower energy, as expected (Fig 3).

Chemical [19] and enzymatic [20] deacetylation of the labelled compound **1** were effected for confirmation of the N-[1-^{11}C]acetyl moiety in the target molecule. Both procedures removed the [1-^{11}C]acetyl residue quantitatively. Since the enzymatic reaction cleaved exclusively the amidic C-N bond, this reaction excluded any O-acetylation in the isolated fraction and served as support for the correct position of the label in the final molecule. Isocratic aqueous rechromatography of the deacetylated mixture (after acidification) on a cationic exchange resin [21] showed carbon-11 labelled acetic acid as the only radioactive compound.

PET-scans with N-[1-^{11}C]acetyl-LTE_4 were performed over periods of 60 min in normal and in mutant rats with deficient transport of cysteinyl leukotrienes from the liver into bile. These results were quantitated in part and were reported elsewhere [12]. The observed elimination of leukotrienes by liver and kidney which could be traced *in vivo* using N-[1-^{11}C]acetyl-LTE_4 may provide an important novel approach to study various human diseases with impaired bile flow and reduced liver function.

EXPERIMENTAL

Reagents. Tetrahydrofuran (THF) and phthaloyl dichloride were from Merck, Darmstadt. These chemicals were distilled under argon immediately before use for rigorous exclusion of oxygen. THF was dried over sodium and checked for the absence of peroxides. Peroxide free media were essential to reduce the formation of sulfoxides with the starting LTE_4 and with the final product. Aliquots of a commercial 3 M solution of CH_3MgCl in THF (Aldrich) were used for trapping [^{11}C]CO_2 by a Grignard reaction.

Leukotriene E_4 was purchased from Amersham Braunschweig as a 0.125 mM solution in methanol/water/acetic acid (70/30/0.1) adjusted to pH 5.4 with ammonium hydroxide. Samples were assessed for purity and standardized by HPLC. LTE_4 was prepared for the labelling reaction by dispensing an amount of $5x10^{-8}$ moles into a conical polyethylene vial and subsequent freeze drying of the substrate. This dry product was flushed with argon and stored at 243 K until use. Immediately before the labelling procedure started, it was redissolved in 20 µl of freshly distilled peroxide free THF. An amount of 20 µl of a $5x10^{-3}$ M solution of 2,6 dimethyl pyridine in THF was added. The polyethylene vial was cut off and placed immediately into the screw capped final reaction vessel (vessel 3 of the apparatus of Fig. 4). It was kept tightly closed in the apparatus at 258 K. Authentic N-acetyl-LTE_4 and N-acetyl-[^{3}H]LTE_4 as reference materials were prepared according to published procedures [20, 15, 13].

The [^{11}C]CO_2 conveniently was obtained by the $^{14}N(p,\alpha)^{11}C$ nuclear reaction from a routine target system [22]. The target contained N_2 at 40 kg/cm^2 and was irradiated for 30 min with a 10 µA proton beam (incident energy 13 MeV). After irradiation the radioactive target gas was expanded via a remote controlled regulator system into a stainless steel capillary, immersed in liquid argon. Typically $4x10^{10}$ Bq of radioactivity were produced.

Chromatography. HPLC was done using a 250x4.6 mm column packed with Shandon ODS Hypersil reversed phase material (5µ). The packing slurry was prepared in isopropanol (12% w/v). Packing pressure was 540 kg/cm^2. The column was conditioned with 50% methanol, then with 43% ethanol in aqueous buffer (10 mM NaH_2PO_4, pH 7.0), containing 1mM EDTA/1000 ml of the ethanolic buffer solution [23]. The same buffer without addition of EDTA (eluant A) was used with a flow rate of 1 ml/min for separation of the labelled product. The retention time of N-[1-^{11}C]acetyl LTE_4 is strongly dependent on the ethanol concentration and pH of the elution buffer. Thus peak shifting may be observed within a retention time window of 4 min [23]. The system described here with eluant A produced a t_R for N-[1-^{11}C]acetyl LTE_4 of 10.5 ± 2 min. Analytical HPLC for quality control was done on a Eurospher C18 column (250x4.6 mm, 5µ, Eurochrom Knauer GmbH, Berlin) using 70% methanol in 0.1% aqueous acetic acid at pH 5.1 adjusted with 20% NH_4OH (eluant B). Column conditioning was as above with the corresponding 1 mM EDTA elution buffer. Flow rate was 1 ml/min, t_R of N-[1-^{11}C]acetyl LTE_4 was 12.3 min. The chromatograms in Fig. 2 and Fig. 3 were performed with this system. UV-absorption was continuously recorded from 200-

360 nm during chromatography using a Merck Hitachi diode array detector (L-3000). The specific activity was quantitated on a Canberra FLOW-ONE A250 detection system (cell size 500 µl) in combination with a Waters 490E multiwavelength detector.

