Mass Spectrometric Analysis of Leukotriene A_4 and Other Chemically Reactive Metabolites of Arachidonic Acid

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The biosynthesis of prostaglandins and leukotrienes proceeds through the formation of chemically reactive intermediates leukotriene A_4 (LTA_4) and prostaglandin H_2 (PGH_2) which in aqueous solutions have chemical half-lives of 3 s and 3 min, respectively. Prostacyclin (PGI_2) is another chemically reactive prostanoid that has a chemical half-life of 3–4 min. The recent development of reversed phase HPLC stationary phases that are stable to elevated pH (pH 10–12) without significant column damage has permitted direct analysis of these acid-sensitive eicosanoids. Using electrospray ionization, molecular anions $[M - H]^-$ of these compounds were observed at *m/z* 317 for LTA_4 and *m/z* 351 for both PGH_2 and PGI_2. The mechanism of formation of ions derived from collisional activation of LTA_4 was studied using stable isotope labeled and chemical analogs of LTA_4 and found to involve formation of highly conjugated anions at *m/z* 261 and 163. The collisional activation of the molecular anion of PGH_2 yielded a product ion spectrum identical to that observed for the isomeric prostaglandins PGE_2 and PGD_2. However, it was possible to baseline separate PGE_2, PDG_2, and PGH_2 by reversed phase HPLC using basic HPLC mobile phases. The collisional activation of PGI_2 led to a family of abundant ions including highly conjugated carbon-centered and oxygen-centered radical species (*m/z* 245 and 205) likely derived from the attack of the carboxylate anion on the cyclic enolether of PGI_2 as well as the most abundant product ion (*m/z* 215) which formed following loss of neutral hexanal and water. The structures of these product ions were consistent with high resolution measurements measured in a quadrupole time-of-flight mass spectrometer. (J Am Soc Mass Spectrom 2002, 13, 1227-1234) © 2002 American Society for Mass Spectrometry

The enzymatic oxidation of arachidonic acid (5,8,11,14-eicosatetraenoic acid) leads to the production of two large families of biologically active compounds, the prostaglandins and leukotrienes [1, 2]. These oxidized fatty acids, more frequently referred to as eicosanoids, are thought to play an important role in intercellular communication as lipid mediators and elicit potent effects in various biological systems mediated through specific protein receptors on the surface of responding cells [3, 4] The biosynthesis of the prostaglandin family of eicosanoids is initiated by the enzyme PGH synthase, more commonly referred to as cyclooxygenase [1]. Recently, two isoforms of this enzyme, COX-1 and COX-2, have been identified as well as specific agents which can block the inducible PGH synthase, COX-2 [5]. In spite of the structural differences between these two COX-isoforms, both enzymes identically catalyze two separate reactions. In the first reaction, two molecules of diatomic oxygen are added to arachidonic acid in what is referred to as the cyclooxygenase reaction; this forms the initial endoperoxide PGG_2. The second catalytic activity of PGH synthase, termed the peroxidase activity, reduces the 15-hydroperoxy substituent of PGG_2 into the 15-hydroxy endoperoxide intermediate PGH_2. It is this intermediate which is acted upon by subsequent auxiliary enzymes expressed in various cells to form the biologically active prostaglandins, including the chemically reactive products PGI_2 (prostacyclin) and thromboxane A_2, following the action of prostacyclin synthase and thromboxane synthase, respectively [6].

The enzyme 5-lipoxygenase (5-LO) also carries out two separate enzymatic reactions [7]. The first involves the stereospecific addition of molecular oxygen to carbon-5 of arachidonic acid, and the second, catalytic conversion of 5-hydroperoxyeicosatetraenoic acid (5-HpETE) into the chemically reactive, conjugated triene epoxide leukotriene A_4 (LTA_4). This intermediate is then substrate for two separate enzymes which form the biologically active leukotrienes, leukotriene B_4, a chemotactic factor for human neutrophils, [8] and the

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cysteinyl leukotrienes (leukotrienes C_4, D_4, and E_4), which are myotropic agents [9].

