ELSEVIER

Journal of Chromatography B, 666 (1995) 197–202

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatography–thermospray mass spectrometry of ω-carboxyleukotriene B_4 and ω-hydroxyleukotriene B_4 from an incubation mixture of human colonic well-differentiated adenocarcinoma homogenate

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First received 11 October 1994; revised manuscript received 6 December 1994; accepted 19 December 1994

Abstract

A method for the analysis of ω-carboxyleukotriene B_4 and ω-hydroxyleukotriene B_4 in human colonic carcinoma homogenate is described. The hydroxy groups of the leukotriene metabolite were acetylated by acetic anhydride, and the mixture was partially purified on a Sep-Pak C_{18} cartridge and analysed by reversed-phase HPLC–thermospray MS. Generally, the base ion, $[MH-2(60)]^+$, is produced through elimination of two acetic acid (60 mass units) molecules from the protonated molecular ion. On selected-ion monitoring, standard curves for ω-carboxy- or ω-hydroxyleukotriene B_4 showed a linear relationship over the range 72–1500 pmol. The assay based on selected-ion monitoring was applied to an extract from human colonic carcinoma homogenate. When a homogenate of human colonic well-differentiated adenocarcinoma was incubated with NADPH and leukotriene B_4 (60.6 nmol) as a substrate, the conversion of precursor leukotriene B_4 to ω-carboxyleukotriene B_4 or ω-hydroxyleukotriene B_4 was 0.33 or 3.17%, respectively. Based on these results, it is suggested that carcinoma cells themselves or leukocytes at the hostsite in a region of human colonic well-differentiated adenocarcinoma are performing ω-oxidation through NADPH-dependent ω-hydroxylation of leukotriene B_4.

1. Introduction

The inflammatory mediator leukotriene B_4 (LTB_4) is a metabolite of arachidonic acid produced by the activation of the 5-lipoxygenase enzyme. LTB_4 is a potent stimulus for chemotaxis and chemokinesis in human polymorphonuclear leukocytes (PMNL), mononuclear cells and others [1–3]. This compound is inactivated by the cytochrome P-450 system through ω-hydroxylation in rat hepatocyte [4–6], human neutrophils [7–9] and guinea pig eosinophils [10], or by the 12-hydroxydehydrogenase system in the kidney [11] or PMNL [12] of porcine.

Although LTB_4, ω-hydroxy-LTB_4 (ω-OH-LTB_4), (ω-1)-OH-LTB_4 and ω-carboxy-LTB_4 (ω-COOH-LTB_4) can be detected by reversed-phase (RP) HPLC with UV detection, there is

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0378-4347/95/$09.50
SSDI 0378-4347(94)00582-6

overlap of (ω-1)-OH-LTB_4 and ω-COOH-LTB_4 on the chromatogram [10]. The RP-HPLC–thermospray MS (RP-HPLC–TSP-MS) technique is able to separate each underivatized component and thus overlap is prevented. However, this procedure suffers from low sensitivity for di- or tri-hydroxy polyunsaturated fatty acids such as ω-COOH-LTB_4 and ω-OH-LTB_4, due to the appearance of numerous fragment ions following elimination of hydroxy groups [14].

The authors recently developed a method for the simultaneous detection of polyhydroxy polyunsaturated fatty acids such as LTB_4, lipoxine A_4 (or B_4), prostaglandins and thromboxanes by RP-HPLC–TSP-MS [13–15]. In this method, hydroxy groups were acetylated with acetic anhydride, partially purified on a Sep-Pak C_{18} cartridge and analysed by RP-HPLC–TSP-MS. Generally, the base ion, $[MH - n(60)]^+$, is produced through elimination of acetic acid (n = number of hydroxyl groups). In this study, the hydroxyl groups of ω-COOH-LTB_4 or ω-OH-LTB_4 were acetylated, the products were partially purified and analysed by RP-HPLC–TSP-MS. This procedure was applied to ω-COOH-LTB_4 and ω-OH-LTB_4 from the homogenate of human colonic well-differentiated adenocarcinoma.

