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Gas chromatographic–mass spectrometric determination of leukotriene E_4 in human urine using deuterium-labelled leukotriene E_4 standards

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ABSTRACT

The utility of two deuterium-labelled leukotriene (LT) E_4 analogs, *e.g.* [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4, as internal standards for the determination of LTE_4 in human urine by gas chromatography–mass spectrometry (GC–MS) was investigated. ^2H-Exchange during hydrogenation occurred both in [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 in an extent of $9.4 \pm 0.5\%$ and $67.3 \pm 0.6\%$ (mean ± S.D., $n = 6$), respectively. The lower extent of ^2H-exchange in [20,20,20-$^2H_3$]LTE_4 allowed a more accurate quantitation than the use of [14,15,17,17,18,18-$^2H_6$]LTE_4. Applying [20,20,20-$^2H_3$]LTE_4 as internal standard the coefficients of variation for the intra- and inter-assay determination of LTE_4 in human urine were 5.7% and 6.2% ($n = 4$), respectively. The inter-assay coefficient of variation for [14,15,17,17,18,18-$^2H_6$]LTE_4 was 15%. Using [20,20,20-$^2H_3$]LTE_4 as internal standard and GC–MS, healthy volunteers were found to excrete 17 ± 10 nmol LTE_4 per mol creatinine (mean ± S.D., $n = 11$). Similar excretion rates for LTE_4 in urine of healthy volunteers were found using GC–tandem MS with [1,1-$^{18}O_2$]LTE_4 as internal standard. Our results demonstrate that [20,20,20-$^2H_3$]LTE_4 is a suitable internal standard for the GC–MS determination of urinary LTE_4.

INTRODUCTION

Cysteinyl leukotrienes are potent mediators of inflammation [1]. They are thermally labile and non-volatile compounds. Their direct analysis by gas chromatography–mass spectrometry (GC–MS) is therefore impossible. However, catalytical reduction and desulphurization of these substances to 5-hydroxyeicosanoic acid (5-HEA) enables cysteinyl leukotrienes to be determined by GC–MS [2]. A prerequisite for their quantitation by GC–MS is the availability of stable isotope-labelled cysteinyl leukotrienes analogs. Recently, the synthesis and the utility of [1,1-$^{18}O_2$]LTE_4 as internal standard for the GC–MS determination of urinary LTE_4 [3], the index metabolite of cysteinyl leukotrienes in humans [4,5], has been described. In the literature the synthesis of [20,20,20-$^2H_3$]LTE_4 [6] and

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[8,9,10,11-$^{13}C_4$]LTE_4 [7] has also been described. From these [20,20,20-$^2H_3$]LTE_4 is commercially available (Biomol, Hamburg, Germany). [14,15,17,17,18,18-$^2H_6$]LTE_4 (99.7 atom% ^2H) was synthesized in our laboratory starting from [14,15,17,17,18,18-$^2H_6$]LTA_4 methylester [8] and cysteine methylester hydrochloride. In particular with respect to the common phenomenon of ^2H-exchange by ^1H during catalytic hydrogenation, which has also been reported for some ^2H-labelled cysteinyl leukotrienes [2], it was of interest to investigate whether the commercially available [20,20,20-$^2H_3$]LTE_4 and the newly synthesized [14,15,17,17,18,18-$^2H_6$]LTE_4 are useful as internal standards for the determination of LTE_4 in human urine by GC–MS. This study demonstrates that despite $^2H/H$-exchange both [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_3$]LTE_4 are suitable internal standards. However, [20,20,20-$^2H_3$]LTE_4 permits more accurate quantification of urinary LTE_4 by GC–MS. Using [20,20,20-$^2H_3$]LTE_4 the urinary excretion rate of LTE_4 in healthy normal volunteers was assessed.

EXPERIMENTAL

Chemicals and reagents

[20,20,20-$^2H_3$]LTE_4 (99.8 atom% ^2H) was obtained from Biomol (Hamburg, Germany). [14,15-$^3H_2$]LTE_4 was obtained from DuPont (Dreieich, Germany). Hydrogen and deuterium gas (99 atom% ^2H) were obtained from Merck (Darmstadt, Germany). The catalyst 5% (w/w) Rh on alumina was obtained from Fluka (Neu-Ulm, Germany) and was washed with ethanol prior to use. Pentafluorobenzyl (PFB) bromide was purchased from Aldrich (Steinheim, Germany). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL, USA). [1,1-$^{18}O_2$]LTE_4 (90 atom% ^{18}O) was synthesized as described previously [3].

