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Enzymic preparation of dioxygen-18 labelled leukotriene E_4 and its use in quantitative gas chromatography–mass spectrometry

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ABSTRACT

A simple and rapid method is described for the preparation of a stable isotope oxygen-18 labelled leukotriene E_4 (LTE_4). Oxygen-18 labelling of LTE_4 methyl ester in oxygen-18 water catalysed by a pig liver esterase resulted in the incorporation of two oxygen-18 atoms in the carboxylic group of LTE_4 to the extent of 89.8% ($[^{18}O_2]LTE_4$) and one oxygen-18 atom to the extent of 9.4% ($[^{16}O^{18}O]LTE_4$), with only 0.7% remaining unchanged ($[^{16}O_2]LTE_4$). $[^{18}O_2]LTE_4$ was found not to back-exchange following incubation in acidified urine (pH 4.0) at 4°C for up to 20 h. $[^{18}O_2]LTE_4$ was demonstrated to be a useful internal standard in a method for the quantitative determination of LTE_4 in human urine involving high-performance liquid chromatography and gas chromatography with negative-ion chemical ionization tandem mass spectrometry: the concentration of LTE_4 in a 24-h urine sample of a healthy subject was determined to be 68.1 pg/ml.

INTRODUCTION

Leukotriene E_4 (LTE_4) is the major urinary metabolite in humans of LTC_4 [1–3], which is one of the most potent lipid mediators of anaphylaxis and inflammation [4]. Quantitative determination of urinary LTE_4 was found to be useful to study the rate of synthesis of cysteinyl leukotrienes in human health and diseases [5–7]. Commonly, quantitation of LTE_4 is performed by radioimmunoassay [8,9] or enzyme-linked immunoassay [10] after its separation by reversed-phase high-performance liquid chromatography (RP-HPLC). Gas chromatography–mass spectrometry (GC–MS), which is widely used for the quantitation of eicosanoids in biological fluids, has recently been reported to be suitable for the analysis of LTC_4, LTD_4, and LTE_4 [11]. This method is based on the catalytic conversion of these compounds into 5-hydroxyeicosanoic acid (5-HEA), which can be sensitively analysed by GC–MS. Accurate and selective quantitation of LTE_4 in urine by this method requires, however, a stable isotope-labelled LTE_4 analogue as internal standard, and separation of LTE_4 by RP-HPLC from other cysteinyl leukotrienes and 5-hydroxyeicosatetraenoic acid (5-HETE) because these are also converted to 5-HEA. The use of deuterated LTE_4 analogues seems to be unsuitable because, during catalytic hydrogenation, exchange of deuterium with hydrogen occurs in these compounds [11]. On the other hand, ^{18}O-labelled 5-HETE as internal standard for LTE_4 [11] is suitable only for semi-quantitative determination, because its HPLC behaviour is different from that of LTE_4.

This paper describes the enzymic preparation of $[^{18}O_2]LTE_4$ from LTE_4 methyl ester, using a commercially available pig liver esterase according to standard methods for the labelling of eicosanoids [12–14]. The usefulness of this stable isotope-labelled LTE_4 analogue in the quantitative GC–MS determination of LTE_4 in human urine is also demonstrated.

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EXPERIMENTAL

Chemicals and reagents

LTE_4 free acid, LTE_4 methyl ester and N,N-diisopropylethylamine were purchased from Sigma (Munich, Germany). [14,15-^{3}H(N)]LTE_4 was obtained from DuPont (Dreieich, Germany). Pig liver esterase (PLE, 130 units/mg) was from Boehringer Mannheim (Mannheim, Germany). $H_2{}^{18}O$ (97.8 atom% oxygen-18) was obtained from MSD Isotopes Merck Frosst Canada (Montreal, Canada). $Li^{18}OH$ (0.36 *M*) was prepared by dissolving the appropiate amount of lithium in $H_2{}^{18}O$. The catalyst used for reduction of LTE_4 was 5% (w/w) Rh on Al_2O_3 and purchased from Fluka (Neu Ulm, Germany). Pentafluorobenzyl (PFB) bromide was obtained from Aldrich (Steinheim, Germany) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL, USA). Acetonitrile and methanol of gradient grade were purchased from Merck (Darmstadt, Germany).

