ELSEVIER

Journal of Chromatography B, 667 (1995) 209–221

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Chemical synthesis of dioxygen-18 labelled ω-/β-oxidized cysteinyl leukotrienes: analysis by gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry

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First received 16 November 1994; revised manuscript received 12 January 1995; accepted 12 January 1995

Abstract

Cysteinyl leukotrienes (LT) C_4, LTD_4 and LTE_4 are potent mediators of anaphylaxis and inflammation. LTE_4 is extensively metabolized in man mainly by ω-oxidation followed by subsequent β-oxidation to more polar and biologically inactive metabolites. This paper describes a method for the synthesis of [1,20-$^{18}O_2$]-carboxy-LTE_4, [1,18-$^{18}O_2$]-carboxy-dinor-LTE_4, and [1,16-$^{18}O_2$]-carboxy-14,15-dihydro-tetranor-LTE_4 starting from the unlabelled dimethyl esters of 20-carboxy-LTA_4, 18-carboxy-dinor-LTA_4 and 16-carboxy-14,15-dihydro-tetranor-LTA_4, respectively, by separate chemical conjugation with cysteine hydrochloride in $H_2{}^{18}O$–methanol followed by alkaline hydrolysis with $Li^{18}OH$. The isotopic purity of the isolated reaction products was 94% at ^{18}O for all three preparations while only 0.3% remained unlabelled as confirmed by negative-ion chemical-ionization gas chromatography–mass spectrometry (GC–NICI-MS) after their catalytical reduction/desulphurization and derivatization. The $^{18}O_2$-labelled compounds are demonstrated to be suitable internal standards for quantification by GC–NICI-MS and GC–NICI-tandem MS. We found by GC–NICI-tandem MS that the excretion rate of 20-carboxy-LTE_4 is comparable to that of LTE_4 (both in nmol/mol creatinine, mean ± S.E.) in healthy children (26.7 ± 4.7 vs. 32.0 ± 6.0, $n = 9$) and adults (13.9 ± 1.1 vs. 27.2 ± 5.4, $n = 3$).

1. Introduction

Leukotriene C_4 (LTC_4) is the first member of the family of cysteinyl leukotrienes which are arachidonic acid metabolites formed in the 5-lipoxygenase pathway by conjugation of the intermediate 5,6-oxide LTA_4 with glutathione. LTC_4 and its products of enzymatic hydrolysis LTD_4 and LTE_4 are powerful vasoconstricting substances and potent mediators of anaphylaxis and inflammation [1]. LTE_4 is extensively metabolized in man by ω-oxidation to 20-carboxy-LTE_4 and by subsequent β-oxidation to 18-carboxy-dinor-LTE_4, 16-carboxy-14,15-dihydro-tetranor-LTE_4, and 14-carboxy-hexanor-LTE_3 [2]. LTE_4 has been identified as the major urinary metabolite of cysteinyl leukotrienes in man [3,4]. It can, therefore, be used to assess whole body cysteinyl leukotrienes synthesis. Based on the measurement of LTE_4 in human

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0378-4347/95/$09.50
SSDI 0378-4347(95)00027-5

urine cysteinyl leukotrienes have been implicated as mediators in several allergic and inflammatory diseases [5–12]. Quantitation of urinary LTE_4 is usually performed by RIA or gas chromatography–negative-ion chemical-ionization mass spectrometry (GC–NICI-MS) following separation by reversed-phase high-performance liquid chromatography (RP-HPLC) [5–15]. The GC–NICI-MS analysis of the thermally labile and non-volatile LTE_4 and its precursors LTD_4 and LTC_4, first described by Balazy and Murphy [16], is based on their catalytical reduction/desulphurization to 5-hydroxyeicosanoic acid (5-HEA).

Recently, urinary LTE_4 metabolites have been suggested as index metabolites instead of LTE_4 itself for cysteinyl leukotrienes because they might better reflect whole body cysteinyl leukotrienes synthesis in man [2]. Due to the potential significance of the ω- and β-oxidized LTE_4 metabolites [2,13] it was of interest to evaluate GC–NICI-MS and GC–tandem MS (GC–NICI-MS–MS) methods for their quantitative analysis. This paper describes the GC–NICI-MS and GC–NICI-MS–MS analysis of a series of cysteinyl leukotrienes including 20-carboxy-LTE_4, 18-carboxy-dinor-LTE_4 and 16-carboxy-14,15-dihydro-tetranor-LTE_4. We also describe here a method for the chemical synthesis of their dioxygen-18 labelled analogs in high isotopic purity starting from the commercially available dimethyl esters of 20-carboxy-LTA_4, 18-carboxy-dinor-LTA_4, and 16-carboxy-14,15-dihydro-tetranor-LTA_4, respectively, by quasi "one-pot" reaction with cysteine in $H_2{}^{18}O$/methanol followed by hydrolysis with $Li^{18}OH$. The excretion rates of LTE_4 and 20-carboxy-LTE_4 in the urine of healthy children ($n = 9$) and adults ($n = 3$) were determined by GC–NICI-MS–MS using [20,20,20-$^2H_3$]LTE_4 and [1,20-$^{18}O_2$]carboxy-LTE_4 as internal standards, respectively.

2. Methods

2.1. Chemicals and reagents

All cysteinyl leukotrienes and the dimethyl esters of 20-carboxy-LTA_4, 18-carboxy-LTA_4 and 16-carboxy-14,15-dihydro-LTA_4, unlabelled LTE_4 and its ω-carboxy metabolites were obtained from Cascade (Reading, UK). [20,20,20-$^2H_3$]LTE_4 was purchased from Biomol (Hamburg, Germany). L-Cysteine hydrochloride and N,N-diisopropylethylamine were purchased from Sigma (Munich, Germany). $H_2{}^{18}O$ (95 atom% ^{18}O) was obtained from CAMPRO Scientific (Emmerich, Germany). $Li^{18}OH$ (0.36 *M*) was prepared by dissolving the appropriate amount of lithium in $H_2{}^{18}O$ (97.8% ^{18}O) which was purchased from MSD Isotopes Merck Frosst Canada (Montreal, Canada). The catalyst used for catalytical hydrogenation (5 wt.% Rh on Al_2O_3) was supplied by Fluka (Neu-Ulm, Germany). Pentafluorobenzyl (PFB) bromide was obtained from Aldrich (Steinheim, Germany) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, USA). Acetonitrile and methanol of gradient grade were purchased from Merck (Darmstadt, Germany).