Catalytical reduction/desulphurization

Catalytical reduction/desulphurization of LTE_4 and labelled-LTE_4, and derivatization of labelled and unlabelled 5-HEA to the corresponding PFB ester trimethylsilyl (TMS) ether derivatives using PFB bromide and BSTFA, respectively, were performed in most experiments as described previously [3,9]. For catalytical reduction/desulphurization hydrogen as well as deuterium gas were used.

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

Endogenous LTE_4 and the stable isotope-labelled LTE_4 (2 ng in most experiments) added to 20-ml urine samples were solid-phase extracted, purified and isolated by reversed-phase high-performance liquid chromatography (RP-HPLC) as described elsewhere [9–11]. The retention times of unlabelled LTE_4, [20,20,20-$^2H_3$]LTE_4, [14,15,17,17,18,18-$^2H_6$]LTE_4 and [1,1-$^{18}O_2$]LTE_4 in this system were (mean ± S.D.): 48.5 ± 1.4 ($n = 10$), 48.1 ± 1.5 ($n = 3$), 47.9 ± 1.4 ($n = 3$), and 48.4 ± 1.5 ($n = 10$) min, respectively. RP-HPLC fractions were collected in 1-min intervals between the 45th and 53rd min and aliquots thereof were counted. Radioactivity containing fractions were pooled and treated further for catalytic reduction/desulphurization.

Gas chromatographic–mass spectrometric conditions

GC–MS was performed on a Hewlett–Packard MS Engine 5989A directly connected with a gas chromatograph HP 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) maintained at a temperature of 100°C. Helium (35 kPa) and methane (200 Pa) were used as carrier and reagent gas for negative-ion chemical ionization (NICI), respectively. The following oven temperature program was used: the column was held for 2 min at 100°C, then the temperature was increased to 250°C at a rate of 25°/min and next to 320°C at a rate of 4°C/min. The interface and ion source were kept at 280°C and 225°C, respectively. Electron energy and electron current were set at 230 eV and 300 μA, respectively. Aliquots of 1 μl were injected in the splitless mode.

GC–tandem MS (GC–MS–MS) was carried

out on a Finnigan MAT gas chromatograph 9611 equipped with a fused silica capillary column OV1, 25 m × 0.25 mm I.D., film thickness 0.25 μm (Macherey–Nagel, Düren, Germany) interfaced with a triple-stage quadrupole mass spectrometer Finnigan MAT TSQ 45 (San Jose, CA, USA) operating in the NICI mode with methane as reagent gas at a pressure of 65 Pa. Helium was used as carrier gas at a pressure of 50 kPa. The ionization energy was 90 eV and the electron current 300 μA. Argon was used for collision activated dissociation (CAD) at a cell pressure of 0.2 Pa. The collision energy was 14 eV. Constant temperatures were maintained at the interface (280°C), ion source (130°C) and injector (280°C). The same oven temperature program was used as described above for GC–MS. Aliquots of 1–2.5 μl were injected in the splitless mode.

RESULTS

Fig. 1 shows NICI mass spectra obtained from the PFB-TMS derivatives of catalytically hydrogenated unlabelled LTE_4 (a), [20,20,20-$^2H_3$]LTE_4 (b), [14,15,17,17,18,18-$^2H_6$]LTE_4 (c), and [1,1-$^{18}O_2$]LTE_4 (d). Catalytic hydrogenation of unlabelled LTE_4 led to a single compound, *e.g.* 5-HEA, with the most intensive signal at *m/z* 399 ($[M - PFB]^-$) (Fig. 1a). The major mass fragment in the mass spectrum derived from [20,20,20-$^2H_3$]LTE_4 (Fig. 1b) is *m/z* 402 which corresponds to [$^2H_3$]-5-HEA. The less intensive signal at *m/z* 399 corresponds to 5-HEA and results from exchange of all three ^2H atoms with ^1H atoms during hydrogenation. The mass spectrum derived from catalytically hydrogenated [14,15,17,17,18,18-$^2H_6$]LTE_4 (Fig. 1c) shows a group of intensive ions at *m/z* 399, 400,

