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Note

Quantitation of sulfidopeptide leukotrienes by reversed-phase high-performance liquid chromatography

MICHAEL MÜLLER and TANIA C. SORRELL*

Department of Medicine, University of Sydney, Westmead Centre, Westmead, N.S.W. 2145 (Australia)

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Leukotrienes (LT), products of the lipoxygenase pathway of arachidonic acid metabolism, exhibit potent proinflammatory, bronchospastic and vasospastic effects when tested in vitro and in vivo [1, 2]. The potential importance of these compounds in the pathophysiology of human disease has led to the development of complementary methods for their identification, purification and quantitation. Of these, reversed-phase high-performance liquid chromatography (HPLC) is the most convenient for determining profiles of lipoxygenase products in biological samples [3]. However, standard reversed-phase HPLC systems cannot be used to quantitate sulfidopeptide leukotrienes because of poor reproducibility of chromatograms and variable recoveries [4]. Ionic interactions between functional groups on the leukotrienes and ionized impurities in the stationary or mobile phase of the chromatographic system have been proposed to explain these findings [4].

Reproducible assays of LTC_4, with a sensitivity of 2—3 ng, have been obtained by treatment of the column with the chelating agent EDTA prior to injection [4, 5]. However, prolonged washing of columns with EDTA followed by incorporation of EDTA into the mobile phase, or the use of daily bolus injections have been required to maintain column performance [4, 5]. In addition, EDTA is insoluble in methanol and acetonitrile, both of which are employed in gradient separations of leukotrienes and in washing reversed-phase columns between injections of biological samples.

Oxalic acid, which is soluble in methanol and acetonitrile, has been used successfully to flush metal ions from ODS columns [6]. In this report, we describe a rapid, convenient method for reproducible HPLC of sulfidopeptide

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leukotrienes, using small bolus injections of oxalic acid, or oxalic acid incorporated into the mobile phase.

MATERIALS AND METHODS

The HPLC system consisted of a Waters Assoc. Model M6000A pump, a U6K injector and M401 UV detector operated at a wavelength of 280 nm, 0.01 a.u.f.s. Reversed-phase chromatography was performed on an Altex Ultrasphere ODS 5-μm column (250 × 4.6 mm) (Beckman Instruments, Sydney, Australia) at a flow-rate of 1 ml min^{-1}. HPLC-grade methanol and oxalic acid were purchased from Mallinckrodt (Oakleigh, Australia), disodium EDTA from Sigma (St. Louis, MO, U.S.A.), and acetic acid from Anax (Ryde, Australia). Pure water was prepared from distilled water using Norganic cartridges (Waters Assoc., Lane Cove, Australia). All solvents were filtered and degassed by vacuum prior to use.

Prostaglandin B_2 (PGB_2, Sigma) and synthetic leukotrienes (LTB_4, LTC_4, LTD_4, LTE_4, a kind gift of Dr. J. Rokach, Merck Frosst, Canada) were used as standards. Biological samples were prepared from murine peritoneal macrophages. Briefly, adherent peritoneal cells were incubated overnight at 37°C in Dulbecco's modification of Eagle medium (DMEM; GIBCO, Long Island, NY, U.S.A.) supplemented with 5% heat-inactivated foetal calf serum (Flow Labs., Sydney, Australia), penicillin 100 U ml^{-1} and streptomycin 100 μg ml^{-1}. Cells were then washed and incubated with 200 μg ml^{-1} zymosan (Sigma) in DMEM for 2 h. Arachidonic acid metabolites were extracted from the supernatant medium by the method of Unger et al. [7], dried under nitrogen and reconstituted in mobile phase. The mobile phase consisted of methanol—water—acetic acid (65:34.95:0.05, v/v/v) adjusted to pH 5.6 with ammonium hydroxide. Oxalic acid was applied to the column either as a 100—200 μl bolus of a 10 m*M* solution or by incorporation into the mobile phase at a concentration of 0.5 m*M*.