2. Experimental[1]

2.1. Standards and reagents

LTB_4, ω-OH-LTB_4 and ω-COOH-LTB_4 were obtained from Cascade Biochem (Reading, UK). Prostaglandin $F_2\alpha$-3,3,4,4-$^2H_4$ (PGF_2 α-d_4) was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). β-NADPH was obtained from Oriental Yeast Co. (Osaka, Japan). Other solvents and reagents were of analytical-reagent grade.

2.2. Extraction from the homogenate of human colonic carcinoma

Specimens of human (male) colonic well-differentiated adenocarcinoma (0.55 g wet weight) were collected upon operation. The tissue was homogenized in a Polytron (Kinematica Switzerland) homogenizer in 10 ml of 50 m*M* Tris-HCl buffer (pH 7.5). The homogenate (4.9 ml), LTB_4 (60.6 nmol, final concentration of 12.1 μM) and β-NADPH (0.1 ml of 50 mg/ml, final concentration of ca. 1 m*M*) were homogenized in a vortex-mixer to disperse the substrate. A control experiment was carried out without LTB_4. Each mixture was incubated at 37°C for 30 min under aerobic conditions in a shaker operated at 120 rpm. The incubation mixture was mixed with 1.387 nmol PGF_2 α-d_4 as the internal standard (I.S.) and 25 ml of ice-cold methanol. The mixture was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was concentrated until water began to distil away under reduced pressure. The concentrate was acidified to ca. pH 3 with 15% formic acid and applied to a Sep-Pak C_{18} cartridge (Waters Co., Milford, MA, USA) equilibrated with water. The cartridge was washed with 5 ml of water. OH-LTB_4 and COOH-LTB_4 in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure.

2.3. Derivatization and RP-HPLC–TSP-MS

OH-LTB_4 and COOH-LTB_4 were derivatized using acetic anhydride in pyridine to obtain the acetic esters, which were then partially purified on a SeP-Pak C_{18} cartridge and analysed by RP-HPLC–TSP-MS as previously described [13–15] except that the acetylation was achieved under argon gas at 25°C in the dark for 4 h, and 0.1 *M* ammonium formate–0.1 *M* formic acid–acetonitrile (8:2:15, v/v) was used as a mobile phase for RP-HPLC-TSP-MS.

[1] Abbreviations used: leukotriene B_4 (LTB_4); human polymorphonuclear leukocyte (PMNL); ω-hydroxy-LTB_4 (ω-OH-LTB_4); ω-carboxy-LTB_4 (ω-COOH-LTB_4); reversed-phase (RP); RP-HPLC–thermospray MS (RP-HPLC–TSP-MS); prostaglandin F_2 α-3,3,4,4-$^2H_4$ (PGF_2 α-d_4); internal standard (I.S.); selected-ion monitoring (SIM).

2.4. Standard curve and recovery experiment

For the standard curve, a fixed aliquot (0.555 nmol) of PGF_2 α-d_4 as I.S. was mixed with various, known quantities of ω-COOH-LTB_4 or ω-OH-LTB_4 ranging from 0.072 to 1.50 nmol, acetylated and analysed by RP-HPLC–TSP-MS in the selected-ion monitoring (SIM) mode as described above. The peak area corresponding to ω-COOH-LTB_4 or ω-OH-LTB_4 on each SIM of $[MH^+ - 120]^+$ ion (m/z 331 or 359) was compared with the peak area corresponding to PGF_2 α-d_4 as I.S. on a SIM of the $[MH^+ - 180]^+$ ion (m/z 305).

For the recovery experiment, ω-COOH-LTB_4 (3.00 nmol), ω-OH-LTB_4 (2.89 nmol), PGF_2 α-d_4 (1.11 nmol) as I.S., 5 ml of 50 m*M* Tris-HCl buffer (pH 7.5) and 25 ml of methanol were mixed. The mixture was concentrated, acidified, and applied to a Sep-Pak C_{18} cartridge as described above. The eluent was evaporated, and the residue was acetylated and analysed by RP-HPLC–TSP-MS as described above.

