Enhancement of Mass Spectrometric Detection of LTC_4, LTD_4, and LTE_4 by Derivatization

Orval A. Mamer
McGill University Biomedical Mass Spectrometry Unit, Montreal, Quebec, Canada

George Just
Department of Chemistry, McGill University, Montreal, Quebec, Canada

Chun-Sing Li, Patrice Préville, Sara Watson, Robert Young, and James A. Yergey
Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada

Several acylating reagents are synthesized and used to introduce quaternary phosphonium or ammonium or ternary sulfonium functions into a simple model of a peptido leukotriene (PLT). One of these reagents was selected for further study with LTE_4, LTD_4, and LTC_4. We demonstrate that acylation of the free amine function of PLTs to produce the 5-triphenylphosphoniumvaleryl-amide (TPPV) derivatives enhances chemical stabilities and significantly increases responses in fast-atom bombardment and continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS) relative to the native PLTs. With high-performance liquid chromatography inlet to CF-LSIMS, we demonstrate the facile detection in selected ion monitoring of the TPPV derivative of 3 pg of LTD_4. *(J Am Soc Mass Spectrom 1994, 5, 292–298)*

Assay of the peptido leukotrienes (PLTs) LTC_4, LTD_4, and LTE_4 by mass spectrometry has several advantages, in principle, over those employing immunological or biological response methodologies. Among these advantages are specificity, freedom from interferences, simplicity, brevity, and a flexibility that permits considerable latitude in choice of sample type and preparation requirements. Principal among the disadvantages is lack of sensitivity, which becomes critical with limited sample size. Even when derivatized in manners that are commonly used to increase thermal stability, volatility, and response in positive and negative ion chemical ionization, the intact PLTs remain unsuitable for gas chromatographic inlet because of their high molecular weights, instability, and polarity.

Balazy and Murphy [1] have reported detection of 10 pg of LTC_4 by negative ion electron capture ionization gas chromatography/mass spectrometry of the pentafluorobenzyl ester, trimethylsilyl ether derivative of 5-hydroxyeicosanoic acid produced by hydrogenolysis of high-performance liquid chromatography (HPLC) fractions containing LTC_4. While this technique is also useful for determination of LTD_4 and LTE_4, these must first be separated by reversed-phase HPLC. The reduction involves noble-metal hydrogenation and lithium dissolution in liquid methylamine, and together with subsequent handling, the procedure may be considered difficult and tedious.

Introduction of continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS) [2] combined with selected ion monitoring (SIM) techniques held promise of fulfilling the requirements for a simple, rapid, flexible, and sensitive assay for intact PLTs. Detection at high signal-to-noise ratio of 50 pg of LTC_4 with batch introduction continuous-flow fast-atom bombardment (CF-FAB) has been reported, along with studies of the collision-induced decomposition (CID) of the $[M + H]^+$ and $[M - H]^-$ ions of LTC_4 and LTE_4 and several derivatives at 7–15 eV in argon [3]. An estimated 40 ng, or less, of LTE_4 was detected by CF-FAB inlet and CID (20 eV argon) of the $[M - H]^-$ ion in human urine following infusion with LTE_4 [4].

More recently, an ion spray liquid chromatography/mass spectrometry assay for urinary LTE_4 has

Address reprint requests to Orval Mamer, McGill University Mass Spectrometry Unit, 1130 Pine Avenue West, Montreal, Quebec, Canada H3A 1A3.

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1044-0305/94/$7.00

Received May 19, 1993
Revised October 29, 1993
Accepted November 1, 1993

demonstrated a lower limit of 40 pg/μL using the MH^+ ion in SIM mode [5]. A study of $[M - H]^-$ ions of a large number of peptido and hydroxylated arachidonic acid metabolites in CF-FAB has shown that significant structural information may be obtained by CID at 8 keV with helium [6].