Preparation of 5(S)-Hydroxy-6(R)-(N-[1-^{11}C]acetyl)cysteinyl-trans-7,9,-cis-11,14-eicosa-tetraenoic acid **1**. The apparatus used for the N-acetylation of LTE_4 is shown in Fig.4. A constant He gas flow, 30 ml/min, carries $[^{11}C]CO_2$ to vessel 1, containing 20 µl of a 3 M solution of CH_3MgCl in THF in an additional amount of 75 µl THF. The solution was cooled by an ice/ethanol cooling bath during trapping of $[^{11}C]CO_2$. When this was completed, phthaloyl dichloride (50 µl) containing traces of CH_3COCl ($1x10^{-9}$ moles) was added through a syringe. The cooling was replaced by a silicon oil bath at 353 K for distillation of instantaneously formed $CH_3[^{11}C]COCl$. Distillation was continued until the excess phthaloyl dichloride turned to an orange to red, viscous residue. The $CH_3[^{11}C]COCl$ was collected in vessel 2 at 195 K within 90 µl of THF. Average distillation time took 5 min for almost quantitative trapping of the labelled acetyl chloride.

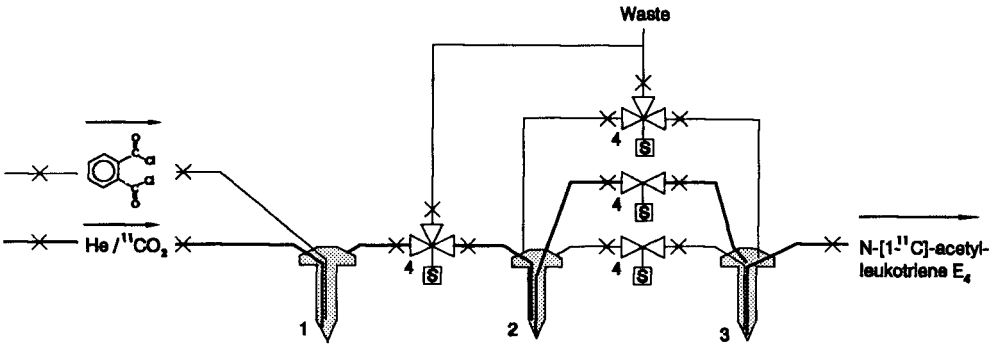


Figure 4: Apparatus for the preparation of **1**; —x— main flow, —x— support lines and reagents; 1, 2, 3 screw capped reaction vessels, 4 solenoid valves; vessel 1 CH_3MgCl, vessel 2 $CH_3[^{11}C]COCl$, vessel 3 polyethylene vial with LTE_4.

The radioactivity was measured in random experiments giving about $1.4x10^{10}$ Bq of $CH_3[^{11}C]COCl$ at about 17 min from end of $[^{11}C]CO_2$ production. The THF solution of

$CH_3[^{11}C]COCl$ was transferred slowly from vessel 2 to vessel 3 containing LTE_4 applying a smooth He pressure [24]. The LTE_4 solution was allowed to warm up to room temperature during collection of the radioactivity and then heated to 318 K for 10 min under a pulsed He flow for completion of the labelling reaction. The reaction mixture was rapidly quenched using 600 µl of water and the resulting solution was loaded on an octadecylsilyl cartridge (SEP-PAK, 10x10 mm, Waters). The cartridge was washed prior to use with 30 ml of CH_3OH followed by 30 ml H_2O, then with 50 ml of a 1 mM EDTA solution containing 10% CH_3OH. This solvent was also used for storage of the cartridges. Just before use 30 ml of water were passed through the cartridge, then the radioactivity was loaded onto it. Rinsing with 50 ml of water removed all of the unreacted radioactivity, whereas the labelled N-[1-^{11}C]Acetyl-LTE_4 was retained quantitatively. A subsequent elution with 0.8 ml of 90% ethanol in a 5 mM solution of NaH_2PO_4 at pH 7.0 delivered a crude radioactive fraction together with unreacted LTE_4 which was diluted with water to a final volume of 2 ml. This was chromatographed under isocratic conditions (1 ml/min) on a Hypersil ODS stationary phase using eluant A. Within that preparative procedure the radioactivity was monitored qualitatively by a simple lead shielded Geiger Müller tube together with the UV absorbance at 282 nm. The fraction with a retention time of authentic N-acetyl-LTE_4 (t_R = 10.5 ± 2 min) was collected in a polyethylene vial. Ethanol was evaporated rapidly from the eluate at 333 K under a strong argon flow. Sterile saline for infusion was used for dilution of the purified product and for preparing injectable doses of ^{11}C-labelled N-acetyl-LTE_4 of a constant specific activity of 2 GBq/µmol. The complete preparation required 50 minutes and yielded $1.9x10^8$ Bq of the labelled product at a purity of 95%. This corresponded to a radiochemical yield of 1.3% of pure 5(S)-hydroxy-6(R)-(N-[1-^{11}C]acetyl)-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid, related to the measured average initial amount of $CH_3[^{11}C]COCl$ (not corrected for decay; average of 6 preparations). The remaining predominant impurity was the 11-trans isomer of **1**, which contributed 1/20 of the final dose.

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[21] EuroKat H^{+} (Eurochrom Knauer GmbH, D-1000 Berlin, Germany), 30 cm x 8 mm. Flow rate 0.7 ml/min deionized water. Detection was by a Waters 410 differential refractometer at 308 K, combined with the Canberra FLOW-ONE radioactivity detector. [1-^{11}C]Acetic acid eluted at 12.5 min.

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[23] Treatment of the reversed phase materials with EDTA improved the recovery of leukotrienes. It is suggested to check for the recovery of leukotrienes with a ^{3}H- or ^{14}C-labelled derivative before the column is finally used with the ^{11}C-labelled compound.

[24] Carrier acetyl chloride was added for obtaining an almost constant specific radioactivity.