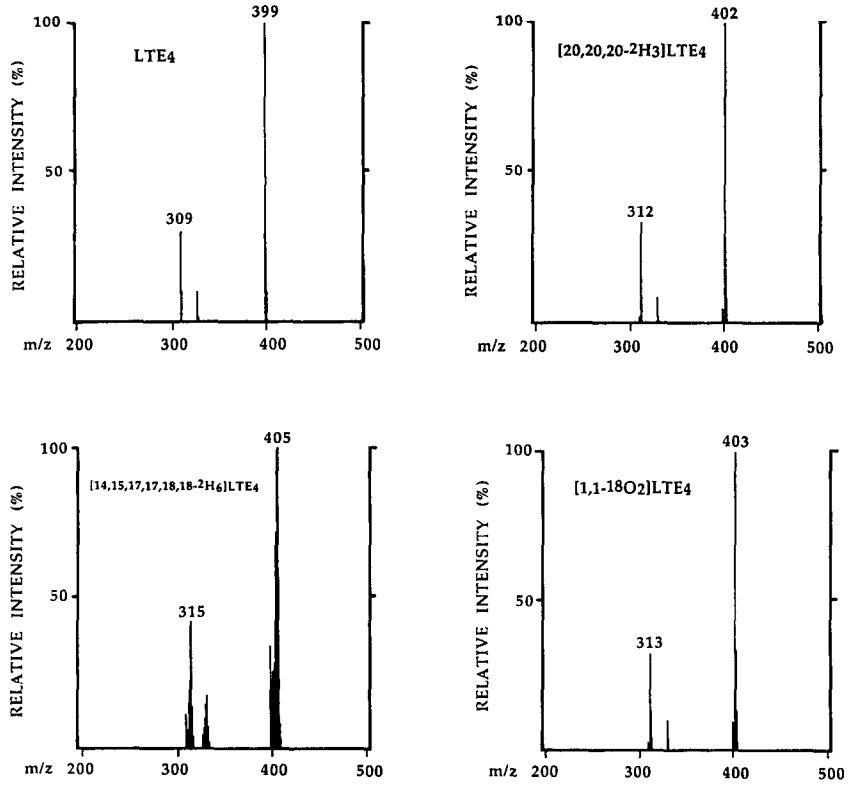

Fig. 1. NICI mass spectra of the PFB-TMS derivatives of reduced/desulphurized LTE_4 (a), [20,20,20-$^2H_3$]LTE_4 (b), [14,15,17,17,18,18-$^2H_6$]LTE_4 (c), and [1,1-$^{18}O_2$]LTE_4 (d).

401, 402, 403, 404, 405, 406 and 407. These signals, except for m/z 406 and 407, correspond to 5-HEA with various ^2H atoms. The signal at m/z 399 corresponds to 5-HEA from exchange of all six ^2H atoms of [14,15,17,17,18,18-$^2H_6$]LTE_4 with ^1H atoms. The major mass fragments in the mass spectrum derived from [1,1-$^{18}O_2$]LTE_4 (Fig. 1d) are m/z 403 and m/z 401 corresponding to [1,1-$^{18}O_2$]-5-HEA and [1,1-$^{18}O^{16}O$]-5-HEA.

The extent of the ^2H/H-exchange and its variability in both [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 are of great importance for quantitation of LTE_4. Six samples of each [20,20,20-$^2H_3$]LTE_4, [14,15,17,17,18,18-$^2H_6$]LTE_4 and [1,1-$^{18}O_2$]LTE_4 (0.5 ng, 1 ng, 2 ng, 5 ng, 10 ng and 50 ng) were subjected separately to catalytical reduction/desulphurization using hydrogen gas. GC–MS analysis of the PFB-TMS derivatives was carried out by selected ion monitoring (SIM) on m/z of the ions $[M - PFB]^-$, *e.g.* m/z 399, 400, 401 and 402 for $^2H_3$-LTE_4, m/z 399 to m/z 405 for $^2H_6$-LTE_4, and m/z 399, 401 and 403 for $^{18}O_2$-LTE_4. The ratio m/z 399 to m/z of $[M - PFB]^-$ (m/z 402 for [20,20,20-$^2H_3$]LTE_4, m/z 405 for [14,15,17,17,18,18-$^2H_6$]LTE_4 and m/z 403 for [1,1-$^{18}O_2$]LTE_4) was determined from the peak areas of the corresponding ions of each LTE_4. Similarly, the relative contributions of the ion at m/z 399 and of the ions $[M - PFB]^-$ were calculated by dividing the peak area of these ions by the sum of the peak areas of all corresponding ions of each LTE_4, respectively. The results of these experiments are summarized in Table I. This table clearly shows that [1,1-$^{18}O_2$]LTE_4 is superior with respect to accuracy over the ^2H-analogs. However, the relative low ^2H/H-exchange in [20,20,20-$^2H_3$]LTE_4 and its constant value should allow its use as an internal standard for LTE_4 in GC–MS. The corresponding data for [14,15,17,17,18,18-$^2H_6$]LTE_4 in Table I suggest that this analogue is less suitable as an internal standard.