While HPLC CF-LSIMS appears to be an optimal choice for PLTs, sensitivity is still marginal when sample sizes are severely limited. If an uncompensated formal cation could be introduced into the PLT being assayed, one may expect increased response in both FAB and LSIMS in the same way that the methyl esters of acylcarnitines have enhanced responses in FAB [7, 8]. Thus, carbodiimides have been used to condense 2-aminoethyltriphenylphosphonium bromide with C_{10} to C_{22} fatty acids [9] and the C-termini of peptides [10] to yield amide derivatives containing the positively charged triphenylphosphonium moiety which have up to two orders of magnitude greater sensitivity for their detection relative to the native compounds. In addition, reaction of 2-bromoethyltriphenylphosphonium bromide with the N-terminal primary amine of peptides [10] produces an analogous triphenylphosphonium derivative, with similar increases in sensitivity.

We report here results of experiments which led to a method for preparation of stable derivatives of the PLTs, which by incorporation of a formal cation also produces a significant increase in sensitivity for mass spectrometric detection. We synthesized and studied four derivatization reagents, 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo)pentyltriphenylphosphonium bromide (I), 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo) pentylmethylphenylsulfonium trifluoromethylsulfonate (II), 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo) pentylmethylphenylsulfonium tetrafluoroborate (III), and 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo) pentylbenzyldimethylammonium hydroxide (IV). The four activated ester reagents (see Figure 1) acylate free primary amines in aqueous solution to produce the corresponding cationic amide.

Each of these four reagents was evaluated on the bases of ease of reaction and the relative stabilities and mass spectrometric properties of the derivatives by using these reagents to derivatize S-benzylcysteine methyl ester as a model. Reagent I was selected for further studies and was used to derivatize LTC_4, LTD_4, and LTE_4, each as the free acids and as the corresponding alkyl esters. If the triphenylphosphoniumvalerylamide (TPPV) derivatives of the esters were found to have significantly better properties than those of the free acids, it would then be advantageous to develop methodology to esterify PLTs on a microscale similar to that reported for N-acetyl LTC_4 [11].

We provide an example of the preparation of the TPPV derivative of LTD_4 with I in aqueous solution on a microscale. Studies of PLT metabolism using this derivatization technique will be published elsewhere.

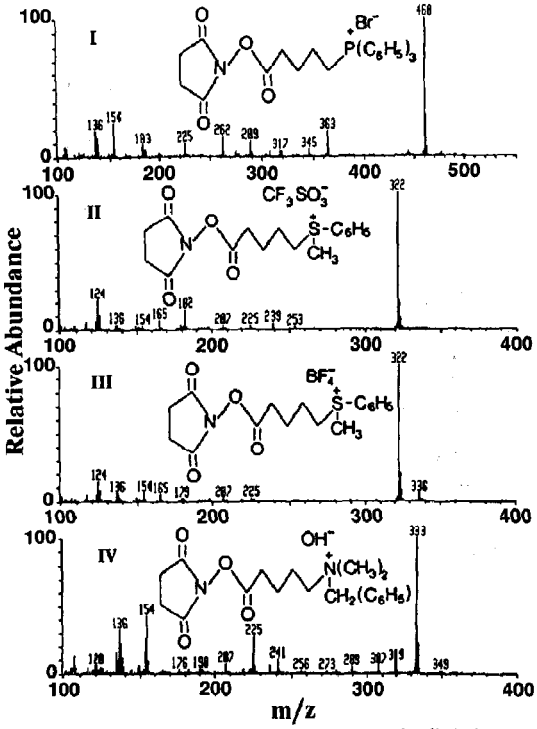

Figure 1. Positive ion FAB spectra in NBA of 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo)pentyltriphenylphosphonium bromide (I), 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo)pentylmethylphenylsulfonium trifluoromethylsulfonate (II), 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo)pentylmethylphenylsulfonium tetrafluoroborate rate (III), and 5-((2,5-dioxo-1-pyrrolidinyl)oxy) (5-oxo)pentylbenzyldimethylammonium hydroxide (IV).

Experimental

General

The leukotrienes LTC_4, LTD_4, and LTE_4 were obtained by synthesis in house. The PLT derivatization reagents were also synthesized in these laboratories [12].

Synthesis of Reagent I

Reagent I was synthesized by stirring at room temperature for 3 hours a mixture of (4-carboxybutyl) triphenylphosphonium bromide (5.0 g, 11.3 mmol), N-hydroxysuccinimide (1.5 g, 13.3 mmol), and dicyclohexylcarbodiimide (2.8 g, 13.6 mmol) in acetonitrile (50 mL). The precipitate formed was removed by filtration and the filtrate concentrated in vacuo. Chromatography over silica gel and elution with CH_3CN: CH_2Cl_2:H_2O 3:1:0.2 yielded I (4.7 g, 77%), which was lyophilized to give a white powder [12].