2.2. Solid-phase extraction and reversed-phase high-performance liquid chromatography

Collection and storage of urine samples as well as solid-phase extraction on 300-mg Sep-Pak octadecylsilica-cartridges (Waters, Hamburg, Germany) of cysteinyl leukotrienes from 20-ml urine aliquots were performed as described recently for LTE_4 [8]. RP-HPLC was carried out on a Spectra Physics delivery system Model SP 8800. The mobile phase, pumped at a flow-rate of 1.0 ml/min, was a ternary linear gradient between solvent A (water–methanol–acetic acid, 70:30:0.005, v/v), solvent B (water–methanol–acetonitrile–acetic acid, 60:20:20:0.005, v/v) and solvent C (water–methanol–acetonitrile–acetic acid, 30:14:56:0.005, v/v) all adjusted to pH 5.4 with ammonia, from 100% A at time zero to 100% B at time 30 min and to 100% C at time 120 min. The effluent was monitored at 280 nm. The retention times (t_R) of the unlabelled ω-carboxy-LTE_4 metabolites were determined to be (mean ± S.D., $n = 6$; min): 20.7 ± 0.4 for 16-carboxy-14,15-dihydro-tetranor-LTE_4, 26.3 ± 0.5 for 18-carboxy-dinor-LTE_4 and 33.4 ± 0.4 for 20-carboxy-LTE_4. The t_R of unlabelled LTE_4 in this

system was (mean ± S.D., $n = 6$) 68.7 ± 0.8 min. In experiments concerning the quantitation of cysteinyl leukotrienes in urine, the peaks with the retention time of the synthetic cysteinyl leukotrienes were collected separately. The organic solvents were then removed under reduced pressure, the remaining aqueous phases were diluted with 900 μl of methanol and the resulting solutions were stored at −80°C until further treatment.

2.3. Chemical preparation of $^{18}O_2$-labelled ω-carboxy-LTE_4 metabolites

$^{18}O_2$-Labelling of ω-carboxy-LTE_4 metabolites was performed by a similar procedure to that described by Prakash et al. [17] for the preparation of C-20 trideuterated cysteinyl leukotrienes as follows. L-Cysteine hydrochloride (34 mg) was dissolved in a mixture of 160 μl of $H_2{}^{18}O$ and 800 μl of methanol and the pH of the resulted solution was adjusted to 8.0 by addition of triethylamine. The organic solvents (diethyl ether/hexane) of the commercial solution of ω-carboxy-LTA_4 dimethyl esters (each 25 μg) were removed under a nitrogen stream and the reaction was started by dissolving the residues in 320 μl of the cysteine-containing $H_2{}^{18}O$–methanol. After one hour of stirring at room temperature the reaction mixtures were cooled to 4°C, then treated with 100 μl of an 0.36 *M* $Li^{18}OH$ solution (cooled at 4°C) and with 200 μl of methanol and stirred for a further 30 min at room temperature. The reaction was stopped by addition of 2.5 *M* phosphoric acid adjusting the pH to 7.0. A 100-μl aliquot of the resulting suspension was injected onto the RP-HPLC system and the peaks with the retention time of the synthetic ω-carboxy-LTE_4 metabolites were collected separately. After evaporation of the organic solvents the remaining aqueous phases were diluted with methanol and the resulting solutions were stored at −80°C until further use. Quantitation of chemically prepared ^{18}O-labelled ω-carboxy-LTE_4 metabolites was performed by UV spectroscopy using a molar absorption coefficient (A) of 40 000 ($\lambda = 280$ nm) for all compounds. Aliquots from stock solutions were taken for analysis by GC–NICI-MS and GC–NICI-MS–MS. Reduction/desulphurization and derivatization to the PFB-TMS derivatives of these samples and of urine extracts were performed as described previously for LTE_4 [14].

2.4. Gas chromatographic–mass spectrometric and gas chromatographic–tandem mass spectrometric conditions

GC–NICI-MS was performed on a Hewlett-Packard MS Engine 5989A directly connected with a gas chromatograph 5890 Series II (Waldbronn, Germany). The gas chromatograph was equipped with a fused-silica capillary column HP1 (12 m × 0.25 mm I.D., 0.25 μm film thickness) which was held at 100°C. Helium (35 kPa) and methane (200 Pa) were used as carrier and reagent gas, respectively. Interface and ion source were kept at 280°C and 225°C, respectively. For ionization in the electron-impact (EI) mode the ion-source temperature was set to 110°C. Electron energy and electron current were set to 230 eV and 300 μA, respectively. The following oven temperature programm was used: 2 min at 100°C, then increase to 250°C at a rate of 25°C/min, followed by an increase to 320°C at a rate of 4°/min. GC–NICI-MS–MS was performed on a Finnigan 9611 gas chromatograph equipped with a fused-silica capillary column DB-1 (15 m × 0.25 mm I.D., 0.25 μm film thickness) from J&W Scientific (Rancho Cordova, CA, USA) connected to a Finnigan MAT TSQ 45 triple-stage quadrupole mass spectrometer (San Jose, CA, USA). The injector was kept at 280°C, the column held at 150°C for 3 min, then programmed to 320°C with an increase of 25°C/min. The column was leading directly into the ion source which was kept at 130°C. Helium was used as a carrier gas at a pressure of 55 kPa. A constant temperature of 280°C was kept at the interface. Methane was used as reagent gas for NICI at a pressure of 65 Pa. The ionization energy was 90 eV for NICI at an electron current of 300 μA. In GC–NICI-MS–MS argon was used for collisionally activated dissociation (CAD) at a collision cell pressure of 0.2 Pa. The collision energy was set to 14 eV. Both in GC–

NICI-MS and GC–NICI-MS–MS, 1 to 2 μl aliquots were injected in the splitless mode.

3. Results

3.1. Gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry

The cysteinyl leukotrienes listed in Table 1 were separately reduced/desulphurized, the corresponding 5-hydroxy-(di)carboxylic acids were converted to the PFB-TMS derivatives and approximately 20 ng of each were analyzed by GC–NICI-MS and GC–NICI-MS–MS. The m/z of the major mass fragments, e.g. $[M - PFB]^-$ (parent ions), and of the corresponding major daughter ions for each cysteinyl leukotriene investigated in this study are summarized in Table 1. Obviously, catalytical reduction/desulphurization of all ω/β non-oxidized cysteinyl leukotrienes results in the same hydroxy-fatty acid, e.g. 5-HEA. In the GC–NICI-MS spectrum of the PFB-TMS derivative of 5-HEA the most intensive mass fragment is due to $[M - PFB]^-$ with m/z of 399. Identical GC–NICI-MS spectra were also observed from hydrogenated 5-, 12- and 15-hydroxy-eicosatetraenoic acid (HETE; not shown). From each cysteinyl leukotriene and HETE a minor ion at m/z 309 due to $[M - PFB - TMSOH]^-$ was observed (see also Ref. [15]). CAD of the parent ion at m/z 399 gave for all ω/β non-oxidized cysteinyl leukotrienes and hydrogenated 5-HETE a major mass fragment with m/z 253 due to $[M - PFB - CH_3CH_2COOTMS]^-$ [14,15]. However, in the GC–NICI-MS–MS spectra of hydrogenated 12- and 15-HETE the daughter ion at m/z 253 was missing. These findings and the observation that this ion was not obtained from the parent ion at m/z 309 ($[M - PFB - TMSOH]^-$) of hydrogenated 5-HETE suggest that the mechanism of the formation of the ion m/z 253 (e.g. $[M - PFB - CH_3CH_2COOTMS]^-$) involves a rearrangement of the TMS group from the hydroxyl-group at position C-5 to one oxygen atom of the carboxylic group following formation and release of the TMS ester of propionic acid.