3. Results and discussion

The acetyl derivatives of ω-COOH-LTB_4 and ω-OH-LTB_4 standards each showed a characteristic MS pattern (Fig. 1A,B) compared with native ω-COOH-LTB_4 and ω-OH-LTB_4 (Fig. 1C,D). As shown in Fig. 1A,B, the base-ion of the acetyl derivative of ω-COOH-LTB_4 or ω-OH-LTB_4 was $[MH - 120]^+$, based on the elimination of two acetic acid (60 mass units) molecules from the protonated molecular ion. Since the rate of elimination of an acetyl group introduced at the ω-position is low, the $[MH - 120]^+$ (m/z 359) is the base-ion of the ω-OH-LTB_4 acetyl derivative. As shown in Fig. 1C,D, the direct analysis of native ω-COOH-LTB_4 and ω-OH-LTB_4 by RP-HPLC–TSP-MS is not satisfactory owing to low sensitivity due to the appearance of many fragment ions following elimination of hydroxyl groups.

The standard curves for the acetyl derivatives of ω-COOH-LTB_4 or ω-OH-LTB_4 are obtained as follows. When the peak area corresponding to the acetyl derivative of ω-COOH-LTB_4 (m/z 331) or ω-OH-LTB_4 (m/z 359) on each SIM chromatogram of $[MH - 120]^+$ is compared with the peak area corresponding to the PGF_2 α-d_4 acetyl derivative (as I.S.) on a SIM chromatogram of $[MH - 180]^+$ (m/z 305), an approximately linear relationship exists between the peak-area ratios of ω-COOH-LTB_4 or ω-OH-LTB_4 to I.S. and the amount of ω-COOH-LTB_4 or ω-OH-LTB_4. The regression equation for ω-COOH-LTB_4 is described as $y = 0.39x$ ($n = 1$) where y is the peak-area ratio of acetylated ω-COOH-LTB_4 to I.S. and x is the amount of ω-COOH-LTB_4 (nmol). The regression equation for ω-OH-LTB_4 is described as $y = 0.42x$ ($n = 1$) where y is the peak-area ratio of acetylated ω-OH-LTB_4 to I.S. and x is the amount of ω-OH-LTB_4 (nmol).

The recovery experiment of ω-COOH-LTB_4 and ω-OH-LTB_4 from the incubation medium gave recoveries of 65.6 and 99.5% of the added amounts, respectively ($n = 1$). It is stipulated that the retention values of ω-COOH-LTB_4 in the first Sep-Pak C_{18} cartridge procedure decreases compared with ω-OH-LTB_4 or I.S.

SIM chromatograms of the acetyl derivatives obtained from the standards or an extract from an incubation of LTB_4 added to human colonic adenocarcinoma homogenate with β-NADPH are shown in Fig. 2. On the basis of m/z 331, 408 or 413, peak A in Fig. 2 was assigned to ω-COOH-LTB_4 (Fig. 1A). On the basis of m/z 359, 436 or 441, peak B in Fig. 2 was assigned to ω-OH-LTB_4 (Fig. 1B). On the basis of m/z 305, peak C in Fig. 2 was assigned to PGF_2 α-d_4 as I.S. as described previously [13,14]. In an extract of an incubation mixture of human colonic well-differentiated adenocarcinoma homogenate with NADPH and LTB_4, acetyl derivatives of ω-COOH-LTB_4 and ω-OH-LTB_4 (Fig. 2,II) were detected in comparison with the control experiment (Fig. 2,III). The amounts of ω-COOH-LTB_4 or ω-OH-LTB_4 from the incubation experiment were 0.202 and 1.92 nmol, and the conversion % of precursor LTB_4 (60.6 nmol) to ω-COOH-LTB_4 or ω-OH-LTB_4 was 0.33 and 3.17%, respectively. However, since slight amounts of acetyl derivatives of ω-COOH-LTB_4