Solid-phase extraction and reversed-phase HPLC

Solid-phase extraction on 300-mg Sep-Pak C_{18} cartridges from Waters and RP-HPLC of enzyme incubation mixtures and urine samples were performed as described previously [7]. Gradient elution at a flow-rate of 1.0 ml/min was used. The effluent was monitored at 280 nm. Quantitation of enzymically synthesized [$^{18}O_2$]LTE_4 was performed by RP-HPLC using appropiate amounts of synthetic LTE_4 and a molar absorptivity (a) of 40 000. Identical retention times for synthetic LTE_4 and [$^{18}O_2$]LTE_4 were found (45.55 min).

Enzymic preparation of [$^{18}O_2$]LTE_4

LTE_4 methyl ester (25 μg in 65% aqueous methanolic potassium phosphate buffer, pH 7.0) and PLE (195 units in ammonium sulphate suspension) were dried under vacuum. The PLE residue was disolved in 90 μl of $H_2{}^{18}O$, and this suspension was used for dissolving the residue of the LTE_4 methyl ester solution. The pH was adjusted to 7.5 by treating the resulting suspension with 5 μl of 0.36 *M* $Li^{18}OH$. The mixture was incubated at 37°C for 16 h. The reaction was stopped by addition of 500 μl of ice-cold ethanol, and the mixture was allowed to stand at −20°C for 30 min. Thereafter, the mixture was diluted with 1 ml of water and centrifuged (2000 *g*, 5 min). Aliquots of 100 μl were injected into the RP-HPLC system, and the peaks with the retention time of synthetic LTE_4 were collected. LTE_4 was recovered by solid-phase extraction and stored in methanol–water (1:1, v/v) at −80°C.

Catalytic reduction and derivatization

LTE_4 and ^{18}O-labelled LTE_4 were converted into the corresponding 5-HEAs by catalytic reduction and desulphurization, using 5% (w/w) Rh/Al_2O_3 and hydrogen gas by the modified method of Balazy and Murphy [11]. Briefly, 5 mg of the catalyst were suspended in a methanolic solution of LTE_4, the suspension was gently shaken and allowed to stand for 5 min on ice, and hydrogen gas was bubbled through the suspension for 20 min at 0°C. Thereafter the sample was centrifuged (2000 *g*, 5 min), methanol was taken up, the catalyst washed with 1 ml of methanol and the suspension centrifuged again. The combined methanol supernatants were evaporated under a stream of nitrogen, the residue was reconstituted in 1 ml of water and extracted twice with 1 ml of ethyl acetate, and the solvent was dried over sodium sulphate and then removed under nitrogen. 5-HEAs obtained from this process were converted by standard derivatization procedures into their PFB ester trimethylsilyl (TMS) ether derivatives using PFB bromide followed by BSTFA.

Gas chromatography–mass spectrometry

GC–MS and GC–MS–MS were performed on a Finnigan 9611 gas chromatograph equipped with a fused-silica capillary column OV-1 (25 m × 0.25 mm I.D., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany) connected to a Finnigan MAT TSQ 45 mass spectrometer (San Jose, CA, USA). The injector was kept at 280°C, and the column held at 100°C for 2 min, then programmed to 320°C at 25°C/min. The column led directly into the ion source which was kept at 120°C. Helium was used as a carrier gas at a pressure of 55 kPa. A constant temperature of 240°C was kept at the interface. Methane was used as reagent gas for negative-ion chemical ionization (NICI) at a pressure of 65 Pa. The ion-

ization energy was 90 eV for NICI and 70 eV for electron impact (EI) at an electron current of 300 μA. In GC–MS–MS experiments, argon was used for collision-activated dissociation (CAD) at a collision cell pressure at 3 mTorr. The collision energy was set at 20 eV. The electron multiplier voltage was 2000 V.