In the GC–NICI-MS spectra of each ω-/β-oxidized cysteinyl leukotriene an intense mass fragment was observed due to $[M - PFB]^-$ (Table 1; see Fig. 2 bottom). CAD of the corresponding parent ion of these cysteinyl leukotrienes gave a major daughter ion due to $[M - PFB - PFBOH]^-$ (Table 1), a typical daughter ion of dicarboxylic acids, and a less intense daughter ion due to $[M - PFB - PFBOH - TMSOH]^-$ (not shown) which was also observed from ω-carboxy-LTB_4 [13].

Table 1
Mass-to-charge (m/z) values of the parent ions ($[M - PFB]^-$) and of the corresponding major daughter ions in the GC–NICI-MS and GC–NICI-MS–MS spectra of the PFB-TMS derivatives of the 5-hydroxy-(di)carboxylic acids obtained from separate reduction/desulphurization of primary and ω-/β-oxidized cysteinyl leukotrienes

Cysteinyl leukotriene	5-Hydroxy fatty acid	m/z	
		Parent ion $[M - PFB]^-$	Major daughter ion
LTC_4, LTD_4, LTE_4, LTF_4	5-Hydroxyeicosanoic acid	399	253
N-Acetyl-LTE_4	5-Hydroxyeicosanoic acid	399	253
LTE_4 sulphoxide/sulphone	5-Hydroxyeicosanoic acid	399	253
20-Carboxy-LTE_4	5-Hydroxyeicosa-1,20-dioic acid	609	411
18-Carboxy-dinor-LTE_4	5-Hydroxyoctadeca-1,18-dioic acid	581	383
16-Carboxy-14,15-dihydro-tetranor-LTE_4	5-Hydroxyhexadeca-1,16-dioic acid	553	355

3.2. Chemical preparation of $^{18}O_2$-labelled ω-carboxy-LTE$_4$ metabolites

Fig. 1 shows a RP-HPLC chromatogram from a mixture of the prepared ^{18}O-labelled ω-carboxy-LTE$_4$ metabolites and the UV spectra of the compounds eluted at 20.7, 26.3 and 33.5 min. The retention times as well as the UV spectra of the ^{18}O-labelled ω-carboxy-LTE$_4$ metabolites are identical with those of the unlabelled compounds, strongly suggesting formation of the ω-carboxy-LTE$_4$ metabolites from the dimethyl esters of the corresponding ω-carboxy-LTA$_4$ precursors by chemical reaction with cysteine followed by alkaline hydrolysis (see also below).

The EI mass spectrum of the PFB-TMS derivative of the catalytically reduced/desulphurized reaction product with the t_R of 33.5 min on RP-HPLC (Fig. 1) from the ^{18}O-labelling experiment of 20-carboxy-LTE$_4$ is shown in Fig. 2 (top). The mass fragment ions at m/z 371 ($[C_5H_7 - TMSO - {}^{18}OOPFB]^+$) and m/z 525 ($[C_{16}H_{29} - TMSO - {}^{18}OOPFB]^+$) representing the two complementary α-fission fragments at the TMSO-group at C-5 were increased each by 2 Da compared with the corresponding signals from the PFB-TMS derivative of catalytically reduced/desulphurized unlabelled 20-carboxy-LTE$_4$ (not shown). In the EI mass spectrum of the PFB-TMS derivative from reduced/desulphurized ^{18}O-labelled 18-carboxy-dinor-LTE$_4$ two intense fragment ions at m/z 371 $[C_5H_7 - TMSO - {}^{18}OOPFB]^+$ and 497 ($[C_{14}H_{25} - TMSO - {}^{18}OOPFB]^+$) besides m/z 181 ($[PFB]^+$) were observed (not shown). The EI mass spectrum obtained from the PFB-TMS derivative of catalytically reduced/desulphurized ^{18}O-labelled 16-carboxy-14,15-dihydro-tetranor-LTE$_4$ showed besides m/z 181 two fragment ions at m/z 371 ($[C_5H_7 - TMSO - {}^{18}OOPFB]^+$) and 469 ($[C_{12}H_{21} - TMSO - {}^{18}OOPFB]^+$) (data not shown).

The EI mass spectra in combination with the data from RP-HPLC analysis and UV spectrometry strongly indicate that (see Fig. 3): (i) in the first reaction step a nucleophilic attack of cysteine with its thiol-group at C-6 of the dimethyl esters of 20-carboxy-LTA$_4$, 18-carboxy-dinor-LTA$_4$ and 16-carboxy-14,15-dihydro-tetranor-LTA$_4$ occurred, respectively, leading to the corresponding ω-carboxy-LTE$_4$ dimethyl esters; and (ii) in the second reaction step one ^{18}O atom from Li^{18}OH was incorporated into each carboxylic group of the ω-carboxy-LTE$_4$ metabolites leading thus to the [1,ω-$^{18}O_2$]-carboxy-LTE$_4$ metabolites. In the EI mass spectra of the [1,ω-$^{18}O_2$]-carboxy-LTE$_4$ metabolites no mass fragments have been found which would correspond to the unlabelled metabolites or to metabolites having both ^{18}O atoms in only one carboxylic group.