Derivatization of LTD_4 with Reagent I

To a solution of LTD_4 (250 μg, 0.5 μmol) in water (100 μL) was added 1 M aqueous sodium hydroxide (0.5

μL, 0.5 μmol) and **I** (544 μg, 1 μmol). The mixture was stirred at room temperature for 2 hours. Analytical HPLC showed complete consumption of LTD_4 and essentially complete conversion to a peak corresponding to the LTD_4-TPPV derivative, which was then isolated by preparative HPLC in the usual manner [12].

The TPPV derivatives of the three PLTs were also synthesized by conventional chemical techniques [12] different from those that are used in a microanalytical application. These syntheses were necessary to obtain the derivatives in pure form in sufficient quantities for chemical characterization and to allow comparisons of their mass spectrometric responses as the acids and as the esters with those of the native PLTs.

Other organic and inorganic materials and solvents were obtained from a variety of sources and were used as received.

Low Resolution FAB Analyses

Analyses were conducted with a VG Instruments (Manchester, UK) ZAB-HS, set to 2000 resolving power, equipped with a xenon gun operating at 8 kV and 1 ma neutral beam current and a stainless-steel sample stage. Samples dissolved in methanol were evaporated in a current of warm air onto the sample stage which was previously moistened with 200 nL of dimethylsulfoxide. The matrix, 600 nL of 3-nitrobenzyl alcohol (NBA), was well mixed into the sample with a coarse glass fiber. Five spectra were obtained over the mass range 1200–100 Da at a scan rate of 10 s dec^{-1}, and these were averaged and plotted. The lower mass cut-off was raised to 315 Da in the analyses of the underivatized PLTs to avoid the intense matrix ions at 307 Da and below. An equimolar mixture was made of methyl S-benzylcysteine and its TPPV derivative and this was analyzed in NBA, as described above. Mixtures were also made of each PLT with its respective TPPV derivative in the free acid and in the esterified forms, and analyzed in the same manner. Finally, an equimolar mixture was made of the TPPV derivatives of the free acid and esterified forms of the three PLTs in methanol (0.39 nmol of each), and aliquots of this mixture were similarly analyzed immediately after mixing, after 3 hours at room temperature in air, and after 2.5 hours and 21 hours at 50 °C in air in a closed vial.

Capillary HPLC/CF-LSIMS Analyses

CF-LSIMS experiments were carried out at a resolving power of 1000 on a JEOL HX-110A mass spectrometer (JEOL, Boston, MA). A Waters 600MS HPLC pump (Waters Chromatography, Milford, MA) supplied the mobile phase at a flow rate of 1 mL/min. The main flow was split to 3 μL/min using an open split at a Valco ZDV tee (Valco Instruments, Houston, TX). The 3 μL/min flow was then passed through a Valco 6-port electrically actuated injection valve, which was controlled by the Waters pump and fitted with a 10 μL injection loop. The exit of the injection valve was fitted with a KAPPA Spherisorb ODS-2 0.3 × 100 mm capillary column (Keystone Scientific, Bellefonte, PA), and a 0.5 m section of 50 μm i.d. fused silica (Polymicro Technologies, Phoenix, AZ) connected the column exit to the JEOL CF-LSIMS probe. The mobile phase consisted of methanol and 20 mM ammonium acetate (pH 5.0), both of which contained 1.5% glycerol which served as the LSIMS matrix. Independent analytical HPLC experiments demonstrated that the presence of glycerol at these concentrations did not affect the separations of the PLTs or their derivatives. A mobile-phase gradient, starting at relatively low organic strength, was used to concentrate the 10 μL injections onto the head of the column. The gradient was started at 50:50 CH_3OH:NH_4OAc, ramped linearly to 70:30 at 5 minutes, then linearly to 90:10 at 20 minutes, and finally held at 90:10 for an additional 5 minutes. The LSIMS source was equipped with a focusing Cs^+ ion gun operated at 10 kV above the 10 kV ion source potential. Full linear scan mass spectra were acquired from 0–1000 Da every 5 seconds for an equimolar mixture of LTD_4 and LTD_4-TPPV. SIM was used to estimate the limits of detection and was performed on the mass of the molecular cation of the LTD_4-TPPV derivative (841.4 Da).