RESULTS

In preliminary experiments, the stability of LTC_4, LTD_4, LTE_4 (8 μg each) was determined in aqueous solutions of 5 m*M* oxalic acid; leukotriene absorption spectra (240—320 nm) and ultraviolet absorbance at 280 nm remained unchanged during incubation at room temperature for up to 90 min. LTB_4 (4 μg) remained stable under similar conditions in a solution of 7 m*M* oxalic acid containing 25% (v/v) methanol.

Tailing of LTC_4, LTD_4 and LTE_4 peaks was evident in chromatograms of standard mixtures applied to columns not treated previously with oxalic acid (Fig. 1, pre-oxalate). The injection of 200 μl of a solution of oxalic acid (10 m*M*) resulted in the elution of material in a small, narrow peak, with a retention time identical to that of LTC_4 (data not shown). Following injection of oxalic acid, the column equilibration time was 14 min. Subsequent chromatograms of LTC_4 and LTE_4 revealed a substantial increase in plate counts, but little change in retention time or selectivity (Fig. 1, post-oxalate, A). The effect of a single bolus of oxalic acid persisted for several hours, depending on column

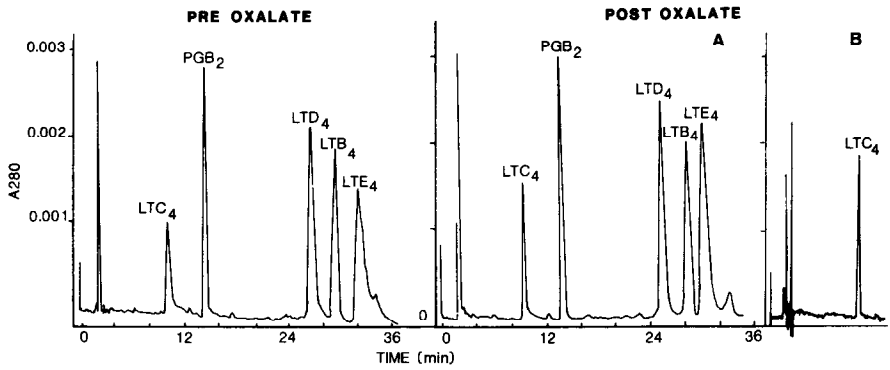

Fig. 1. Representative chromatograms of LTC_4 (20 ng), PGB_2 (20 ng), LTD_4 (30 ng), LTB_4 (10 ng) and LTE_4 (30 ng) before (pre-oxalate) and after (post-oxalate, A) injection of a 200-μl bolus of oxalic acid. Peak width and tailing were decreased following the use of oxalic acid. The ratios of plate counts (post-oxalate:pre-oxalate) were increased as follows: 2.07 (LTC_4), 1.53 (PGB_2) 1.19 (LTD_4), 1.20 (LTB_4) and 2.03 (LTE_4). After exposure of the column to the bolus of oxalic acid, residual peak tailing observed with LTC_4 (post-oxalate, A) was eliminated when 0.5 m*M* oxalic acid was incorporated into the mobile phase (post-oxalate, B).

usage and length of methanol washes between samples. Subsequent injections of oxalic acid did not cause release of LTC_4-like material from the column. The oxalate effect on plate counts could be maintained by supplementary injections of 10 m*M* oxalic acid (100 μl) and maximized by inclusion of 0.5 m*M* oxalic acid into the mobile phase (shown for LTC_4 in Fig. 1, post-oxalate, B). The incorporation of oxalic acid into the mobile phase also led to excellent reproducibility of chromatograms obtained on different days. Standard curves for the sulfidopeptide leukotrienes are shown in Fig. 2. After exposure of the column to a 200-μl bolus of oxalic acid, peak heights were increased by 36% for LTC_4, 14% for LTD_4 and 41% for LTE_4. The resultant limit of sensitivity of the assays approximated 1 ng for LTC_4 and 1—2 ng for the other 20:4 metabolites. The elution characteristics of LTC_4 extracted from biological samples were also improved by the use of oxalic acid (Fig. 3A and B).