In the GC–NICI-MS spectrum of the PFB-TMS derivative of reduced/desulphurized [1,20-$^{18}O_2$]-20-carboxy-LTE$_4$ (Fig. 2; bottom) the most intense ion was observed at m/z 613 ($[M - PFB]^-$). This ion was increased by four Da with respect to the corresponding ion fragment at m/z 609 of unlabelled 20-carboxy-LTE$_4$ (Table 1) indicating incorporation of two ^{18}O atoms. Similar results have been obtained with [1,18-$^{18}O_2$]-18-carboxy-dinor-LTE$_4$ and [1,16-$^{18}O_2$]-16-carboxy-14,15-dihydro-LTE$_4$. The most intense mass fragments found in the GC–NICI-MS spectra of the prepared $^{18}O_2$-labelled ω-carboxy-LTE$_4$ metabolites are summarized in Table 2. This table also gives the extent of the incorporation of ^{18}O atoms in the ω-20-carboxy-LTE$_4$ as determined on the basis of peak area by selected-ion monitoring (SIM) on m/z of the ions $[M - PFB]^-$ given in Table 2. The 5-hydroxy-eicosa-1,ω-dioic acid derivatives from unlabelled ω-carboxy-LTE$_4$ as well as from [1,ω-$^{18}O_2$]-carboxy-LTE$_4$ metabolites emerged from the GC column at the same time. The total yield of the $^{18}O_2$-labelled ω-carboxy-LTE$_4$ metabolites was determined by UV spectroscopy and found to be 29%, 35% and 25% for the 20-, 18- and 16-carboxy-LTE$_4$ metabolite, respectively.

The applicability of the $^{18}O_2$-labelled ω-carboxy-LTE$_4$ metabolites as internal standards was tested by separate catalytical reduction/desulphurization of varying amounts of the unlabelled compounds (0–200 ng) and at a fixed amount of 20 ng for each $^{18}O_2$-labelled ω-carboxy-LTE$_4$ metabolite followed by GC–NICI-MS analysis of their PFB-TMS derivatives by SIM of the corre-

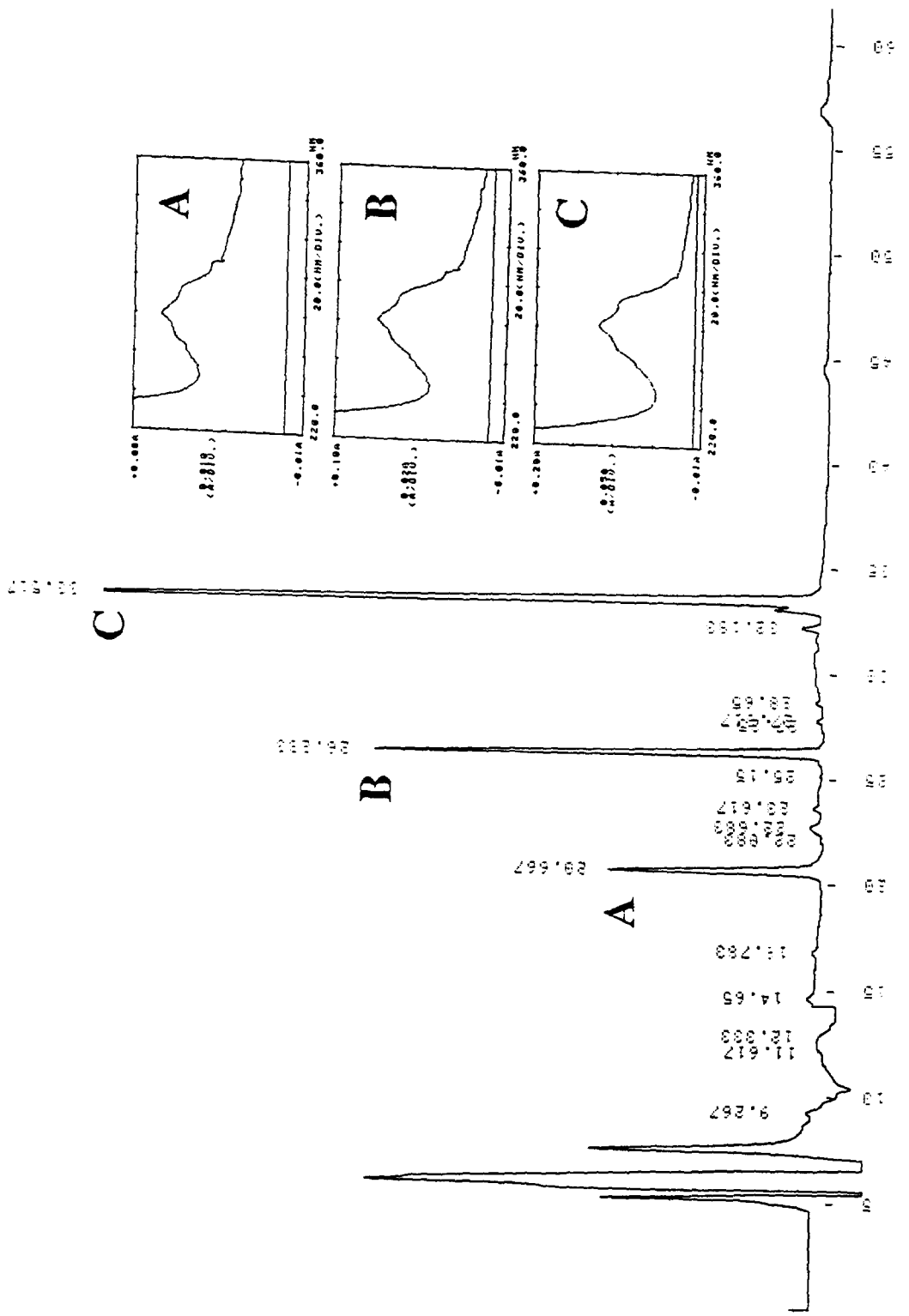

Fig. 1. RP-HPLC chromatogram from the analysis of a mixture containing the $^{18}O_2$-labelled ω-carboxy-LTE_4 metabolites. The UV spectra generated from the corresponding peaks show the characteristic UV absorbance of cysteinyl leukotrienes due to a conjugated triene. The peaks were identified by GC–MS (see Fig. 2 and the text) as [1,16-$^{18}O_2$]-carboxy-14,15-dihydro-tetranor-LTE_4 (A, t_R 20.7 min), [1,18-$^{18}O_2$]-carboxy-dinor-LTE_4 (B, t_R 26.3 min) and [1,20-$^{18}O_2$]-carboxy-LTE_4 (C, t_R 33.5 min).

