Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 5

SYNTHESIS OF [14,15,17,17,18,18-$^2H_6$]-LEUKOTRIENE B4

Dimitrios Tsikas*, Joachim Fauler and Jürgen C. Frölich

Department of Clinical Pharmacology, Hannover Medical School, P.O. Box 610180, W-3000 Hannover 61, Germany

Summary

Incubation of [14,15,17,17,18,18-$^2H_6$] leukotriene A4 obtained by hydrolysis of chiral [14,15,17,17,18,18-$^2H_6$] leukotriene A4 methylester with human monocytes resulted in the formation of [14,15,17,17,18,18-$^2H_6$] leukotriene B4 (d6-LTB4) and its trans isomers 6-trans-d6-LTB4 and 12-epi-6-trans-d6-LTB4 in high isotopic purity (99.4 % ^2H). d6-LTB4 was separated from its trans-isomers by reversed-phase high-performance liquid chromatography and identified by gas chromatography mass spectrometry. d6-LTB4 is shown to be a useful internal standard for gas chromatographic mass spectrometric analysis of LTB4 in biological matrix.

Key words

$^2H_6$-Leukotriene A4; $^2H_6$-leukotriene B4; human monocytes; gas chromatography mass spectrometry

Introduction

Leukotriene A4 (LTA4) is a key-intermediate in the 5-lipoxygenase cascade formed from arachidonic acid by the catalytic action of 5-lipoxygenase [1]. Enzymatic

0362–4803/92/050341–08$05.00
© 1992 by John Wiley & Sons, Ltd.
Received 21 October, 1991
Revised 26 November, 1991

hydratation and glutathione conjugation of LTA4 leads to the formation of leukotriene B4 (LTB4) and the cysteinyl leukotriene C4, respectively, which are potent inflammatory mediators [1]. Gas chromatography-mass spectrometry (GC-MS) of LTB4 and the cysteinyl leukotrienes has been shown to be a powerful tool for their quantitative determination in biological fluids [2,3]. However, this technique requieres analogues labelled with stable isotopes such as deuterium, oxygen-18 or carbon-13. Recently, the chemical synthesis of deuterium-labelled LTA4 standards [4,5] and deuterated cysteinyl leukotrienes [6,7] has been reported. Deuterated LTA4 could also be a useful starting material for the preparation of deuterated LTB4. As LTA4 is an extremely unstable compound in aqueous buffered solutions, which spontaneously hydrolyzes to the trans-isomers of LTB4 and 5,6-dihydroxyeicosatetraenoic acid but not LTB4, the only way to obtain deuterium-labelled LTB4 from deuterated-LTA4 is by enzymatic reaction as has been previously demonstrated for unlabelled LTA4 [8]. Here we describe the preparation of [14,15,17,17,18,18-$^2H_6$] LTB4 (d_6-LTB4) in high isotopic purity from the precursor [14,15,17,17,18,18-$^2H_6$] LTA4 (d_6-LTA4) using human monocytes and show its applicability as an internal standard for the GC-MS determination of LTB4. Human monocytes were chosen because they have a small capacity for ω-oxidation of LTB4 [11].

Experimental

General

Chiral d_6-LTA4 methylester (>99% ^2H, 1 mg/ml in acetonitrile) and unlabelled LTA4 methylester were kindly provided by Prof. H.J. Bestmann and Dr. T. Röder, University of Erlangen, Germany. d_6-LTA4 free acid was prepared from d_6-LTA4 methylester as described previously [8]. Unlabelled LTB4 was kindly provided by Dr. J. Rockach, Merck Frosst, Canada. Unlabelled 6-trans-LTB4 and 6-trans-12-epi-LTB4 were obtained by hydrolysis of LTA4 methylester according to [10]. Pentafluorobenzyl (PFB) bromide was obtained from Aldrich, Steinheim, Germany. N,O-Bis(trimethylsilyl)trifluoroacetamide was purchased from Pierce,

Rockford, IL, USA. Reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out using an LKB solvent delivery system and a Kratos UV-VIS detector as described in details elsewhere [11]. All organic solvents used were of HPLC quality. The retention times of unlabelled 6-trans-LTB4, 6-trans-12-epi-LTB4 and LTB4 were 48.5, 50.1 and 52.8 min, respectively. Quantitation of deuterated LTB4 was employed by RP-HPLC and UV detection (270 nm) using a molar coefficient of extinction of ε=50,000. For GC-MS analysis labelled and unlabelled LTB4 were converted to their PFB ester trimethylsilyl (TMS) ether derivatives as described previously [11]. GC-MS of the PFB-TMS derivatives was performed on a Finnigan MAT TSQ 45 in the negative ion chemical ionization (NICI) mode, operated in the single quadrupole mode, after gas chromatographic separation on a Finnigan gas chromatograph model 9611 equipped with an OV1, 25 m x 0.25 mm I.D., 0.25 mm film thickness, fused silica capillary column from Macherey-Nagel, Düren, Germany.