RESULTS AND DISCUSSION

The EI mass spectrum of the PFB-TMS derivative of ^{18}O-labelled LTE$_4$ is shown in Fig. 1. The ion at *m/z* 313 results from fragmentation between carbon atoms 4 and 5 ($C_{16}H_{32}$ – OTMS)$^+$, and was also observed from unlabelled LTE$_4$. In contrast, the ion at *m/z* 373 was observed only from ^{18}O-labelled LTE$_4$ and results from fragmentation between carbon atoms 5 and 6 (C_5H_7 – TMSO – $^{18}O_2$PFB)$^+$, and is increased by four atom mass units (a.m.u.) with respect to the corresponding ion (*m/z* 369) of unlabelled LTE$_4$. The EI mass spectrum of enzymically ^{18}O-labelled LTE$_4$ clearly indicates that this compound is [1,1-$^{18}O_2$]5-hydroxyeicosanoic acid, which is formed from enzymically produced [1,1-$^{18}O_2$]LTE$_4$ by catalytic reduction and desulphurization.

In Fig. 2 the NICI mass spectrum of enzymically ^{18}O-labelled LTE$_4$ is shown. The most intense ions are *m/z* 403 (M – PFB)$^-$ and *m/z* 313 (M – PFB – TMSOH)$^-$. They are increased by four a.m.u. with respect to unlabelled LTE$_4$ (*m/z* 399 and 309, respectively). These findings indicate that two ^{18}O atoms were incorporated in the molecule of LTE$_4$. Furthermore, the NICI mass spectrum of ^{18}O-labelled LTE$_4$ shows a significantly less intensive signal at *m/z* 401 (incorporation of one ^{18}O atom) and no signal at *m/z* 399 (no incorporation of ^{18}O atoms). The extent of the incorporation of ^{18}O atoms into LTE$_4$ was determined by NICI selected-ion monitoring (SIM) on *m/z* 403, *m/z* 401 and *m/z* 399. Fig. 3 shows a partial GC–MS chromatogram from the NICI-SIM analysis at these ions of enzymically prepared [$^{18}O_2$]LTE$_4$ after RP-HPLC separation. Integration of the area of the peaks with scan number 2792 gave 89.8% [$^{18}O_2$]LTE$_4$, 9.4% [$^{16}O^{18}O$]LTE$_4$ and 0.7% unlabelled LTE$_4$. The total yield of [$^{18}O_2$]LTE$_4$ was determined to be 16% (4 μg) by HPLC and UV detection (280 nm, a = 40 000).

The applicability of [$^{18}O_2$]LTE$_4$ as an internal standard was checked by catalytic hydrogenation of various amounts of LTE$_4$ (zero to 10 ng) each

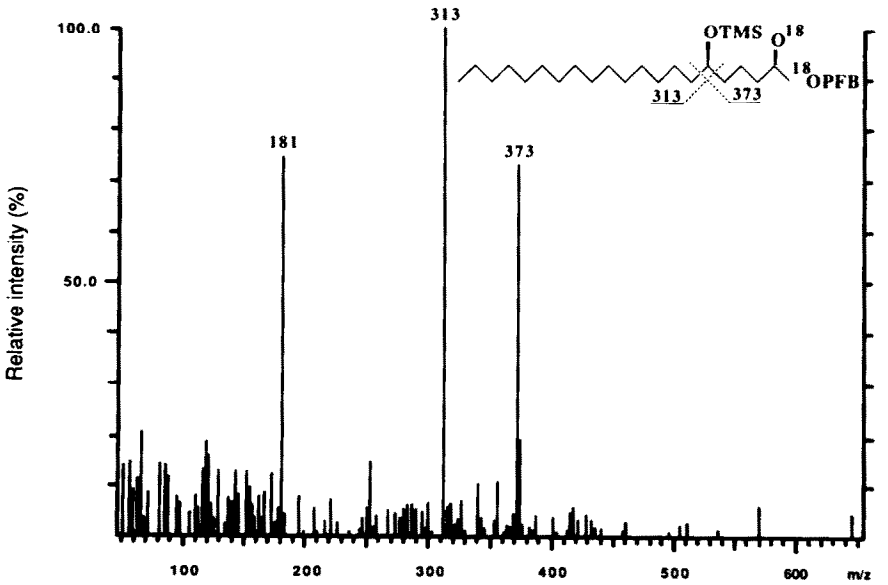

Fig. 1. EI mass spectrum of the PFB ester TMS ether derivative of [1,1-$^{18}O_2$]5-HEA obtained from enzymically prepared [$^{18}O_2$]LTE$_4$ after HPLC analysis, catalytic reduction and desulphurization.