Quantitation of LTC_4, LTD_4 and LTE_4 eluted from oxalic acid-treated columns was limited initially by non-linearity of standard curves obtained by measurement of peak height (Fig. 2). This problem, also noted when LTC_4 was chromatographed on columns pretreated with 3% EDTA, was not evident with PGB_2 and LTB_4, nor was it noted when peak area was used for construction of standard curves. It was found to depend on the methanol concentration in the sample solvent, as summarized in Table I and shown chromatographically in Fig. 3. The problem was corrected by the use of mobile phase as the sample solvent; linear standard curves were then obtained for sulfidopeptide leukotrienes tested in the range 5—90 ng. We also noted that with sample solvents containing high concentrations of methanol, the broadened, shorter leukotriene peaks occurred at a critical sample volume, which was related inversely to the methanol concentration. Critical volumes of 40 and 10 μl, respectively, were noted with sample solvents containing 75% and 100% methanol in water. Such

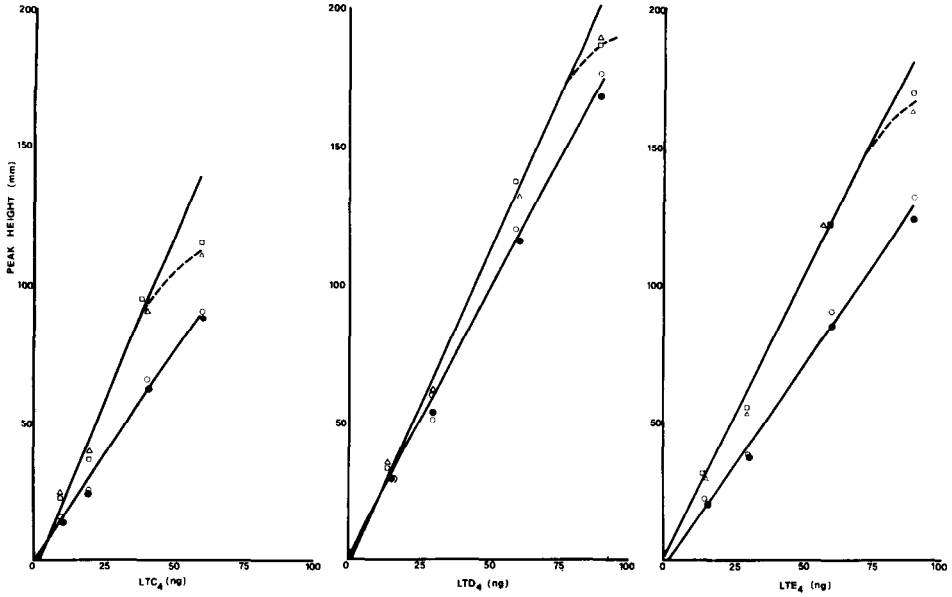

Fig. 2. Standard curves for LTC_4, LTD_4 and LTE_4 injected in 5—60 μl of methanol—water (75:25, v/v). Regression lines were derived from duplicate data points, before (lower curves) and after (upper curves) injection of a 100-μl bolus of 10 m*M* oxalic acid. The non-linearity of the post-oxalate standard curves at amounts greater than 40 ng (dotted lines) was found subsequently to be due to the nature of the sample solvent (see text).