Enzymatic synthesis of deuterated leukotriene B4

LTA4 methylester has been shown not to be a substrate for the enzymatic conversion to LTB4 or to LTB4 methylester in several cell systems [9]. Therefore, d6-LTA4 methylester was hydrolyzed to its free acid form immediately prior to incubation according to a method described in [8]. Briefly, 10 µl of the d6-LTA4 methylester solution in acetonitrile were evaporated under nitrogen, the residue was treated with 500 µl of a solution of acetone/0.25 N NaOH, 8:2, v/v, and allowed to stay at 25 °C for 60 minutes. As LTA4 is highly unstable in aqueous buffers of neutral pH [8,9] this solution was stored at -80 °C until use. Immediately prior to incubation acetone was evaporated under a stream of nitrogen. Human monocytes were isolated from blood of a healthy volunteer and prepared as previously described [11]. Monocytes ($5x10^6$ cells/ml) were incubated in Hank's balanced salt solution containing 0.1 % bovine serum albumin for one hour prior to incubation at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The reaction was started by addition of 25 µl-aliquots of the sodium hydroxide solution of freshly prepared d6-LTA4 (approx. 2.5 µg/10^6 cells) and the mixture was allowed to react

for four hours. The reaction was terminated by addition of ice-cold methanol and the mixture was cooled at -20 °C. After centrifugation (200 xg, 10 min) the supernatants were dekanted, combined and 200 µl-aliquots were analyzed by RP-HPLC. The RP-HPLC fractions eluted 0.5 min earlier than unlabelled LTB4 and its isomers (Fig. 1). They were collected individually and the organic solvent was removed under a stream of nitrogen. The residue was acidified to pH 4.0 and the compounds were finally extracted with ethylacetate. After solvent evaporation the residue was reconstituted in 1 ml of methanol from which aliquots were taken for GC-MS analysis and quantification by RP-HPLC.

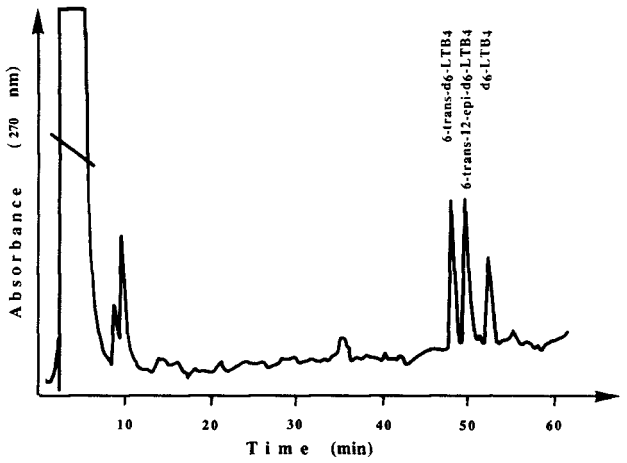

Figure 1: RP-HPLC analysis of the supernatant of d6-LTA4 incubated with human monocytes at 37 °C. For more details see text

Results and discussion

Fig. 1 shows a typical RP-HPLC chromatogram from the analysis of the supernatant of d6-LTA4 free acid incubated with human monocytes. The compounds eluted at 48.0, 49.6 and 52.2 min were identified by RP-HPLC and

NICI GC-MS analysis of the unlabelled synthetic standards 6-trans-LTB4, 6-trans-12-epi-LTB4 and LTB4.