Results

FAB Analyses

The mass spectra of the four derivatizing agents are reproduced in Figure 1. The most prominent ions have masses corresponding to the respective onium ions. The NBA FAB spectra obtained for the model compound, methyl S-benzylcysteine (**V**), and for its derivatives made with the four reagents studied are reproduced in Figure 2. Very abundant ions are found that correspond to the intact cationic derivatives of this model compound.

The equimolar mixture of **V** and its TPPV derivative (**VI**) produced a mass spectrum in NBA (Figure 3) that clearly demonstrates the gain in sensitivity produced by the making of the TPPV derivative in the benzylcysteine model system.

Figure 4 shows the FAB spectra obtained in this study for the ester and acid forms of the TPPV derivatives of LTC_4, LTD_4, and LTE_4. These produce very prominent even-electron ions at the concentration of 1 nmol in 600 nL of NBA matrix, whereas the native PLTs yield poor responses in FAB in NBA matrix (glycerol is worse) at levels of 1 to 3 nmol on the probe (Table 1).

When the abundances of a PLT MH^+ and the molecular cation of the corresponding TPPV derivative are measured in FAB relative to chemical noise and background in a 20 Da range centered on the ions of

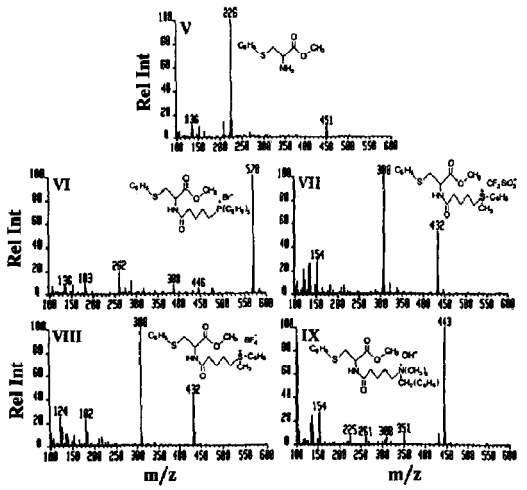

Figure 2. FAB spectra of the model compound methyl S-benzylcysteine (V) and of its triphenylphosphonium bromide (VI), phenylmethylsulfonium trifluoromethylsulfonate (VII), phenylmethylsulfonium fluoroborate (VIII), and dimethylbenzylammonium hydroxide (IX) derivatives made with the four reagents studied.

interest (Table 1), it is clear that the making of the derivative provides a considerable enhancement in analyte-related signal relative to noise and background. For example, for LTC_4, m/z 626 has roughly twice the average abundance of the background in the range m/z 616 to 636. On the other hand, an equal amount of the TPPV derivative produces a signal at m/z 970 that is about 25 times the background in the range m/z 960 to 980, and the cation of a similar amount of esterified LTC_4-TPPV is roughly 100 times the background in its 20 Da range. LTD_4-TPPV and LTE_4-TPPV esters produce responses apparently not as

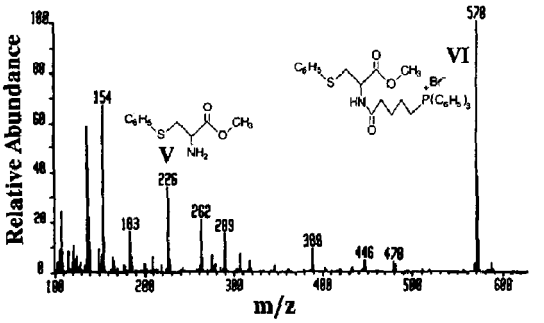

Figure 3. FAB spectrum in NBA of an equimolar mixture of V and VI demonstrating their relative responses (m/z 226 and 570, respectively).