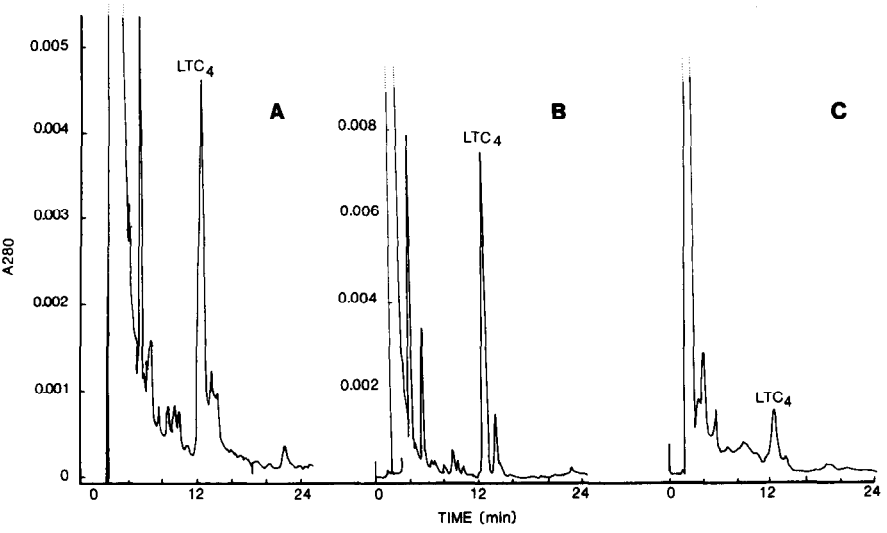

Fig. 3. Chromatograms of supernatant extracts from zymosan-stimulated peritoneal macrophages. The sample solvent consisted of mobile phase. The plate count of the major lipoxygenase product, LTC_4, on a column not previously exposed to oxalic acid (A) was improved substantially by inclusion of 0.5 m*M* oxalic acid into the mobile phase (shown for a different biological sample in B). Quantitation of plate counts revealed values of 1708 and 3844, respectively. Selectivity and retention time remained unchanged after exposure of the column to oxalic acid. Chromatograms of LTC_4 extracted from the same biological sample injected in 90 μl of mobile phase (B) and methanol (C) reveal marked degradation in peak shape with the use of methanol as sample solvent. The LTC_4 peak in B represents 45 ng of LTC_4, produced by approximately $2.4 \cdot 10^6$ macrophages.

TABLE I

EFFECT OF METHANOL CONTENT OF THE SAMPLE SOLVENT ON THE QUANTITATION OF LTC_4 BY THE MEASUREMENT OF PEAK HEIGHT

Each sample contained 50 ng of LTC_4 injected in 50 μl of solvent. The peak height of samples injected in mobile phase was similar to that observed with sample solvents containing 20—60% methanol in water (data not shown).

Percentage methanol (v/v) in water	Peak height (mm)
0	98
20	133
40	134
60	137
80	102
100	38

a critical volume was not identified when up to 100 μl of mobile phase was used as the sample solvent. All subsequent samples and standards were dissolved in mobile phase prior to HPLC.

Column life, determined by serial plate counts and measurement of column back-pressure, was not affected by the use of oxalic acid.

DISCUSSION

Our data indicate that sensitive (1—2 ng) and reproducible assays of sulfidopeptide leukotrienes can be obtained by exposure of reversed-phase HPLC columns to dilute oxalic acid. A similar improvement in the resolution and recovery of these leukotrienes has been observed with the use of the chelating agent, EDTA [4, 5]. However, in contrast to oxalic acid, EDTA is insoluble in methanol and acetonitrile, both of which may be used to flush columns between injections of biological samples and in gradient elution chromatography. In addition, we have found that the column equilibration time is much shorter following injection of oxalic acid (14 min after a bolus) than following injection of EDTA (more than 2 h after the recommended overnight flushing [4, 5] of the column).

The mechanism(s) of the oxalic acid effect may be similar to those proposed for EDTA. Both agents remove metal ions from ODS columns [5, 6]. EDTA may also displace leukotrienes bound by negatively charged functional groups to cations retained on the stationary phase [5]. Metal ions may originate from stainless steel in the HPLC apparatus [8], the sample, or poorly prepared mobile phase. Our data showing release of LTC_4-like material after injection of oxalic acid, suggest that small quantities of LTC_4 are retained on ODS columns, due to ionic interactions with cations bound to underivatized silanyl groups. We propose that these interactions are also responsible for the peak tailing observed in routine HPLC of sulfidopeptide leukotrienes and that they are prevented by pretreating the column with oxalic acid.

The use of sample solvents containing higher concentrations of methanol than were present in the mobile phase, resulted in significant band spreading and asymmetrical peaks. These phenomena have been noted by others when

the sample has been injected in a solvent that is stronger than the mobile phase [9]. The problem was corrected by the use of mobile phase as the sample solvent. Our data suggest that sample solvents containing 20—60% methanol in water should also be satisfactory.

Although metallic salts of oxalic acid are poorly soluble in water, we obtained no evidence of salt precipitation on columns treated with oxalic acid. In addition, the use of oxalic acid under our conditions had no effect on column life. We conclude that oxalic acid is an acceptable, more convenient alternative to EDTA for optimizing quantitation of sulfidopeptide leukotrienes by reversed-phase HPLC.

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