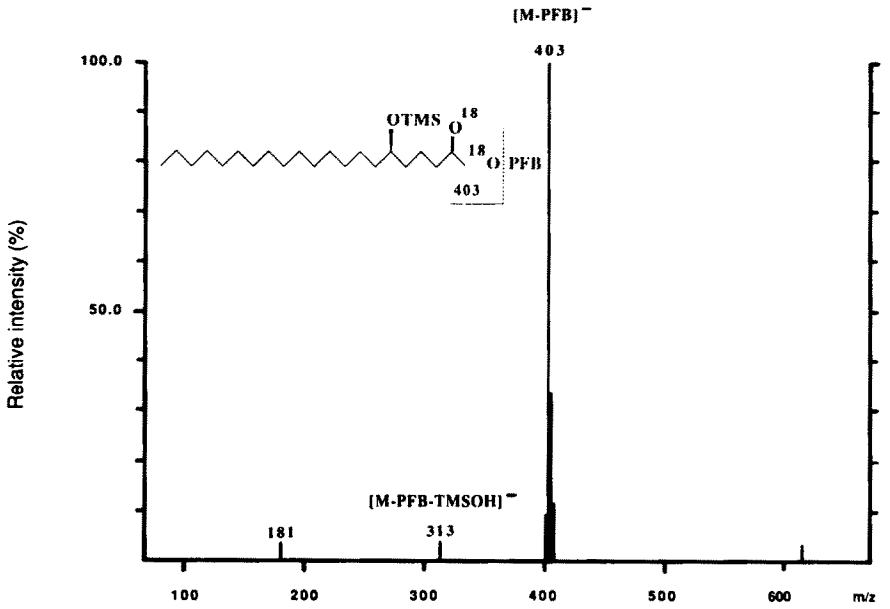

Fig. 2. NICI mass spectrum from the PFB ester TMS ether derivative of enzymically prepared $[^{18}O_2]LTE_4$ after HPLC analysis, catalytic reduction and desulphurization.

spiked with 0.55 ng of $[^{18}O_2]LTE_4$. Linear regression of the ratio m/z 399 to 403 (y) on the amount of LTE_4 (x) gave a straight line, with the regression equation $y = 0.084 + 1.678x$ ($r > 0.985$).

The usefulness of $[^{18}O_2]LTE_4$ as an internal standard in the quantitative determination of LTE_4 in human urine by GC–MS–MS was investigated by analysis of 20-ml urine samples spiked with $[^{18}O_2]LTE_4$ and $[^{3}H]LTE_4$ as described in Experimental and in ref. 7. Fig. 4 shows a partial NICI GC–MS–MS chromatogram from a human urine sample spiked with 3 ng of $[^{18}O_2]LTE_4$ obtained by SIM at m/z 253. This ion was also observed from CAD of the parent ions $(M - PFB)^-$ on m/z 399 (LTE_4) and m/z 403 ($[^{18}O_2]LTE_4$) (Fig. 5). As CAD of the parent ions $(M - PFB)^-$ of the deuterated internal standards $[20,20,20\text{-}^2H_3]LTE_4$ and $[14,15,17,17,18,18\text{-}^2H_6]LTE_4$ leads to the forma-