While the enzymes PGH synthase and 5-lipoxygenase catalyze very different oxidative events with arachidonic acid, they have one common feature in that they catalyze the formation of chemically reactive intermediates that are converted into the biologically active lipid mediators. The epoxide LTA_4 reacts with water rapidly at physiological pH (pH 7.4) so that it has a half-life of less than 3 s at 37 °C [10]. The unstable endoperoxide PGH_2 is somewhat more stable, but nonetheless, at physiological pH it has a half-life of approximately 3 min at 37 °C [11]. While both these reactive intermediates are substrates for further enzymatic processing, chemically stable products can result from acid catalyzed hydrolysis. An additional biologically active eicosanoid, also chemically reactive, is PGI_2 which at pH 7.4 has a half-life of 3–4 min at 37 °C [12]. The instability of these three reactive compounds at physiological pH has made their separation and direct measurement by physical chemical methods quite problematic.

Recent advances in HPLC column packing materials have resulted in products which are quite stable at pH extremes including high pH (pH 10–12) without deterioration of column performance [13, 14]. These developments have now permitted the use of online HPLC separation and direct mass spectrometric characterization of eicosanoids that typically decompose during normal chromatographic separation with mobile phases at pH 8 or less. This has made it possible to carry out direct mass spectrometric analysis of reactive eicosanoids which are stable to base, but unstable to neutral or acidic mobile phases.

Materials and Methods

Materials

Leukotriene A_4 ethyl ester was a generous gift from Dr. Joseph Mancini at Merck-Frosst Canada (Pointe-Claire, Quebec). The [3,3,4,4-$^2H_4$]6-keto-$PGF_{1\alpha}$ and [2,3,14,15-$^2H_4$]LTA_4 were purchased from Cayman Chemical Company (Ann Arbor, MI) and each were greater than 98% atom % labeled. All other eicosanoids used were also obtained from Cayman. All other solvents were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ).

Hydrolysis of LTA_4 Ethyl Ester and Related Compounds

The free acid of LTA_4 was generated from the ester as previously described [15]. Briefly, LTA_4 ethyl or methyl ester (20 μg) was taken to dryness under vacuum, then resuspended in 4:1 ice-cold acetone:0.25 M aqueous NaOH (20 μl total volume). This mixture was then allowed to incubate for 1 h at room temperature and subsequently stored at −70 °C until use.

^{18}O Labeling Experiments

The [^{18}O]labeled LTA_4 was prepared by using 0.25 M $Na^{18}OH$ during the hydrolysis of the ethyl ester. The $Na^{18}OH$ was made from the reaction of sodium with 99.9 atom% $H_2^{18}O$ obtained from Isotec Inc. (Miamisburg, OH). The hydrolysis of LTA_4 ethyl ester employed the same conditions as described above and resulted in 48% incorporation of one oxygen-18 atom into the carboxyl group.

Reversed Phase HPLC/MS/MS

A reversed phase XTerra MS column (1.00 × 50 mm, 3.5 μm C_{18}; Waters Corporation, Milford, MA) was used at a flow rate of 50 μL/min with a linear gradient using a mobile phase A consisting of either 10 mM triethylamine at pH 11 or 8.3 mM acetic acid adjusted to pH 5.7 with ammonium hydroxide and a mobile phase B consisting of acetonitrile:methanol (65:35 vol/vol). For samples run at high pH, 10 mM triethylamine was also added to mobile phase B. The gradient started at 15% B for initial conditions and increased to 80% B in 17 min. For the isocratic separations, an XTerra MS column (2.1 × 100 mm, 3.5 μm C_{18}) was used at a flow rate of 200 μL/min. The mobile phase for these separations was 10 mM triethylamine in 25% acetonitrile:methanol (65:35 vol/vol), 75% water. The effluent from these HPLC separations was introduced into a Sciex API-3000 (PESciex, Thornhill, Ontario, Canada). The ion spray for these experiments was −3400 V and the entrance potential was 10 V. The collision gas used for the MRM experiments was nitrogen at a pressure of 2.8×10^{-3} torr. The collision energy for these experiments was −22 V and the collision cell exit potential was between −11 and −17 V, depending upon the compound. The transitions monitored during these experiments were determined from MS/MS spectra obtained on the Sciex API-III$^+$.

Mass Spectrometry

Negative ion electrospray spectra were obtained on a Sciex API-III$^+$; samples (5–10 ng/μL) were infused at 10 μL/min in a solution of 10 mM triethylamine: methanol (1:1). Ions were detected by scanning from *m/z* 50 to 400 with a scan speed of 2 s/scan. The ion spray (−2800 V) was used with an orifice voltage of −60 V. Collisional activation experiments were carried out with a collisional offset potential of 30 eV; argon was used in the second quadrupole region as collision gas with a pressure of 2.88×10^{-3} torr. Collisional activation during electrospray ionization was performed with the orifice voltage at −100 V. LC/MS conditions using the API-3000 are described above.