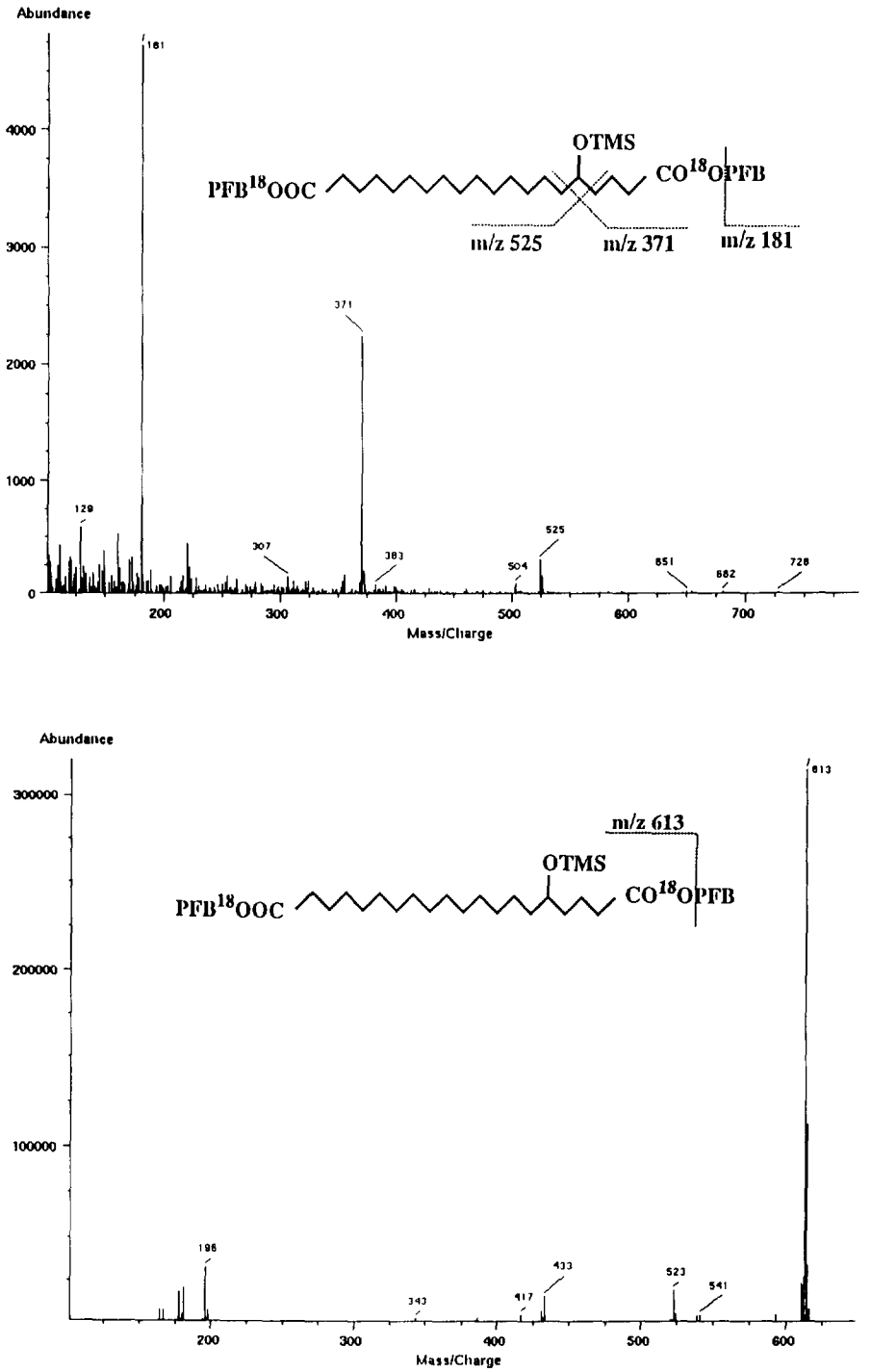

Fig. 2. EI (top) and NICI (bottom) mass spectra of the PFB-TMS derivative of [1,20-$^{18}O_2$]-5-hydroxy-eicosadioic acid obtained from chemically prepared [1,20-$^{18}O_2$]-carboxy-LTE$_4$ after RP-HPLC analysis (Fig. 1; peak C at t_R 33.5 min), catalytical reduction/desulphurization and chemical derivatization.

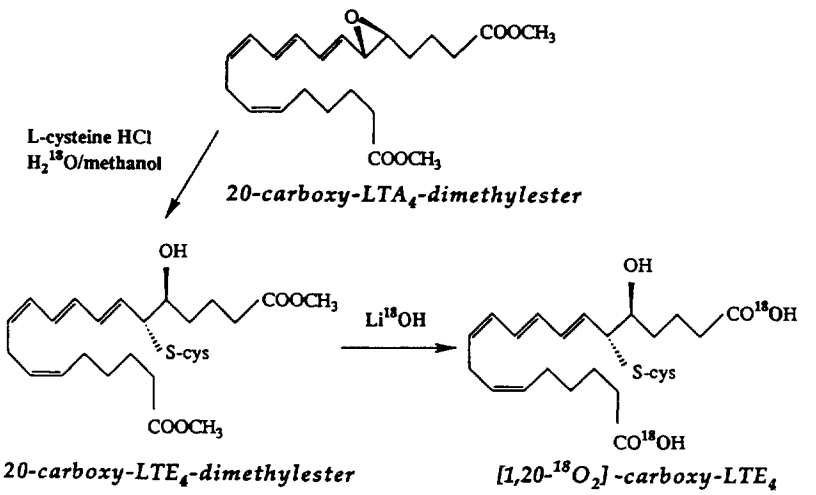

Fig. 3. Schematic of the chemical synthesis of [1,20-$^{18}O_2$]-carboxy-LTE$_4$ from 20-carboxy-LTA$_4$-dimethyl ester and L-cysteine in $H_2{}^{18}O$.

sponding parent ions $[M - PFB]^-$. Linear relationships ($r > 0.995$) were observed by regression analysis of the measured m/z ratio of unlabelled to labelled metabolites vs. the added amounts of the unlabelled compounds for all ω-carboxy-LTE$_4$ metabolites. Linearity was also observed

Table 2
Major mass fragments in the GC–NICI-MS spectra the PFB-TMS derivatives of reduced/desulphurized $^{18}O_2$-labelled ω-carboxy-LTE$_4$ metabolites and extent of ^{18}O incorporation