Linearity was checked by separate catalytic reduction/desulphurization of synthetic LTE_4 up to 10 ng using 2 ng each of [20,20,20-$^2H_3$]LTE_4, [14,15,17,17,18,18-$^2H_6$]LTE_4, and [1,1-$^{18}O_2$]-LTE_4. For all three internal standards linear relationships ($r^2 > 0.989$) were obtained by SIMs on m/z 399, 402, 405, and 403, respectively. Furthermore, linearity ($r^2 > 0.957$) was also observed for LTE_4 added (up to 1000 pg/ml) to human urine samples (20 ml) using 2 ng of [20,20,20-$^2H_3$]LTE_4 or 2 ng of [14,15,17,17,18,18-$^2H_6$]LTE_4, as observed by GC–MS–MS in the selected reaction monitoring (SRM) mode of the daughter ions ($[M - PFB - CH_3CH_2COO\text{-}TMS]^-$) at m/z 253 for LTE_4, m/z 256 for [20,20,20-$^2H_3$]LTE_4 and m/z 259 for [14,15,17,17,18,18-$^2H_6$]LTE_4 [3].

Based on the data of Table I (1st and 2nd line from the 2nd and 3rd entry) the following equations were developed and used to calculate the concentration of urinary LTE_4 (C_{LTE_4}) from the measured ratio m/z 399/402 or 253/256 for [20,20,20-$^2H_3$]LTE_4 (eqn. 1) and m/z 399/405 or 253/259 for [14,15,17,17,18,18-$^2H_6$]LTE_4

TABLE I

EXTENT AND VARIABILITY OF THE ^2H/H-EXCHANGE DURING CATALYTIC ONE-STEP REDUCTION/DESULPHURIZATION OF [20,20,20-$^2H_3$]LTE_4 AND [14,15,17,17,18,18-$^2H_6$]LTE_4

Values are mean ± S.D. from six determinations.

LTE_4	Ratio m/z 399 to m/z of $[M - PFB]^-$ [a]	Percentage of m/z 399 from total[b]	Percentage of m/z $[M - PFB]^-$ [a] from total[b]
$^2H_3$-LTE_4	0.059 ± 0.024	4.17 ± 0.14	90.6 ± 4.8
$^2H_6$-LTE_4	0.190 ± 0.030	5.32 ± 2.34	32.7 ± 5.6
$^{18}O_2$-LTE_4	0.002 ± 0.0007	0.70 ± 0.02	89.8 ± 1.2

[a] $[M - PFB]^-$ corresponds to the ions at m/z 402, 405 and 403 for $^2H_3$-LTE_4, $^2H_6$-LTE_4 and $^{18}O_2$-LTE_4, respectively.
[b] Total is the sum of the peak areas of the $[M - PFB]^-$ ions for the corresponding LTE_4 analog as described in the text.

(eqn. 2) and from the concentration in the urine of the weighted internal standard ($C_{[20,20,20\text{-}^2H_3]LTE_4}$ or $C_{[14,15,17,17,18,18\text{-}^2H_6]LTE_4}$).

$$C_{LTE_4} = \{(m/z\ 399/402S \times 0.906) - 0.042\} \times C_{[20,20,20\text{-}^2H_3]LTE_4} \quad (1)$$

$$C_{LTE_4} = \{(m/z\ 399/405 \times 0.327) - 0.053\} \times C_{[14,15,17,17,18,18\text{-}^2H_6]LTE_4} \quad (2)$$