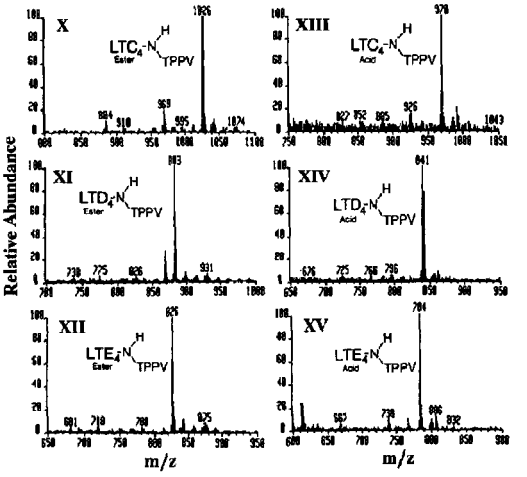

Figure 4. Positive ion FAB spectra in NBA of 1 nmol of the TPPV derivatives of the following leukotrienes: LTC_4 ester (X); LTD_4 ester (XI); LTE_4 ester (XII); LTC_4 acid (XIII); LTD_4 acid (XIV); and LTE_4 acid (XV).

Table 1. Comparison of FAB ion abundances of PLT TPPV derivatives as the acids and esters relative to the free PLT

Analyte	m/z of MH^+ or Ph_3RP^+	Nanomoles on probe	Signal/ background[a]	Relative intensity (%)[b]
Free LTC_4	626	1	2	
LTC_4-acid-P^+ (XIII)	970	1	25	
LTC_4-ester-P^+ (X)	1026	1	100	
LTC_4 /XIII /X mixture		0.8 /0.8 /0.8		1.57 /51.2 /72.82
Free LTD_4	497	3	not detected	
LTD_4-acid-P^+ (XIV)	841	1	70	
LTD_4-ester-P^+ (XI)	883	1	50	
LTD_4 /XIV /XI mixture		0.8 /0.8 /0.8		0 /7.4 /30.4
Free LTE_4	440	3	3	
LTE_4-acid-P^+ (XV)	784	1	90	
LTE_4-ester-P^+ (XII)	826	1	60	
LTE_4 /XV /XII mixture		1.5 /0.3 /0.3		2.7 /10.8 /28.9

[a] For single analytes, indicated intensities are the approximate ratios of analyte peak intensities to chemical background intensities averaged over a 10 Da range on either side of the ion cluster of interest.

[b] For the mixtures, intensities presented are relative to the base peak in the spectrum in the order indicated.

Table 2. Dependency of molecular cationic abundances[a] on aging of a mixture of TPPV derivatives of LTC_4, LTD_4, and LTE_4 as the esters and as the acids

Time (h)	Temperature (°C)	LTC_4	LTD_4	LTE_4
		%TIC[b] Ester, %TIC Acid, (Ratio E /A)		
0	25	0.82 0.34 (2.4)	1.00 0.65 (1.5)	1.71 0.96 (1.8)
3	25	1.01 0.34 (3.0)	0.96 0.65 (1.5)	1.18 0.74 (1.6)
2.5	50	1.22 0.46 (2.7)	0.93 0.58 (1.6)	2.23 0.80 (2.8)
21	50	1.18 0.27 (4.4)	0.89 0.55 (1.6)	1.44 0.51 (2.8)

[a] Measured as the percent total ion current recorded in FAB in the scan range 315 to 1200 Da.
[b] TIC = total ion current.

intense relative to their neighboring backgrounds as do the derivatives of the free acids; this is a direct result of the more intense background at the lower masses presenting with anomalously abundant ions in the vicinity of the esters but not of the free acids. However, the signal-to-background ratios for these esters and acids are comparable. When an equimolar mixture of a PLT and the TPPV derivatives of the free acid and esterified forms of that PLT are analyzed in FAB, substantial signal strength improvements are found first for the free acids and additionally for the esters (last column, Table 1). Thus, in the LTC_4 mixture, these ions produce intensities 1.57%, 51.2%, and 72.8%, respectively, of the base ion in the spectrum. The TPPV derivatives of the esterified PLTs produced responses that are between 1.5 and 4 times the responses of the TPPV derivatives of the free acids in the equimolar mixtures.