[1,ω-$^{18}O_2$]-Carboxy-LTE$_4$	GC–NICI-MS spectrum (m/z) (intensity, %; ion)	Distribution[a] of $^{18}O_2$-labelled ω-carboxy-LTE$_4$ (m/z; % of ^{18}O)
[1,20-$^{18}O_2$]-Carboxy-LTE$_4$	613 (100; P)[b] 541 (10; $[P - TMSH]^-$) 523 (10; $[P - TMSOH]^-$)	609; 0.3% $^{18}O_0$ 611; 5.0% $^{18}O_1$ 613; 94.4% $^{18}O_2$ 615; 0.3% $^{18}O_3$ 617; 0.0% $^{18}O_4$
[1,18-$^{18}O_2$]-Carboxy-dinor-LTE$_4$	585 (100; P^-) 511 (10; $[P - TMSH]^-$) 495 (10; $[P - TMSOH]^-$)	581; 0.2% $^{18}O_0$ 583; 5.1% $^{18}O_1$ 585; 94.5% $^{18}O_2$ 587; 0.2% $^{18}O_3$ 589; 0.0% $^{18}O_4$
[1,16-$^{18}O_2$]-Carboxy-14,15-dihydro-tetranor-LTE$_4$	557 (100; P^-) 483 (10; $[P - TMSH]^-$) 467 (10; $[P - TMSOH]^-$)	553; 0.3% $^{18}O_0$ 555; 5.0% $^{18}O_1$ 557; 94.4% $^{18}O_2$ 559; 0.3% $^{18}O_3$ 561; 0.0% $^{18}O_4$

[a] The distribution was calculated as described in the text.
[b] P^- is the parent ion $[M - PFB]^-$.

for urinary LTE_4 and 20-carboxy-LTE_4 when measured by GC–NICI-MS–MS. Quantitation of 18-carboxy-dinor-LTE_4 and 16-carboxy-14,15-dihydro-tetranor-LTE_4 even by GC–NICI-MS–MS in human urine was difficult due to interferences by unknown co-eluting compounds. Back-exchange of ^{18}O by ^{16}O in [1,20-$^{18}O_2$]-20-carboxy-LTE_4 incubated in urine at room temperature for up to three hours was not observed.

3.3. Excretion of LTE_4 and 20-carboxy-LTE_4 in human urine

The parent and the daughter ions used for quantitative measurement of urinary LTE_4 by GC–NICI-MS–MS were m/z 399 and 253 for unlabelled and m/z 402 and 256 for [20,20,20-$^2H_3$]LTE_4, respectively. The corresponding ion pairs for 20-carboxy-LTE_4 and [1,20-$^{18}O_2$]-20-carboxy-LTE_4 were m/z 609/411 and m/z 613/415, respectively. The concentration of LTE_4 in urine was determined as described previously [15]. Because CAD of the $^{18}O_2$-labelled standards gave two major daughter ions with the same intensity (due to the loss of PFBOH and PFB^{18}OH from the parent ions $[M - PFB]^-$) the measured m/z-ratio of the daughter ions of 20-carboxy-LTE_4 to [1,20-$^{18}O_2$]-20-carboxy-LTE_4 was divided by two in quantitative measurements. Fig. 4 shows representative GC–NICI-MS–MS partial chromatograms from the analysis of LTE_4 and 20-carboxy-LTE_4 in a 20-ml urine sample of a healthy volunteer spiked with a mixture of [20,20,20-$^2H_3$]LTE_4 (2 ng) and [1,20-$^{18}O_2$]-20-carboxy-LTE_4 (10 ng). The intra-assay relative standard deviation ($n = 6$) of the method was 5.5% for LTE_4 and 6.2% for 20-carboxy-LTE_4. The corresponding values for the inter-assay relative deviation ($n = 6$) were 6.5% and 8.5%, respectively. In Table 3 the urinary excretion rates for LTE_4 and 20-carboxy-LTE_4 with respect to creatinine and the 24-h output of nine healthy children and three healthy adults are summarized as measured by GC–NICI-MS–MS. Comparable urinary excretion rates for LTE_4 and 20-carboxy-LTE_4 in healthy children have been recently reported [13].

4. Discussion

In this paper we describe the analysis by GC–NICI-MS and GC–NICI-MS–MS of primary and ω-/β-oxidized cysteinyl leukotrienes, a conventional method for the preparation of the [1,ω-$^{18}O_2$]-labelled ω- and β-oxidized LTE_4 metabolites, e.g. 20-carboxy-LTE_4, 18-carboxy-dinor-LTE_4 and 16-carboxy-14,15-dihydro-tetranor-LTE_4. We also describe the quantitative determination of LTE_4 and 20-carboxy-LTE_4 in urine of healthy children and adults by GC–NICI-MS–MS.

Catalytical reduction/desulphurization of ω- and/or β-non-oxidized cysteinyl leukotrienes with identical or similar fatty acid moiety, e.g. LTC_4, D_4, E_4, LTE_4 sulphoxide and sulphone, N-acetyl-LTE_4, and LTF_4, leads to the formation 5-HEA. Analysis of the PFB-TMS derivative of 5-HEA obtained from these cysteinyl leukotrienes results in identical mass spectra both in GC–NICI-MS and GC–NICI-MS–MS with most intense mass fragments at m/z 399 ($[M - PFB]^-$) and 253 ($[M - PFB - CH_3CH_2COOTMS]^-$), respectively. Thus, quantitation of a certain cysteinyl leukotriene requires chromatographic separation from other cysteinyl leukotrienes and 5-HETE, the hydrogenation of which leads also to 5-HEA. In particular when GC–NICI-MS–MS is applied other HETE isomers such as 12- and 15-HETE do not interfere with the measurement of these cysteinyl leukotrienes. The GC–NICI-MS spectra of the PFB-TMS derivatives of reduced/desulphurized ω-/β-oxidized cysteinyl leukotrienes are characterized substantially by a single intense mass fragment due to $[M - PFB]^-$. CAD of these parent ions results in the formation of the characteristic major daughter ion due to $[M - PFB - PFBOH]^-$ and of a less intense daughter ion from consecutive loss of the TMSOH group. SIM of the most intensive mass fragments ($[M - PFB]^-$) at m/z 399 in GC–NICI-MS or selected-reaction monitoring (SRM) of the characteristic daughter ion at m/z 253 in GC–NICI-MS–MS of ω-/β-non-oxidized cysteinyl leukotrienes allow quantitative determination of less than 5 pg/ml urine of LTE_4. The detection limits for other