Tandem mass spectrometry was also carried out using a Finnigan LCQ ion trap mass spectrometer (San

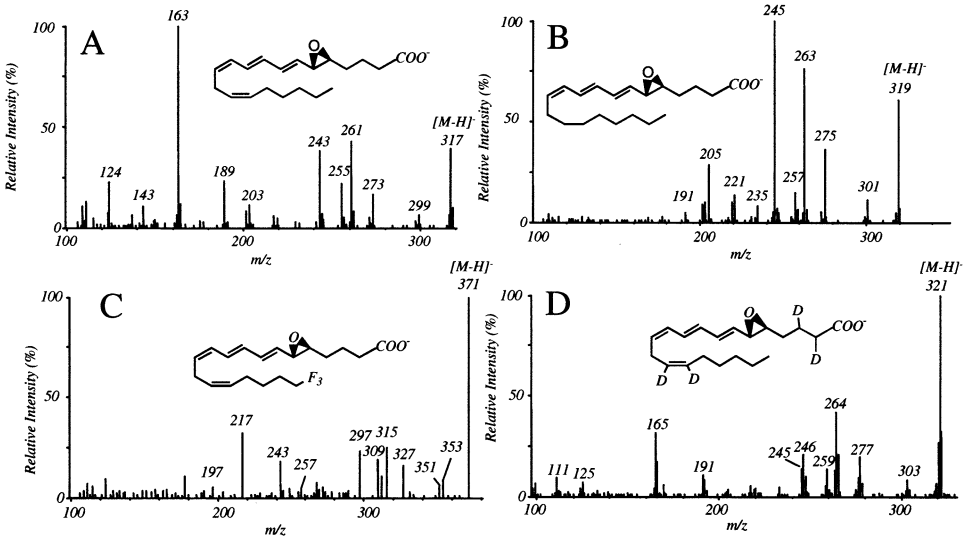


Figure 1. Negative ion electrospray and tandem mass spectrometry following collisional activation of the carboxylate anion $[M - H]^-$ of (**a**) LTA_4 (*m/z* 317), (**b**) LTA_3 (*m/z* 319), (**c**) 20-trifluoro-LTA_4 (*m/z* 371), and (**d**) D_4-LTA_4 (*m/z* 321). Samples were introduced by infusion.

Jose, CA), where samples were infused in the above described isocratic mobile phase. The spray needle was set to −3.5 kV. The stainless steel capillary held a potential of −28 V, the heated capillary set at 150 °C, and the tube lens offset was 10 V. The sheath gas on this instrument was nitrogen and collision gas was helium. MS/MS and MS^n experiments were carried out with a scan speed of 3 s and a mass isolation width of 3 Da; the typical collision energy for these experiments was between 25–40% relative collision energy.

High resolution electrospray tandem mass spectrometry was performed using a Micromass Q-TOF Ultima Global (Micromass, Inc., Manchester, UK) operated at 5000 resolving power. The sample (10 ng/mL) was infused into the machine at 10 μL/min in a solution of 10 mM NH_4OH:methanol (50:50). The capillary voltage was set to −2.5 kV and the cone voltage set to 31 V. The source temperature was 80 °C and the desolvation temperature set to 150 °C. The cone gas flow was 50 L/h and the desolvation gas flow was 150 L/h. The collision energy was set to 30 V and the collision gas used was argon. A two point mass calibration using two internal reference masses was employed for the TOF measurement with an expected RMS error better than 1.5 mDa.

Results and Discussion

LTA_4 and Related Compounds

The analysis of LTA_4 by LC/MS and LC/MS/MS was made possible by using mobile phases containing triethylamine to reduce hydrogen ion concentrations to greater than pH 8. Using these conditions the free acid of LTA_4, LTA_3, D_4-LTA_4, and 20-trifluoro-LTA_4 were found to chromatograph without extensive hydrolysis (see below). Analysis by LC/MS revealed that the compounds generated carboxylate anions at *m/z* 317, 319, 321, and 371, respectively. Collisional activation of each of these $[M - H]^-$ ions yielded similar high mass product ions in the tandem quadrupole mass spectrometer. The collisional induced decomposition of *m/z* 317 (Figure 1a) yielded product ions at *m/z* 299 and 273, corresponding to the neutral loss of water and carbon dioxide. These ions were shifted in the collisional activation of LTA_3 (Figure 1b) to *m/z* 301 and 275, *m/z* 353 and 327 for 20-trifluoro-LTA_4 (Figure 1c), and *m/z* 303 and 277 for D_4-LTA_4 (Figure 1d). Also, additional losses were seen at higher masses for the 20-trifluoro-LTA_4 (Figure 1c), including the ion at *m/z* 351 corresponding to the loss of hydrofluoric acid.