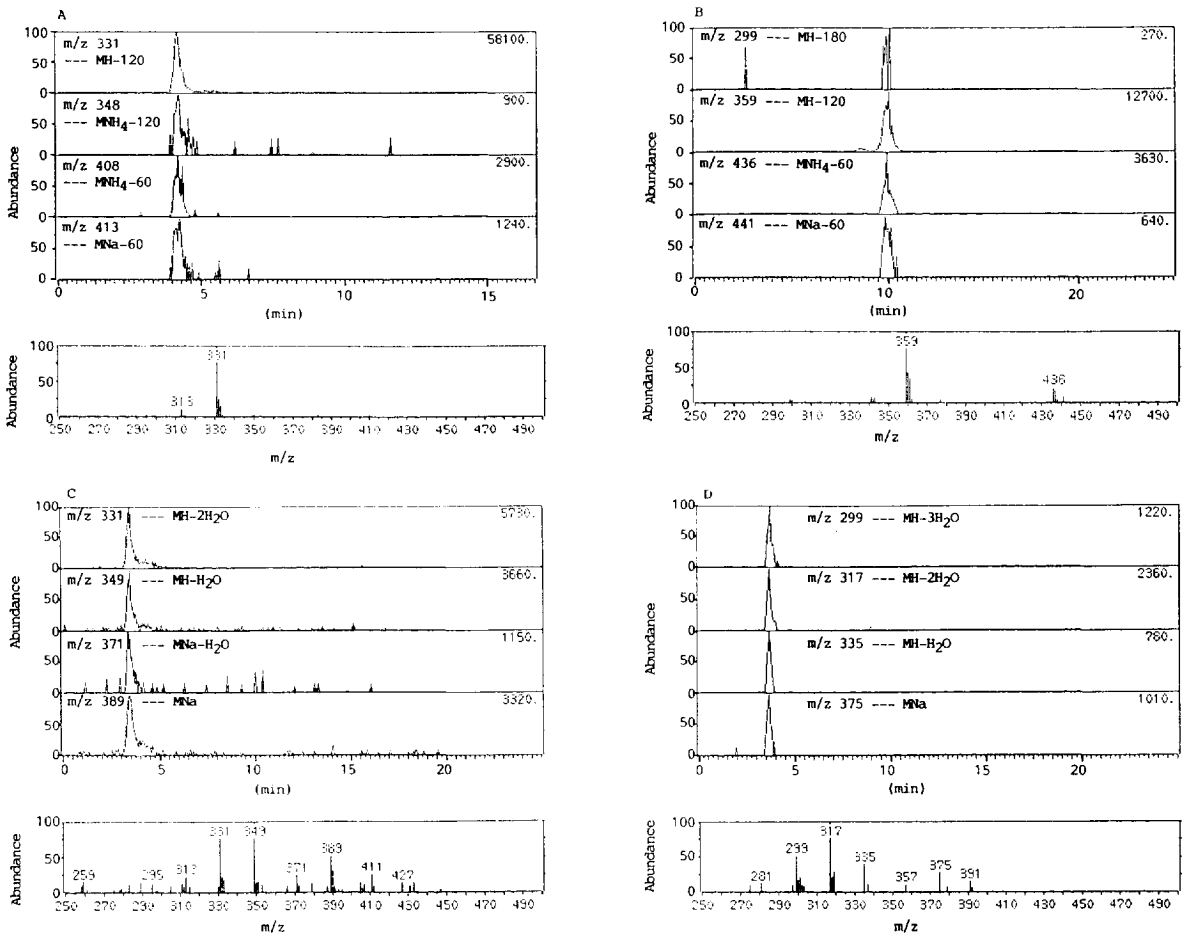

Fig. 1. Mass chromatogram profile on each m/z number and mass spectra obtained from ω-COOH-LTB$_4$ or ω-OH-LTB$_4$ acetyl derivatives and underivatized ω-COOH-LTB$_4$ or ω-OH-LTB$_4$. HPLC and TSP conditions as described in Experimental. Scan speed, 1.0 scan/s from m/z 250 to 500. Filament off. Spectral patterns corresponding to the main peak on each mass chromatogram are shown below. (A) ω-COOH-LTB$_4$ acetyl derivative, ca. 3 nmol; (B) ω-OH-LTB$_4$ acetyl derivative, ca. 1 nmol; (C) ω-COOH-LTB$_4$, ca. 3 nmol; (D) ω-OH-LTB$_4$, ca. 3 nmol. The number in the upper right-hand corner of each chromatogram is the ion count.

and ω-OH-LTB$_4$ (Fig. 2,III) were detected in the control experiment, it is thought that the homogenate contained endogenous LTB$_4$, ω-COOH-LTB$_4$ or ω-OH-LTB$_4$. Based on these results, it is suggested that carcinoma cells themselves or leukocytes at the host site in a region of human colonic well-differentiated adenocarcinoma are performing ω-oxidation through NADPH-dependent ω-hydroxylation of LTB$_4$.

We recently found a high activity of NADPH-dependent ω-hydroxylation of docosahexaenoic acid or arachidonic acid in human colonic well-differentiated adenocarcinoma homogenate and rat colonic mucosa homogenate (unpublished data). We recently reported on the high activity of NADPH-dependent ω-hydroxylation of (ω-3)-system fatty acids such as docosahexaenoic acid or eicosapentaenoic acid in rat brain or large intestine homogenate [16]. Since the central nervous system and mucosal cells of colonic well-differentiated adenocarcinoma are systems arising from epithelium and epithelial cells, respectively, these ω-oxidation activities may be related to functions characteristic of epithelial cells.