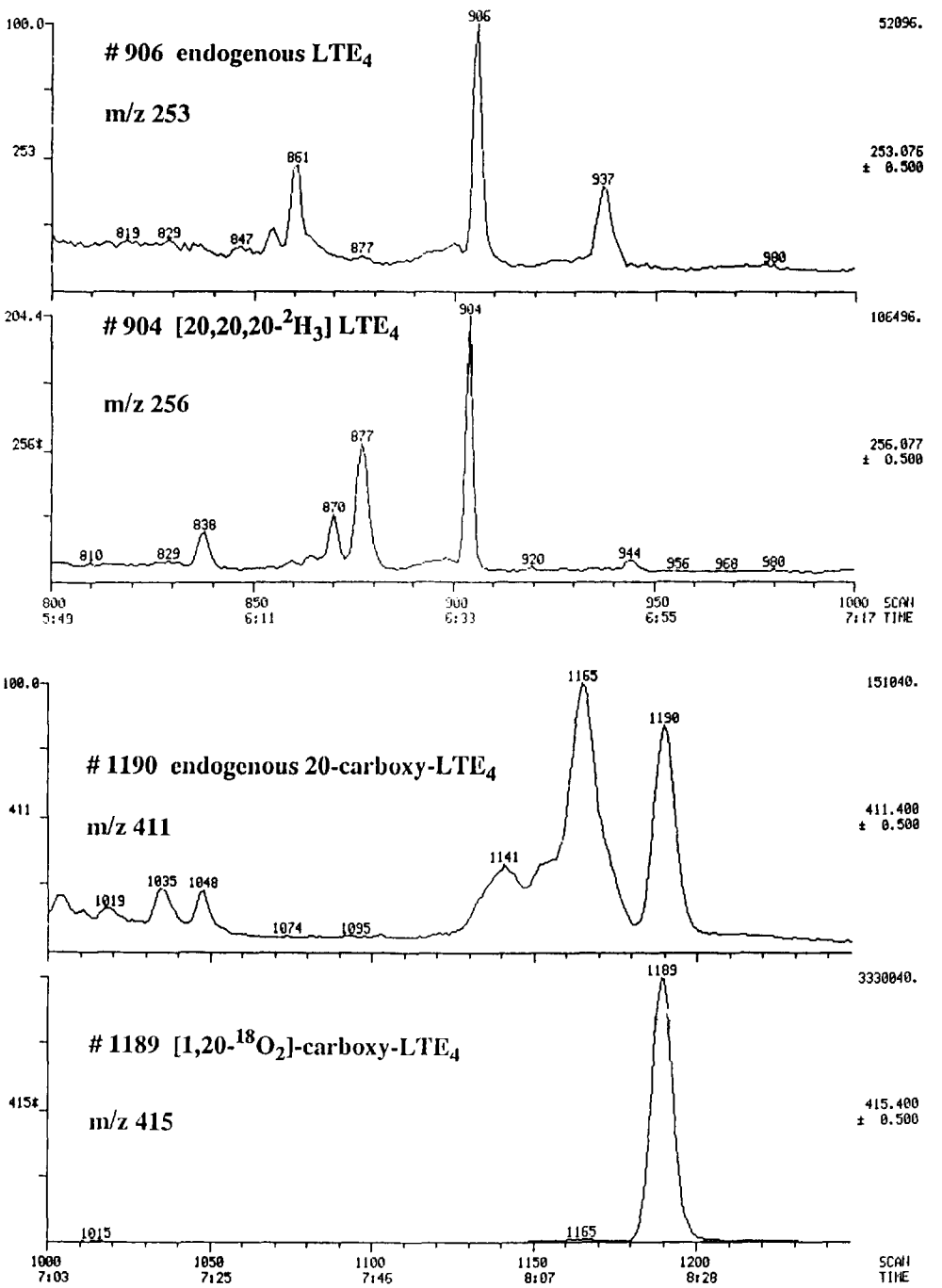

Fig. 4. Partial GC–NICI-MS–MS chromatograms from the analysis for LTE_4 (top) and 20-carboxy-LTE_4 (bottom) in a human urine sample (20 ml) spiked with 2 ng of [20,20,20-$^2H_3$]LTE_4 and 10 ng of [1,20-$^{18}O_2$]-carboxy-LTE_4 followed by solid-phase extraction, RP-HPLC isolation, catalytical reduction/desulphurization and chemical derivatization. Top: SRM of the daughter ions at *m/z* 253 and 256 derived from the parent ions at *m/z* 399 and 402 for endogenous LTE_4 and [20,20,20-$^2H_3$]LTE_4, respectively. Bottom: SRM on the daughter ions at *m/z* 411 and 415 derived from the parent ions at *m/z* 609 and m/z 613 for endogenous 20-carboxy-LTE_4 and [1,20-$^{18}O_2$]-carboxy-LTE_4, respectively; *y*-axis: relative intensity in %.

Table 3
Excretion rates of LTE_4 and ω-carboxy-LTE_4 in urine of nine healthy children and three healthy adults as measured by GC–NICI-MS–MS

Subject	Age (yr)	LTE_4 (nmol/mol cre.)	20-Carboxy-LTE_4 (nmol/mol cre.)
1. M.F. (m)[a]	4	64.5 (0.137)[b]	44.4 (0.094)[b]
2. S.W. (f)	3	37.7 (0.050)	49.4 (0.065)
3. L.B. (f)	5	51.6 (0.109)	8.7 (0.018)
4. U.B. (f)	5	37.8 (0.134)	25.4 (0.090)
5. K.B. (f)	5	32.9 (0.105)	27.9 (0.089)
6. M.M. (m)	10	17.1 (0.136)	7.7 (0.061)
7. M.M. (m)	8	19.1 (0.071)	31.1 (0.117)
8. K.M. (f)	7	15.1 (0.050)	22.1 (0.074)
9. V.M. (f)	6	12.4 (0.039)	23.6 (0.075)
Mean ± S.E., $n = 9$		32.0 ± 5.97 (0.092 ± 0.013)	26.7 ± 4.66 (0.076 ± 0.009)
10. W.J. (m)	32	37.7 (0.630)	12.2 (0.204)
11. J.F. (m)	41	23.8 (0.105)	13.6 (0.060)
12. U.F. (f)	36	20.0 (0.151)	16.1 (0.121)
Mean ± S.E., $n = 3$		27.2 ± 5.38 (0.295 ± 0.168)	13.9 ± 1.14 (0.128 ± 0.042)
Mean ± S.E., $n = 12$		30.8 ± 4.59	23.5 ± 3.83

[a] m = male, f = female.
[b] Values in parenthesis represent nmol/24 h.

ω-/β-oxidized cysteinyl leukotrienes are higher. We found that 20-carboxy-LTE_4 can be detected by GC–NICI-MS–MS in concentrations down to 20 pg/ml urine.