A major product ion derived from LTA_4 observed at *m/z* 261 was present in each of the LTA_4 analogs and corresponded to a loss of 56 mass units from the carboxylate anion of LTA_4. This loss of 56 u was observed for the LTA_3 (*m/z* 263, Figure 1b) as well as 20-trifluoro-LTA_4 analog (*m/z* 315, Figure 1c). However, this ion derived from D_4-LTA_4 was observed at *m/z* 264 corresponding to a loss of 57 u, corresponding to transfer of one of the deuterium atoms from either the C14–C15 labeled positions or the C2–C3 position during the formation of this ion species. Labeling of one of the carboxylic acid oxygen atoms by hydrolysis of the ethyl ester of LTA_4 in $H_2^{18}O$ made it possible to ascertain whether this ion retained the carboxylate oxygen atoms during formation of *m/z* 261. The corresponding product ion from collisional activation of 1-[^{18}O]LTA_4 (*m/z* 319) (Figure 2) was observed at *m/z* 263 and not a doublet at *m/z* 261/263 which would be expected for loss of only one of the carboxylate oxygen atoms. These observations led to the hypothesis that the carboxylate anion could undergo a facile attack of the epoxide at carbon-6, leading to a

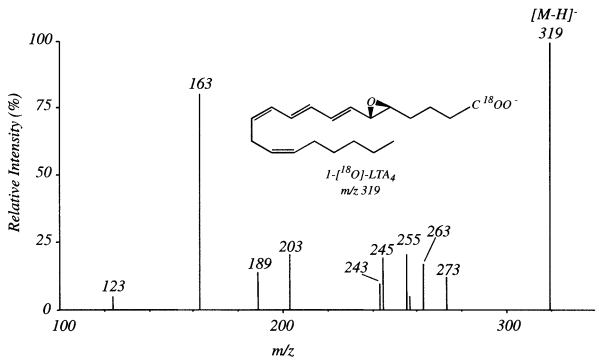
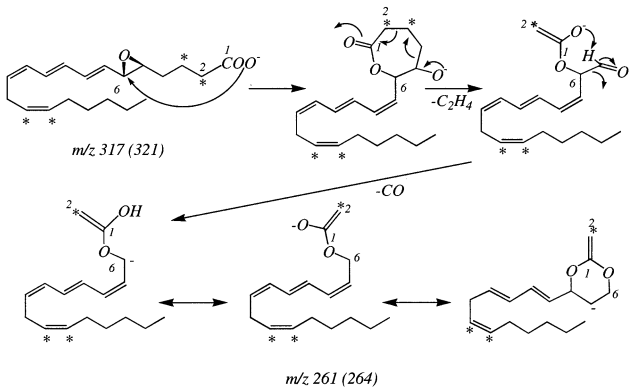


Figure 2. Negative ion electrospray and tandem mass spectrometry following collisional activation of the carboxylate anion [M − H]$^-$ of 1-[^{18}O]LTA$_4$ (*m/z* 319). Sample was introduced by infusion.



Scheme 1

cyclic alkoxide anion that could lose neutral ethylene and carbon monoxide (Scheme **1**). Clearly, the resultant ion was derived from a proton rearrangement, but since there was a loss of only one deuterium atom, the formation of this ion had to involve loss of either carbon-2 or carbon-3 (proposed as ethylene). While the formation of the seven-membered ring and exocyclic alkoxy anion may be energetically less favorable than a six-member ring, perhaps the higher energy content of such an intermediate results in facile loss of the neutral ethylene. The product ion *m/z* 243 likely results from the loss of water from the ion at *m/z* 261. This ion was a doublet in the D_4-LTA_4 mass spectrum because of the loss of either H_2O or HOD since carbon-2 has both a hydrogen and deuterium atom.