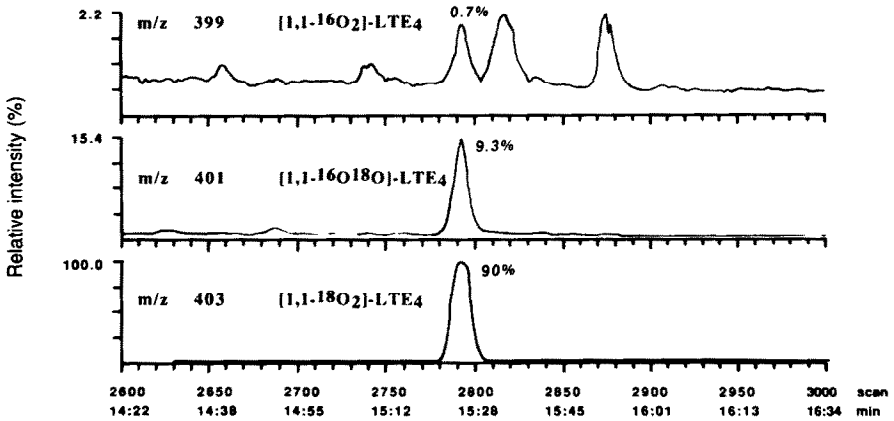

Fig. 3. Partial GC–MS chromatogram obtained from enzymically prepared $[^{18}O_2]LTE_4$ by SIM on m/z 403 ($[^{18}O_2]LTE_4$), m/z 401 ($[^{16}O^{18}O]LTE_4$) and m/z 399 ($[^{16}O_2]LTE_4$).

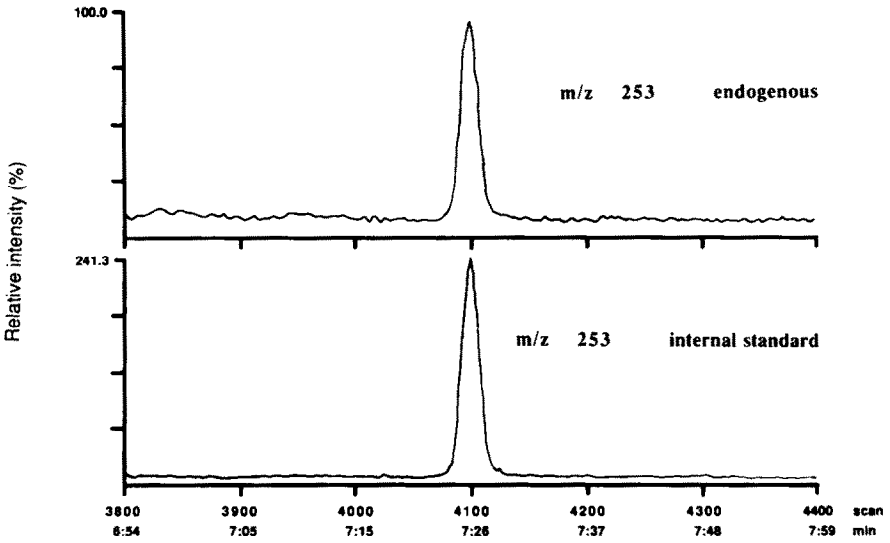

Fig. 4. Partial GC–MS–MS chromatogram from the analysis of a human urine sample (20 ml) spiked with 3 ng of $[^{18}O_2]LTE_4$. SIM on *m/z* 253, the same daughter ion of endogenous LTE_4 and $[^{18}O_2]LTE_4$ (see also Fig. 5). The concentration of endogenous LTE_4 in this urine sample was 68.1 pg/ml.

tion of daughter ions with *m/z* 256 and *m/z* 259 respectively, due to the number of deuterium atoms, these daughter ions of LTE_4s probably result from loss of a moiety containing both oxygen atoms of the carboxylic group [15]. The 5-HEA derivatives of endogenous LTE_4 as well as $[^{18}O_2]LTE_4$ emerged from the column at the same time. The concentration of endogenous LTE_4 in this urine sample was determined to be 68.1 pg/ml.