LTE_4 was determined by GC–MS in 20-ml urine samples of a healthy volunteer spiked separately with 2 ng of each [1,1-$^{18}O_2$]LTE_4, [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 as the internal standards. Applying eqns. (1) and (2) the following concentrations of LTE_4 (pg/ml) in the urine were observed (mean ± S.D., $n = 4$): 60 ± 3 using [1,1-$^{18}O_2$]LTE_4, 65 ± 4 using [20,20,20-$^2H_3$]LTE_4 and 58 ± 10 using [14,15,17,17,18,18-$^2H_6$]LTE_4. The corresponding coefficients of variation (C.V.) were 4.8%, 5.7% and 15%, respectively. Using [20,20,20-$^2H_3$]LTE_4 as internal standard the inter-assay C.V. for the determination of LTE_4 in human urine was 6.2% ($n = 4$).

Healthy normal volunteers were found to excrete 17 ± 10 nmol LTE_4 per mol creatinine (mean ± S.D., $n = 11$) as measured by GC–MS using [20,20,20-$^2H_3$]LTE_4. Representative tracings from the GC–MS and GC–MS–MS analysis of urinary LTE_4 of a healthy volunteer using [20,20,20-$^2H_3$]LTE_4 are shown in Fig. 2. Linear regression analysis of the data obtained by GC–MS and those obtained by GC–MS–MS of the same samples gave a correlation coefficient of 0.958. The lower values from the GC–MS analyses probably result from the fact that the peak for endogenous LTE_4 is not base-line resolved (Fig. 2a) so that integration of the peak area as well as the peak height gave lower values compared to GC–MS–MS (Fig. 2b). The urinary LTE_4 concentrations in healthy volunteers measured by GC–MS are within the range for healthy volunteers as measured by GC–MS–MS using [1,1-$^{18}O_2$]LTE_4 [12] and by radioimmunoassay [9,10]. An example for the utility of [14,15,17,17,18,18-$^2H_6$]LTE_4 as internal standard is shown in Fig. 3.

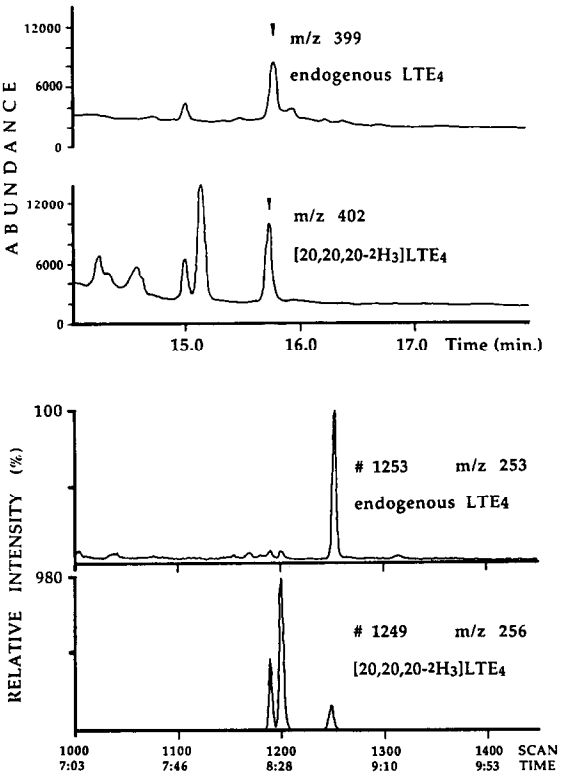

Fig. 2. Partial GC–MS (a) (Hewlett-Packard instrument) and (b) GC–MS–MS (Finnigan MAT instrument) chromatograms from the analysis of urinary LTE_4 in a 20-ml urine sample of a healthy volunteer spiked with 2 ng of [20,20,20-$^2H_3$]LTE_4. In GC–MS the ions at m/z 399 and m/z 402 for endogenous and [20,20,20-$^2H_3$]LTE_4 were detected in the SIM mode. In GC–MS–MS the daughter ions at m/z 253 and m/z 256 derived from the corresponding parent ions at m/z 399 and m/z 402 were detected in the SRM mode. The concentration of LTE_4 in this urine sample was determined to be 49 pg/ml by GC–MS and 50 pg/ml by GC–MS–MS.