Figure 5 and Table 2 relate the dependency of mass spectral response for the TPPV derivatives to storage conditions that normally result in rapid degradation of the native PLTs. After 21 hours at 50 °C in methanol solution sealed in a vial with air, the ion abundances appear relatively unchanged (Figure 5b) compared to the spectrum obtained immediately following mixing (Figure 5a). Only fairly minor losses in intensity have occurred to the acid forms of the phosphonium derivatives.

Capillary HPLC/CF-LSIMS Analyses

Figure 6 illustrates the total ion current and extracted ion chromatogram for the M^+ and MH^+ ions of LTD_4-TPPV and LTD_4, respectively, obtained by capillary HPLC/CF-LSIMS determination of an equimolar mixture. Figure 7 represents subtracted mass spectra taken from the same determination. A significant increase in sensitivity was realized by using CF-LSIMS, as demonstrated by the ability to obtain good full scan spectra for 5 pmol each of LTD_4 and LTD_4-TPPV (2.5 and 4.2 ng, respectively) in the experiment shown in Figures 6 and 7. The LTD_4-TPPV derivative produces a signal 2.2 times greater than for underivatized LTD_4 on the basis

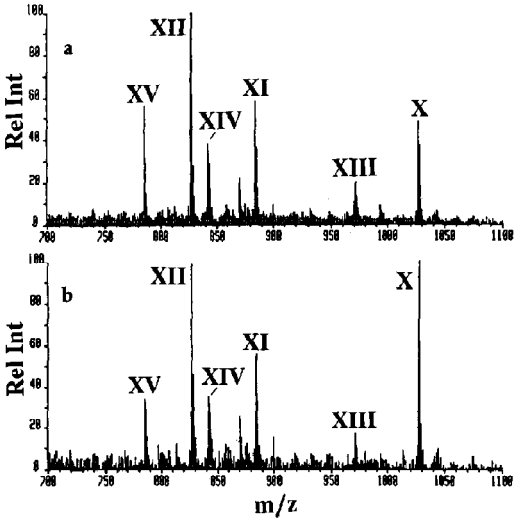

Figure 5. Mass spectra obtained for an equimolar mixture of the phosphonium derivatives described in Figure 4 (0.39 nmol each) immediately following mixing at room temperature (**a**) and after 21 hours at 50 °C in 15 μL of methanol in a 200 μL vial containing room air (**b**). The numbering is the same as in Figure 4.

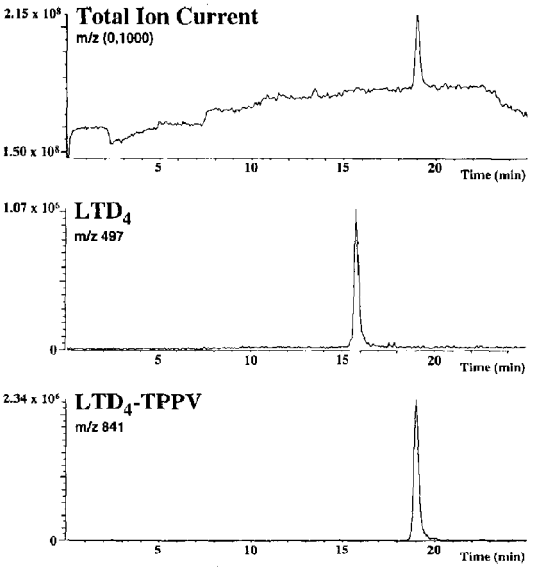

Figure 6. CF-LSIMS of 5 pmol each of LTD_4 and LTD_4-TPPV. Total ion current from m/z 0–1000, and extracted ion currents for m/z 497 and 841.