Back-exchange of ^{18}O with ^{16}O in ^{18}O-labelled eicosanoids is a common phenomenon, which mainly depends on esterase activities in biological materials and the ability of eicosanoids to form lactones [14]. Under the conditions used in our method for solid-phase extraction of LTE_4 from urine and HPLC separation [7], and catalytic reduction and desulphurization as described, no back-exchange was observed following separate incubation of 3 ng of $[^{18}O_2]LTE_4$ in 20 ml of acidified urine (pH 4.0) at 4°C for 30 min and 20 h. The ratios of the peak areas of $[^{16}O_2]LTE_4$ to $[^{18}O_2]LTE_4$ were determined to be 0.454 and 0.481, respectively. This difference lies within the variance of the reproducibility of the method. Also, $[^{18}O_2]LTE_4$ may be useful for GC–MS quantitation of LTE_4 in other biological materials when esterase-catalysed back-exchange is avoided by enzyme denaturation, as has been previously reported for other ^{18}O-labelled eicosanoids [12–14].

The use of pig liver esterases and $H_2{}^{18}O$ to introduce ^{18}O atoms into the carboylic group of LTE_4 methyl esters is an elegant method to prepare rapidly and easily $[^{18}O_2]LTE_4$ under mild conditions with sufficient yield. $[^{18}O_2]LTE_4$ is stable in urine for several hours and during catalytic hydrogenation/desulphurization, has the same retention time on RP-HPLC as endogenous LTE_4, and is therefore a useful internal standard in the quantitative determination of endogenous LTE_4 in human urine by GC–MS. Other ^{18}O-labelled cysteinyl leukotrienes could be prepared by this method and used as internal standards in GC–MS quantitation of their endogenous analogues.

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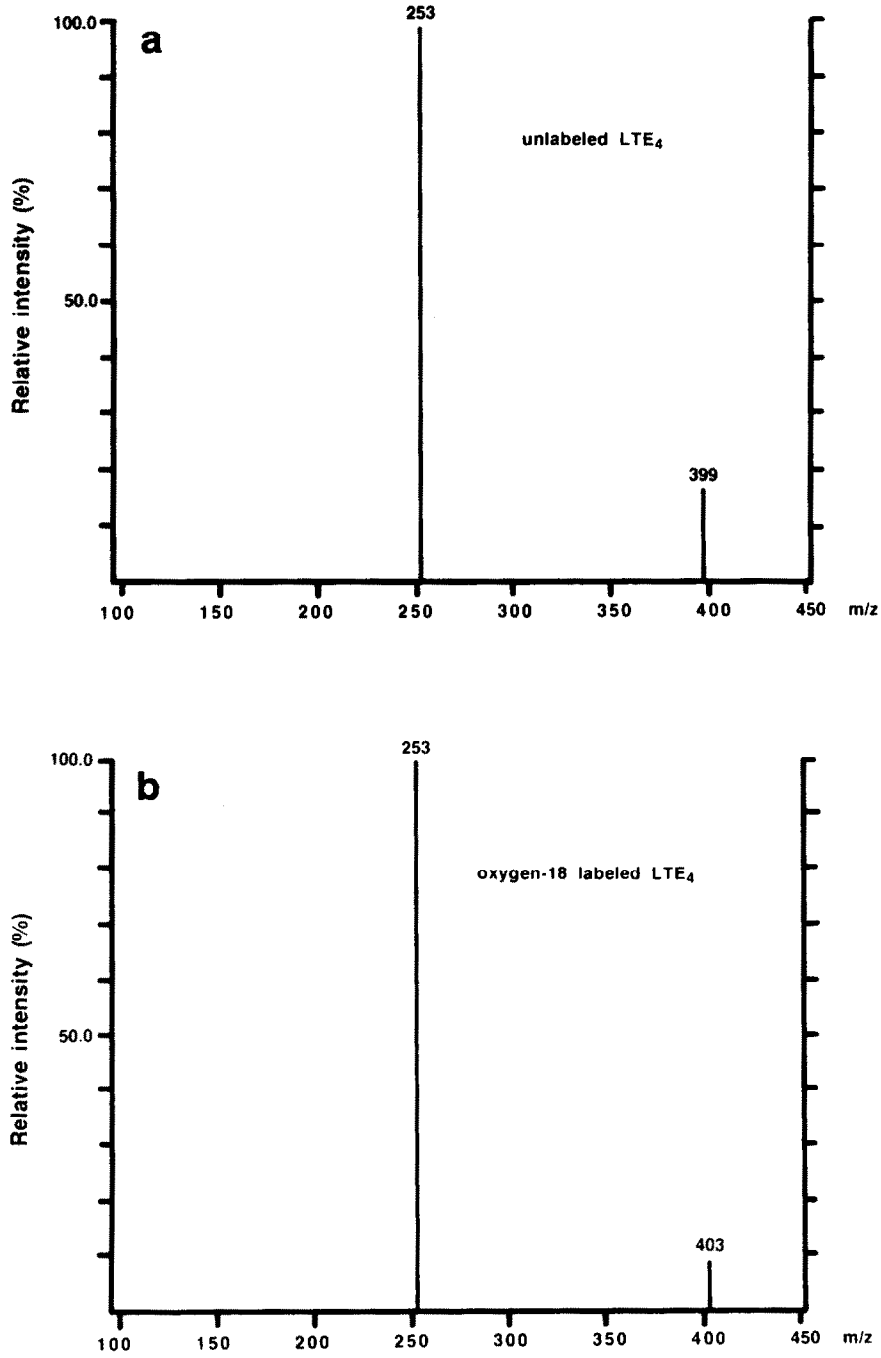