The most prominent ion seen in the collision induced decomposition of LTA_4 in the tandem quadrupole mass spectrometer was observed at *m/z* 163 and appeared to arise following a further rearrangement of the ion at *m/z* 261. When the orifice potential of the triple quadrupole instrument was raised to −100 volts, it was possible to obtain reasonable ion currents of *m/z* 261 that could be selected in the first quadrupole portion of the instrument for a subsequent collision induced decomposition study. The product ions derived from *m/z* 261 (Figure 3a) included an abundant *m/z* 163. However, when collisional activation of the [M − H]$^-$ from LTA_4 was carried out in an ion trap mass spectrometer (Figure 3B), the carboxylate anion (*m/z* 317) did not decompose to any significant quantities of ions at *m/z* 163 in spite of the fact that the most abundant product ion, aside from the loss of water, was observed at *m/z* 261. Performing a MS^3 experiment in the ion trap mass spectrometer with *m/z* 261 did not result any significant abundance of ions at *m/z* 163 (Figure 3b, inset). These results would suggest that the formation of *m/z* 163 was likely the result of multiple collisions taking place within the

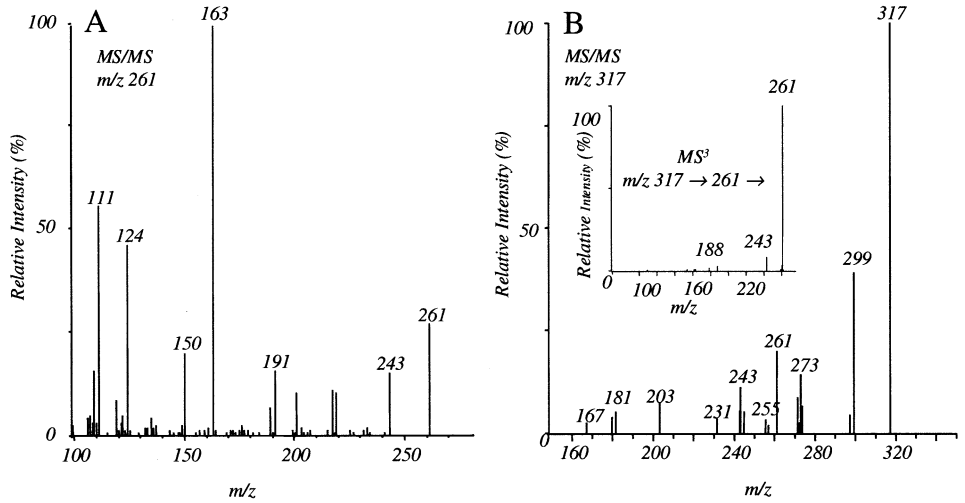
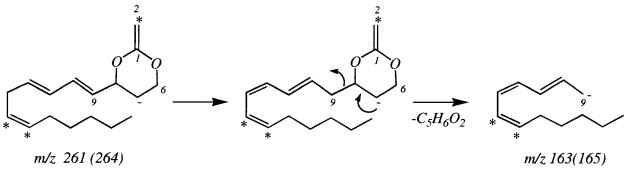


Figure 3. (**a**) Tandem mass spectrometry of *m/z* 261 following formation by in-source decomposition of the [M − H]$^-$ from LTA_4. (**b**) Collision-induced decomposition of the [M − H]$^-$ of LTA_4 generated by electrospray ionization in an ion trap mass spectrometer. Inset shows the product ions (MS^3) following collisional activation of *m/z* 261 generated from the [M − H]$^-$ of LTA_4 in an ion trap mass spectrometer. Samples were introduced by infusion.



Scheme 2

triple quadrupole instrument which could not be duplicated by multi-stage mass spectrometry in the ion trap mass spectrometer. Certainly the number of collisions, the collision gas (argon or helium) as well as the time frame of these experiments were different in both instruments. From the shift of the ion to *m/z* 165 in the deuterated LTA_4 (Figure 1d) and *m/z* 217 in the 20-trifluoro-LTA_4 (Figure 1c) the structure of this product ion involved the additional loss of five carbons from the carboxy terminus of the original LTA_4 structure after isomerization to a conjugated triene as proposed in Scheme **2**.

Other abundant product ions following collision activation of the LTA_4 carboxylate anion were observed at *m/z* 203 and 189 and likely arose from cleavage of carbon-carbon bonds of the eicosanoid backbone. These ions were produced at much lower abundance than the previous described ions. The ion at *m/z* 203 has been observed in the collisional activation of other eicosanoids with a single oxygen substitution at carbon atom 5 such as 5-HETE [16] and 5-oxo-ETE [17]. The appearance of *m/z* 189 has been previously observed in the mass spectra of other leukotrienes which contain a conjugated triene motif [18].