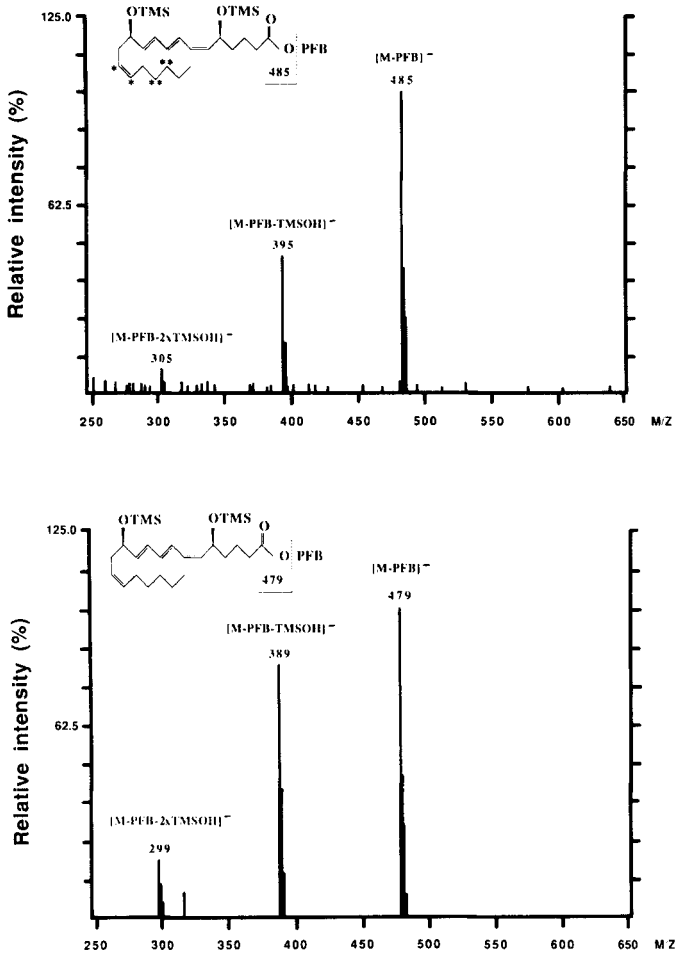

Figure 2: NICI mass spectra of the PFB-TMS derivatives of the enzymatic product eluted at 52.2 min on RP-HPLC (top) and unlabelled LTB4 (bottom). The asterisks indicate the number and the position of the deuterium atoms

The PFB-TMS derivative of the compound eluted on RP-HPLC at 52.2 min emerged from the GC column nearly at the same time as unlabelled LTB4. The

NICI mass spectrum of this compound (Fig. 2, top) showed characteristic mass fragments at m/z 485 [M-PFB]$^-$, m/z 395 [M-PFB-TMSOH]$^-$ and m/z 305 [M-PFB-2xTMSOH]$^-$ which are increased by six atom mass units (amu) compared to the corresponding derivative of unlabelled LTB4 (Fig. 2, bottom; m/z 479, 389, and 299). The absence of the mass fragments at m/z 479, 389 and 299 in the mass spectrum shown in Fig. 2a indicates that no LTB4 was synthesized from endogenous arachidonic acid. The PFB-TMS derivatives of the compounds eluted on RP-HPLC at 48.0 and 49.6 min emerged from the GC column (later than d6-LTB4) at nearly the same time as the corresponding derivatives from the unlabelled standards and had mass spectra identical to d6-LTB4 (data not shown). These findings strongly suggest that the compound enzymatically formed from d6-LTA4 is [14,15,17,17,18,18-^{2}H6]-LTB4. The trans-isomers 6-trans-[14,15,17,17,18,18-^{2}H6]-LTB4, and 6-trans-12-epi-[14,15,17,17,18,18-^{2}H6]-LTB4 and were formed non-enzymatically.

GC-MS analysis of the compound eluting from RP-HPLC at 52.2 min by selected ion monitoring (SIM) on m/z 485 for d6-LTB4 and m/z 479 for unlabelled LTB4 (Fig. 3) and integration of the peak areas showed 99.4 % d6-LTB4 (scan number 4337). Similar results were also obtained for the trans-isomers. Quantitation of isolated d6-LTB4 and its trans-isomers employed by RP-HPLC and UV detection (270 nm, ε=50,000) gave 321 ng d6-LTB4 and each 524 ng for the trans-isomers.