Fig. 5. Daughter mass spectra of unlabelled LTE_4 (a) and [$^{18}O_2$]LTE_4 (b) as their 5-HEAs PFB-TMS derivatives obtained by CAD of the corresponding parent ions $(M - PFB)^-$ on *m/z* 399 and *m/z* 403, respectively. The collision cell pressure was 3 mTorr and the collision energy 20 eV.

REFERENCES

1 M. Huber, S. Kästner, J. Schölmerich, W. Gerok and D. Keppler, *Eur. J. Clin. Invest.*, 19 (1989) 53.
2 N. H. Maltby, G. W. Taylor, J. M. Ritter, K. Moore, R. W. Fuller and C. T. Dollery, *J. Allergy Clin. Immunol.*, 85 (1990) 3.
3 L. Örning, L. Kaijser and S. Hammarström, *Biochem. Biophys. Res. Commun.*, 130 (1985) 214.
4 B. Samuelsson, *Science* (Washington, D.C.), 220 (1983) 568.
5 M. Huber, J. Müller, I. Leier, G. Jedlitschky, H. A., Ball, K. P. Moore, G. W. Taylor, R. Williams and D. Keppler, *Eur. J. Biochem.*, 195 (1990) 309.
6 A. P. Sampson, D. A. Spencer, C. P. Green, P. J. Piper and J. F. Price, *Br. J. Clin. Pharmacol.*, 30 (1990) 861.
7 J. Fauler, D. Tsikas, M. Holch, A. Seekamp, M. L. Nehrlich, J. Sturm and J. C. Frölich, *Clin. Sci.*, 80 (1991) 497.
8 L. Levine, R. A. Morgan, R. A. Lewis, K. F. Austen, D. A. Clark, A. Marfat and E. J. Corey, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 7692.
9 E. C. Hayes, D. L. Lombardo, Y. Girard, A. Maycock, J. Rokach, A. S. Rosenthal, R. N. Young and H. J. Zweerink, *J. Immunol.*, 131 (1983) 429.
10 P. Pradelles, J. Grassi and J. Maclont, *Anal. Chem.*, 57 (1985) 1170.
11 M. Balazy and R. C. Murphy, *Anal. Chem.*, 58 (1986) 1098.
12 W. C. Picket and R. C. Murphy, *Anal. Biochem.*, 111 (1981) 115.
13 J. Y. Westcott, K. L. Clay and R. C. Murphy, *Biomed. Mass Spectrom.*, 12 (1985) 714.
14 H.-J. Leis, W. Welz and E. Malle, *J. Chromatogr.*, 526 (1990) 169.
15 D. Tsikas, J. Fauler and J. C. Frölich, in preparation.