DISCUSSION

Accurate determination of urinary LTE_4 by GC–MS requires several procedures for its extraction from urine, chromatographic separation from other cysteinyl leukotrienes and interfering compounds, and also a stable isotope-labelled LTE_4 analogue. The synthesis of ^{18}O-, ^2H-, and ^{13}C-labelled cysteinyl leukotrienes has been recently reported by various groups [3,6,7]. [1,1-$^{18}O_2$]LTE_4 was demonstrated to fulfill all the criteria for an ideal internal standard for the GC–MS determination of LTE_4 in human urine

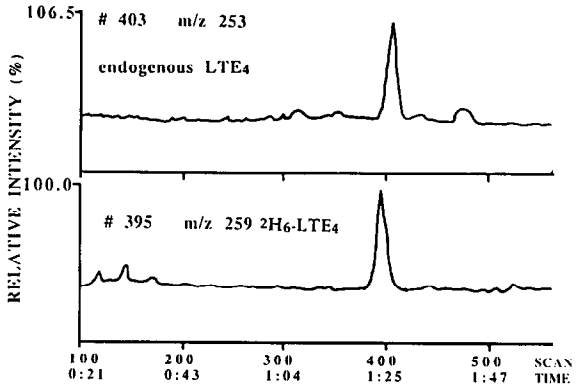

Fig. 3. Partial GC–MS–MS chromatogram from the analysis of urinary LTE_4 in a 20-ml urine sample of a healthy volunteer spiked with 4 ng of [14,15,17,17,18,18-$^2H_6$]LTE_4. SRM of *m/z* 253 and 259 for endogenous LTE_4 and the internal standard, respectively. The concentration of LTE_4 in this urine was determined to be 55 pg/ml.

[3]. In particular with respect to the well known ^2H/H-exchange during catalytic hydrogenation we investigated the usefulness of the commercially available [20,20,20-$^2H_3$]LTE_4 and the newly synthesized [14,15,17,17,18,18-$^2H_6$]LTE_4 as internal standards for quantitative measurements by GC–MS. Our study demonstrates that, despite ^2H/H-exchange in [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 (Figs. 1 and 2; Table I) under the conditions needed for sufficient recovery of 5-HEA [2,3], these compounds can be used as internal standards for LTE_4 in GC–MS (Fig. 2). The ^2H/H-exchange is more reproducible for [20,20,20-$^2H_3$]LTE_4 than for [14,15,17,17,18,18-$^2H_6$]LTE_4 (Table I). This is also reflected in the C.V. for the quantification by GC–MS of urinary LTE_4 applying these standards: The intra-assay C.V. using [20,20,20-$^2H_3$]LTE_4 (5.7%) is only little higher than the corresponding C.V. value using [1,1-$^{18}O_2$]LTE_4 (4.8%), but significantly lower than that using [14,15,17,17,18,18-$^2H_6$]LTE_4 (15%).

The differences in the extent of the ^2H/H-exchange in [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 result apparently from the position of ^2H in the molecules of the deuterated cysteinyl leukotrienes. As two ^2H atoms in [14,15,17,17,18,18-$^2H_6$]LTE_4 are olefinic and the other four are in their close proximity, these ^2H atoms seem to be more activated during the catalytic process than the three end-standing ^2H atoms in [20,20,20-$^2H_3$]LTE_4. Similar results were observed when [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 were subjected to catalytic reduction/desulphurization using deuterium gas (data not shown). Balazy and Murphy have reported significant loss of ^2H through H-atom scrambling in various ^2H-labelled cysteinyl leukotrienes without however giving the position of the ^2H atoms in these standards [2].

Analogous to LTC_4, LTD_4 and LTE_4 the metabolites of ω- and β-oxidation such as 20-carboxy-LTE_4, 18-carboxy-dinor-LTE_4, 16-carboxy-Δ^{13}-tetranor-LTE_4 and 14-carboxy-LTE_3, which also appear in human urine [13], can be measured by GC–MS as their monohydroxy dicarboxylic acids [13]. The results from our investigation on the utility of [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 as internal standards suggest that if ^2H-labelled analogs have to be used for quantitative measurements the label must stay preferably at positions 2, 3 and/or 4. Since cysteinyl leukotrienes are not ω- and β-oxidized from C-1 [14] enzymatic tools could be employed for the preparation of ^2H-labelled ω- and β-oxidized cysteinyl leukotrienes starting from chemically synthesized precursors such as [2,2,3,3-$^2H_4$]LTC_4. Alternatively, ^{18}O labelling could be employed starting from unlabelled materials [3,15].

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