The high isotopic purity of the enzymatically synthesized d6-LTB4 fullfilles the requirement for an internal standard for quantitation of endogenous LTB4 by GC-MS. This was checked by derivatization of unlabelled LTB4 (0 to 1000 pg) with each 500 pg of d6-LTB4, GC-MS analysis in the SIM on m/z 479 and m/z 485 and linear regression analysis of the ratio m/z 479 to 485 (y) vs. m/z 479 (x) gave a straight line with the equation $y=0.054+0.00194x$, $r>0.994$. Because of the favorable position of the deuterium atoms in the molecules of the deuterated LTB4 and its trans-isomers these compounds could also be useful starting materials to

prepare enzymatically their corresponding deutererated metabolites of ω- and β-oxidation as well as reduction using different cells [11-14].

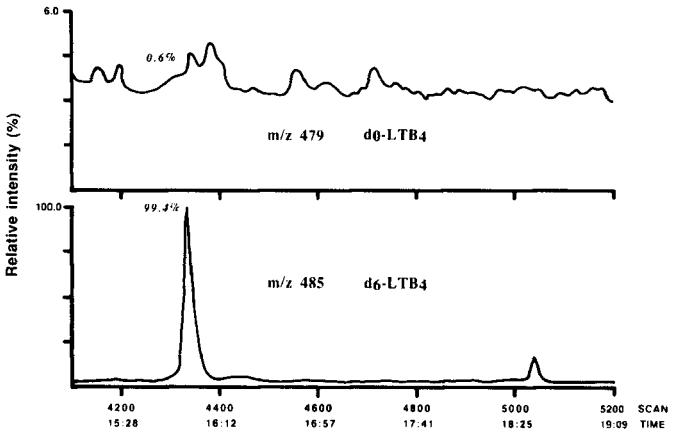

Figure 3: Partial NICI GC-MS chromatogram obtained from enzymatically prepared d6-LTB4 (retention time on RP-HPLC 52.2 min, Fig. 1) by SIM on m/z 485 (d6-LTB4, scan number 4337; bottom) and m/z 479 (d0-LTB4, scan number 4344; top). Note different scales for ordinates

Conclusions

Enzymatic hydratation by human monocytes of d6-LTA4, which was obtained by alkaline hydrolysis of its stable methylester form, is an efficient method to prepare d6-LTB4 in high isotopic purity. d6-LTB4 is suitable as an internal standard for GC-MS quantitation of endogenous LTB4 in various biological fluids. Non-enzymatic hydratation of d6-LTA4 leads to the formation of the corresponding hexadeuterated trans-isomers of LTB4 in high isotopic purity.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (Grant Fr 366/5). The authors wish to thank Prof. H.J. Bestmann and Dr. Th. Röder for providing [14,15,17,17,18,18-$^2H_6$] LTA4 methylester

References

1 Samuelsson B.-Science 200: 568 (1973)

2 Lellouche J.P., Aubert F. and Beaucourt J.P.-Tetrahedron Lett. 29: 3069 (1988)

3 Hughes H., Mitchell J.R. and Gaskell S.J.-Anal. Biochem. 179: 304 (1989)

4 Balazy M. and Murphy R.C.-Anal. Chem. 58: 1098 (1986)

5 Bestmann H.J., Meese C.O. and Röder T.-J.Lab.Comp.Radiopharm. 27: 1325 (1989)

6 Lellouche J.P., Aubert F., Noel J.P. Boullais C. and Beaucourt J.P.-J.Lab.Comp.Radiopharm. 27: 473 (1989)

7 Röder T.-personal communication

8 Carrier D.J., Bogri T., Cosentino G.P., Guse I., Rakhit S. and Singh K.-Prostagl. Leukotr. Essent. Fatty Acids 34: 27 (1988)

9 Maycock A.L., Anderson M.S., DeSousa D.M. and Kuehl F.A., Jr.-J.Biol.Chem. 257: 13911 (1982)

10 Breuer O. and Hammarström S.-Biochem.Biophys.Res.Commun. 142: 667 (1987)

11 Fauler J., Marx K-H., Kaever V. and Frölich J.C.-Prostagl.Leukotr.Essent.Fatty Acids 37: 193 (1989)

12 Powell W.S. and Gravelle F.-J.Biol.Chem. 263: 2170 (1988)

13 Powell W.S. and Gravelle F.-J.Biol.Chem 264: 5364 (1989)

14 Tsikas D. and Frölich J.C.-Naunyn-Schmiedebergs Arch.Pharmacol. Suppl. to Vol. 341: R38 Abstract Nr. 151, p.190 (1990)