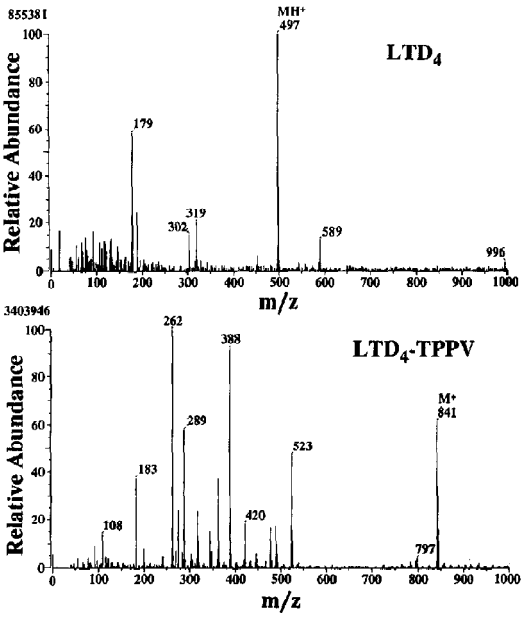

Figure 7. Background subtracted mass spectra taken from the analysis in Figure 6 for LTD_4 and for LTD_4-TPPV (15.8 and 19.0 minutes elution times, respectively).

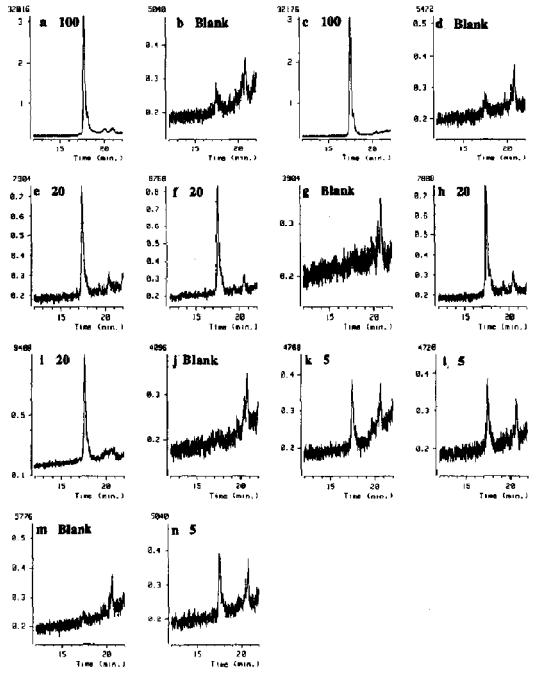

Figure 8. CF-LSIMS determination of LTD_4-TPPV using single ion monitoring (m/z 841.4). Panels (a) through (n) correspond to serial injections of LTD_4-TPPV interspersed with blank solvent injections. Quantities injected are noted on each chromatogram in picograms. The injection of 5 pg of LTD_4-TPPV corresponds to 3 pg of native LTD_4.

of the M^+ and MH^+ ions, respectively. But comparing these intensities to the background ion intensities over a 6 Da range centered on these analyte ions (i.e., m/z 838–844 and 494–500, respectively) in the time interval between the elutions of the analyte peaks shows that derivatization produces a factor of 8.9 enhancement of signal-to-background. Both compounds produce the common effect of suppression of the glycerol background observed in FAB and LSIMS analyses, and as a result the total ion trace does not reflect the proper relative intensities of the LTD_4 and LTD_4-TPPV sample signals. In this analysis, background suppression and sample signal are comparable for LTD_4, while for LTD_4-TPPV, the sample signal more than offsets background suppression.

The limits of detection for LTD_4-TPPV using capillary HPLC/CF-LSIMS and SIM detection are shown in Figure 8. The LTD_4-TPPV derivative was detected with a signal-to-noise ratio greater than 5 at the level of 5 pg/10 μL injected, corresponding to 3 pg of underivatized LTD_4. Interspaced blank injections show that there was no carryover between sample injections.

Discussion

FAB analyses of methyl S-benzylcysteine derivatized by the four reagents described here (Figure 1) demonstrate substantial increases in sample signal over that for the native model compound (Figures 2 and 3). The phosphonium reagent I was selected for further study for several reasons, among them being ease of synthesis of the reagent, stability of the derivatives to chemical degradation, and ease of use. The sulfonium reagents II and III produced derivatives having the same apparent signal response independent of whether the anion was tetrafluoroborate or triflate. The principal disadvantage with the sulfonium derivatives is the loss of thioanisole from the ion of interest which divides the signal into two ions, neither of which seemed to produce analyte signal as well as the TPPV derivative. The ammonium reagent was discounted on the basis of difficulty of synthesis and the observation that it also failed to produce signal strength greater than for I.