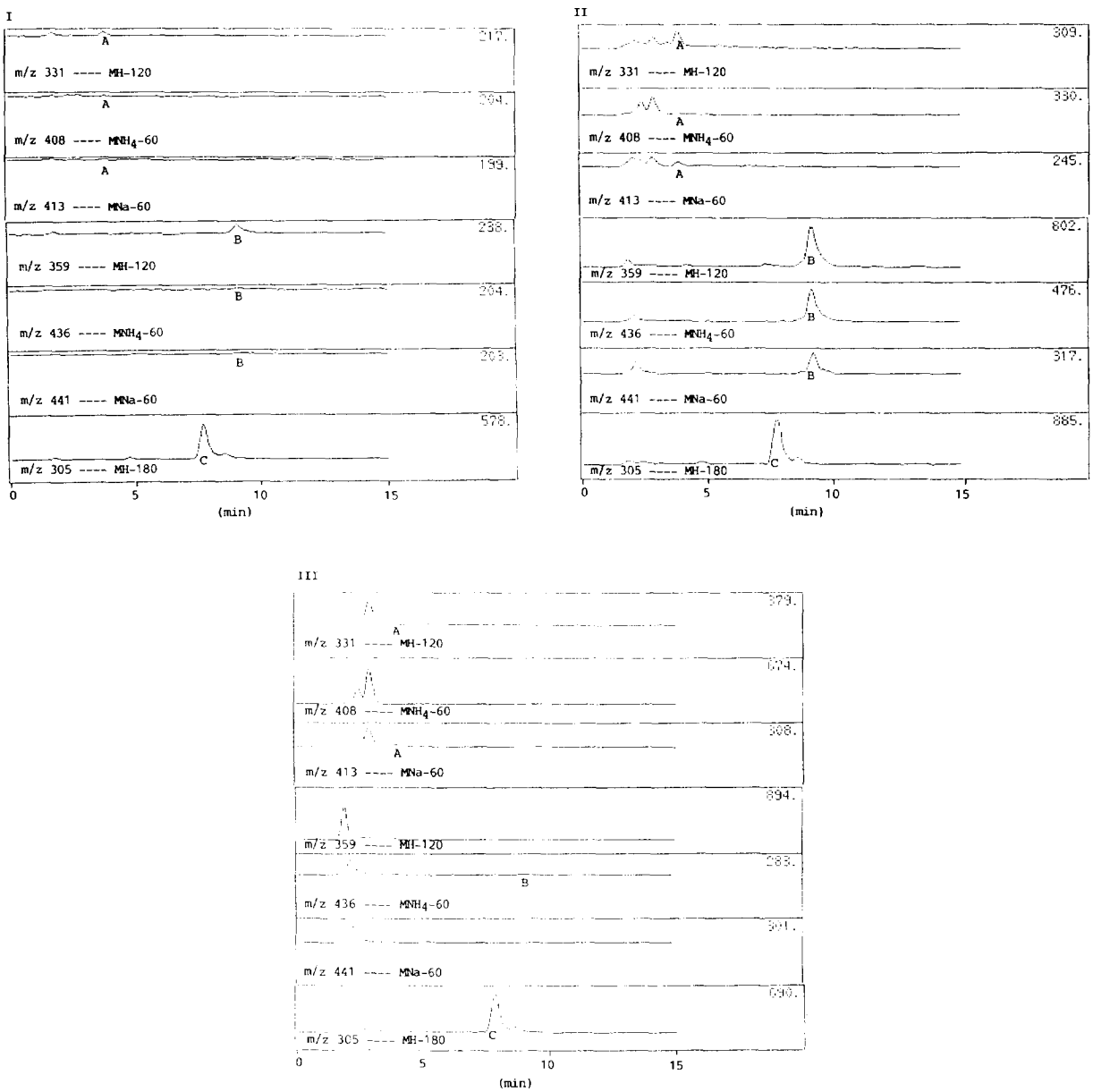

Fig. 2. SIM chromatograms of acetyl derivatives of authentic materials (ω-COOH-LTB$_4$, ω-OH-LTB$_4$ and PGF$_2$ α-d$_4$), an extract from an incubation mixture of LTB$_4$ with human colonic adenocarcinoma homogenate and an extract from an incubation mixture of only human colonic adenocarcinoma homogenate as the control experiment. The number in the upper right-hand corner of each chromatogram is the ion count. (I) authentic ω-COOH-LTB$_4$, ω-OH-LTB$_4$ and PGF$_2$ α-d$_4$; (II) an incubation mixture of LTB$_4$ with human colonic adenocarcinoma homogenate; (III) an incubation mixture of only human colonic adenocarcinoma homogenate as the control experiment.

The commonly used methods for the analysis of LTB$_4$ metabolites consist of the separation of each radioactive LTB$_4$ metabolite from a precursor radioactive LTB$_4$ by a RP-HPLC method with radioactive detection, the preparation of the methyl ester (or pentafluorobenzyl ester) and

trimethylsilyl ether derivative of the metabolite and GC–MS analysis [10,17–19]. Although this method is the most reliable method at present, it is a tedious method for the purposes of a simple screening test. Since the hydroxy groups of the LTB_4 metabolites are acetylated and a strong base-ion of each LTB_4 metabolite was thus detected in RP-HPLC-TSP-MS, the present method is well suited for the simultaneous measurement of ω-COOH-LTB_4 and ω-OH-LTB_4 in a simple screening test in vitro.

4. Conclusions

Since the acetyl derivatives of ω-COOH-LTB_4 and ω-OH-LTB_4 were characterized by the high intensity of the $[MH - 120]^+$ ion in RP-HPLC–TSP-MS analysis, SIM using these ions could be used to selectively detect ω-COOH-LTB_4 or ω-OH-LTB_4. ω-COOH-LTB_4 and ω-OH-LTB_4 from a precursor LTB_4 were detected in the extract of an incubation mixture of human colonic well-differentiated adenocarcinoma with NADPH. The carcinoma cells themselves or leukocytes at the host-site in a region of human colonic well-differentiated adenocarcinoma thus appear to exhibit ω-oxidation system functions through NADPH-dependent ω-hydroxylation of LTB_4.

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