In a quasi "one-pot" reaction we synthesized [1,ω-$^{18}O_2$]-labelled LTE_4 metabolites. Separate chemical reaction of the commercially available dimethyl esters of the unlabelled ω-carboxy-LTA_4 compounds with cysteine in ^{18}O-water–methanol resulted exclusively in the formation of the dimethyl esters of the C-6 cysteinyl leukotrienes the alkäline hydrolysis ($Li^{18}OH$) of which gave the free acids [1,ω-$^{18}O_2$]-labelled LTE_4 metabolites at high isotopic purity (94% at ^{18}O) and sufficient yield. An alternative route to prepare $^{18}O_2$-labelled LTE_4 metabolites is the enzymic labelling of the corresponding unlabelled compounds using esterases. This procedure leads, in contrast to the chemical synthesis described here, to [1,1-$^{18}O_2$]-labelled LTE_4 metabolites as has been demonstrated for [1,1-$^{18}O_2$]-carboxy-LTE_4 [13]. Because of the high isotopic purity with only 0.3% of unlabelled materials, and of the lack of $^{18}O/^{16}O$ back-exchange during incubation in urine and also no loss of ^{18}O during catalytical hydrogenation–in contrast to ^{2}H-labelled standards [15]–these [1,ω-$^{18}O_2$]-labelled compounds are excellent internal standards in GC–NICI-MS and GC–NICI-MS–MS analysis.

The urinary excretion rates of LTE_4 and 20-carboxy-LTE_4 by healthy children were in the same order as measured recently by us in another group of healthy children [13]. No significant differences were found in the creatinine-corrected excretion rates of these metabolites between children and adults. With respect to the 24-h output, however, adults ex-

creted significantly higher amounts (nmol/24 h, mean ± S.E.) of LTE_4 (0.295 ± 0.168) and 20-carboxy-LTE_4 (0.128 ± 0.042) than children (0.092 ± 0.013 for LTE_4 and 0.076 ± 0.009 for 20-carboxy-LTE_4).

Despite use of high-resolving RP-HPLC and GC–NICI-MS–MS 18-carboxy-14,15-dihydro-LTE_4 and 16-carboxy-14,15-dihydro-tetranor-LTE_4 could not be accurately measured in the urine due to interferences by co-eluting unknown compounds. This is not surprising because of the possible high number of closely related compounds derived from C-20, C-18 and C-16 fatty acids and other substances. Our results suggest that quantitation of urinary 18-carboxy-dinor-LTE_4 and 16-carboxy-14,15-dihydro-tetranor-LTE_4, and likely of 14-carboxy-hexanor-LTE_3 requires development of improved extraction and purification procedures including HPLC systems.

5. Conclusions

This and previous studies demonstrate that GC–NICI-MS and GC–NICI-MS–MS are excellently suitable techniques to determine quantitatively cysteinyl leukotrienes in human urine [13–15]. The chemical or enzymic synthesis of $^{18}O_2$-labelled analogs from commercially available precursors is easy. The use of $^{18}O_2$-labelled cysteinyl leukotrienes as internal standards is superior over ^2H-labelled analogs. Alternatively, ^{13}C-labelled cysteinyl leukotrienes [18] should also be suitable internal standards. As we further showed (Fig. 5) cysteinyl leukotrienes can be simultaneously determined by GC–NICI-MS–MS provided they are present in a single matrix which does not contain interfering compounds. However, this is at the present not possible. LTE_4 seems to be, at least from the analytical point of view, the most reliable index metabolite

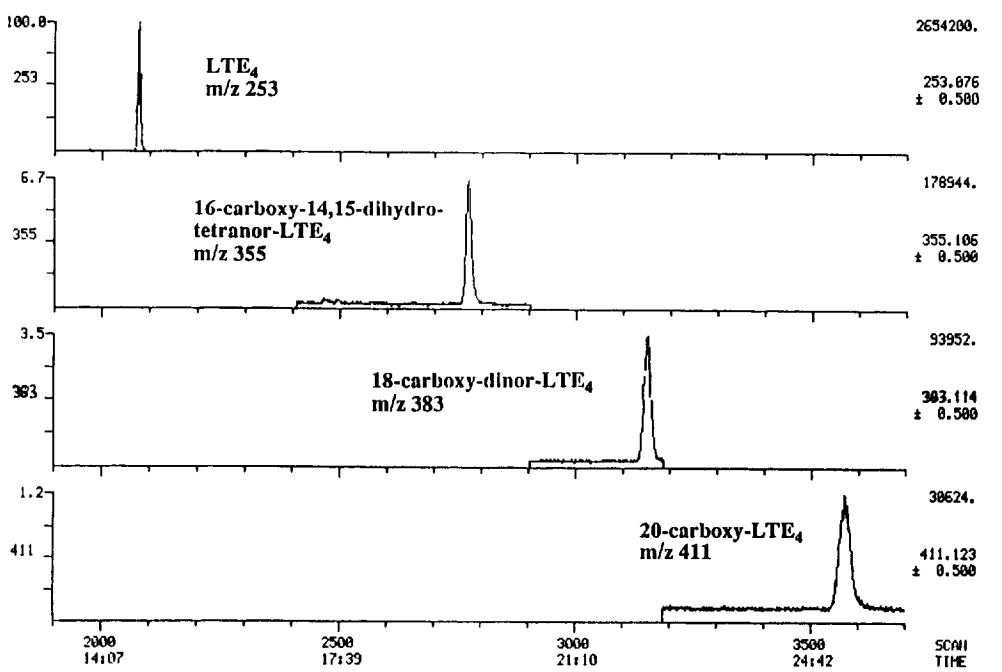

Fig. 5. Simultaneous GC-NICI-MS–MS analysis of a mixture containing the PFB-TMS derivatives of catalytically reduced and desulphurized LTE_4 (2 ng), 20-carboxy-LTE_4 (20 ng), 18-carboxy-dinor-LTE_4 (20 ng) and 16-carboxy-14,15-dihydro-tetranor-LTE_4 (10 ng). A so-called multi-experiment consisting of four consecutively performed experiments was applied. In each experiment the values for the individual parent and daughter ions were set. The collision energy was set to 14 eV for all cysteinyl leukotrienes; *y*-axis: relative intensity in %.

for estimating cysteinyl leukotrienes synthesis in man by GC–NICI-MS and GC–NICI-MS–MS. This suggestion is supported by the following facts: (1) Most of the interfering compounds in the analysis of LTE_4 are known and can be easily eliminated; (2) LTE_4 can be extracted and isolated from urine at a higher selectivity than ω-/β-oxidized cysteinyl leukotrienes [19]; (3) in particular when GC–NICI-MS–MS is applied LTE_4 can be determined at substantially higher selectivity and sensitivity due to its more favourable chromatographic behavior and mass spectrometric properties.

Acknowledgements

This work was supported by a grant from the Bundesministerium für Forschung und Technologie 01VM90020. The excellent laboratory assistance of Miss I. Fuchs is gratefully acknowledged. Also the authors wish to thank Mr. F.M. Gutzki for his excellent assistance in GC–MS.

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