Large enhancements in FAB were observed in the signal-to-background ratio for the TPPV derivatives of the free acid and esterified PLTs in comparison to the native PLTs (Table 1). These improvements (twelvefold and fiftyfold for free and esterified LTC_4, for example) are due in part to an increase in the signal and in part to a general reduction of background ions at the higher masses. It is apparent from the equimolar mixture data of Table 1 (free PLT, PLT-TPPV, and the TPPV derivative of fully esterified PLT) that esterification produces a modest additional gain in sensitivity (1.4-fold to fourfold) relative to the acid TPPV derivatives. Unfortunately, straightforward alkylation of the PLTs to their corresponding methyl or ethyl esters in a

manner analogous to that reported for N-acetyl-LTE_4 [11] was unsuccessful, and indirect syntheses were necessary to make these esters [12]. Therefore, it was decided that the modest additional gains in sensitivity were not worth the losses and difficulties that would be associated with preparing the alkyl esters of PLTs in physiological sample analyses prior to acylation of the residual amine function.

The TPPV derivatives of LTE_4, LTD_4, and LTC_4 are substantially more stable to chemical degradation than the native PLTs. This is shown by comparing the spectrum of an equimolar mixture of the TPPV derivatives of the free acid and esterified forms measured immediately after mixing, with the spectrum measured after heating in air at 50 °C for 21 hours (Figure 5). There is very little change, indicating that the derivatives are stable under conditions that quickly degrade the native PLTs. The ratio of the intensities (as percent total ion current, Table 2) of the ester and acid TPPV derivative molecular cations for LTD_4 also shows virtually no change with heating at 50 °C in air, and while the LTE_4 and LTC_4 derivatives show some change, the relatively minor changes appear not to be simply progressive with severity of conditions. In general, the acid TPPV derivatives seem to be slightly less stable than the esters.

CF-FAB determination of PLTs has previously been demonstrated to provide a significant increase in sensitivity when samples are injected in a batch mode [3, 4]. However, this is the first report of the capillary HPLC introduction and detection of PLTs using CF-LSIMS. Their detection in full-scan mode at the low nanogram level, as exemplified in Figure 6, is orders of magnitude better than observed in full-scan FAB (see Table 1). At the same time, however, the relative sensitivity increases for the making of the TPPV derivatives are comparable with those observed in FAB. An apparent further increase in signal-to-noise for the CF-LSIMS analyses was obtained with background subtraction of the glycerol matrix ions. Three factors probably contribute to the sensitivity differences between the FAB and CF-LSIMS analyses. First, the matrix layer on the probe in the flow system is much thinner, thereby increasing the relative surface-to-volume ratio for ionization. Second, the CF system delivers the sample as a transient chromatographic peak, during which the analyte concentration is significantly increased on the surface. Finally, two different instruments were used, and no attempt was made to compare their relative sensitivities.

Instrumental differences are also evident in the spectra obtained for LTD_4-TPPV. The CF-LSI mass spectrum with the JEOL instrument shows a considerable amount of fragmentation when compared to the FAB spectrum obtained with the ZAB instrument. This implies that substantial additional gains in sensitivity would be possible with reduced fragmentation. Coupled with greater stability, these derivatives clearly have great potential use in quantitative PLT analyses.

The detection limits for the TPPV derivatives of PLTs measured by CF-LSIMS are in the low picogram region (Figure 8). This level of sensitivity would be sufficient for their quantitation as the TPPV derivatives in physiological fluids such as human urine where control levels have been reported to be 17 ± 5 pg/mL [13]. We expect it would be difficult to detect native LTD_4 in biological samples even with CF-LSIMS for two reasons; first, a nearly ninefold lower signal-to-background ratio was found for native LTD_4 relative to the TPPV derivative, and second, additional chemical interferences introduced from biological samples will be more severe at the lower mass.

The use of a relatively large sample loop (10 μL) should aid in the development of methodology for routine PLT measurements. Compared to typical capillary HPLC injection volumes of 50–100 nL, injections of 10 μL should allow a significant portion of the sample to be injected. Further work with appropriate isolation techniques is currently in progress to extend this methodology to the routine determination of PLTs in biological fluids.

Acknowledgments

Support of the Medical Research Council of Canada is acknowledged by O. A. M., and The Natural Sciences and Engineering Research Council of Canada by G. J.

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