The extent of hydrolysis of LTA_4 during reversed phase HPLC separation was assessed in separate experiments using the collision induced formation of *m/z* 163 from the $[M - H]^-$. An isobaric internal standard (15-oxo-ETE) was added to determine relative retention times of LTA_4 and degradation products eluting from the column. The internal standard was monitored following the transition *m/z* 317 → 113. At pH 5.7 virtually no LTA_4 was found to elute from the column (Figure 4a), but only the internal standard and LTA_4 hydrolysis products (6-trans-LTB_4, *m/z* 335 → 195) were observed. However, when the mobile phase was adjusted to pH 11, the internal standard retention time shifted by 40 s and an abundant transition indicative of the elution of LTA_4 at 12.4 min was observed (Figure 4b). Flow injection analysis of LTA_4 prior to HPLC revealed that the hydrolysis products seen at pH 11 were present in the sample and were not a result of hydrolysis on the column.

Prostaglandin H_2 (PGH_2)

Collisional activation of the carboxylate anion (*m/z* 351) derived from the chemically unstable endoperoxide

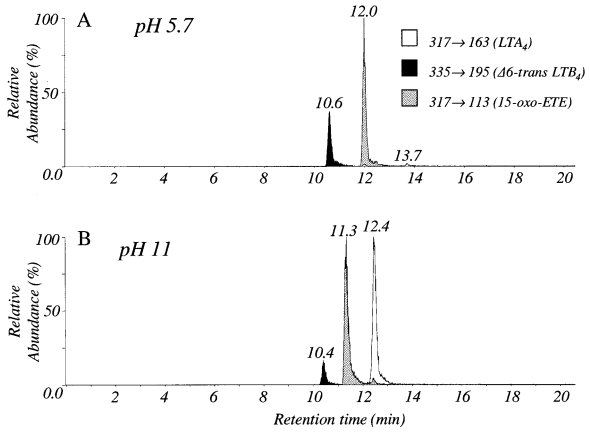


Figure 4. Gradient elution RP-HPLC separation and LC/MS/MS analysis of LTA_4 (8 ng) as detected by multiple reaction monitoring (*m/z* 317 → 163), the LTA_4 nonenzymatic hydrolysis products, Δ6-trans-LTB_4 (*m/z* 335 → 195), and an internal standard 15-oxo-ETE (10 ng) (*m/z* 317 → 113) with mobile phase at (**a**) pH 5.7 and (**b**) pH 11.

PGH_2 produced a product ion spectrum (Figure 5) indistinguishable from the negative ion MS/MS spectra for prostaglandin E_2 and prostaglandin D_2 previously reported [19, 20]. Briefly, collisional activation resulted in the loss of one and two molecules of water observed at *m/z* 333 and 315 as well as the loss of water and carbon dioxide resulting in product ions at *m/z* 289 and 271. The abundant ion at *m/z* 233 corresponded to the loss of hexanal and water from the methyl terminus (ω-end) of PGH_2 as had been previously described for other prostanoids [21]. An additional loss of carbon dioxide from this ω-end truncated fragment ion produced the ion at *m/z* 189. The identity of the MS/MS mass spectra from PGH_2 to both PGE_2 and PGD_2 suggests that collisional activation likely involved sufficient energy to effect isomerization to common structures.

While collisional activation of the corresponding carboxylate anions derived from the three isobaric

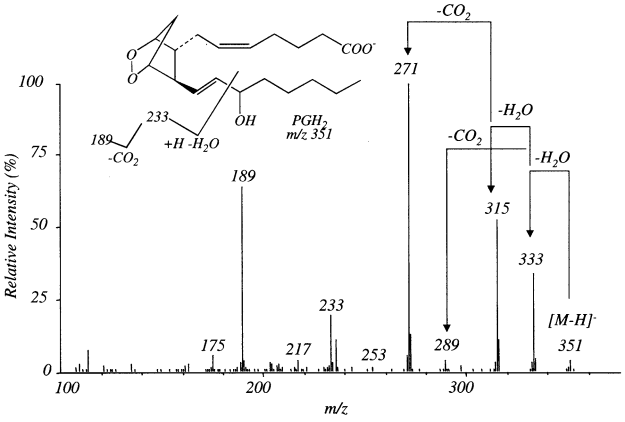


Figure 5. Negative ion electrospray and tandem mass spectrometry following collisional activation of the carboxylate anion $[M - H]^-$ of PGH_2 (*m/z* 351). Sample was introduced by infusion.

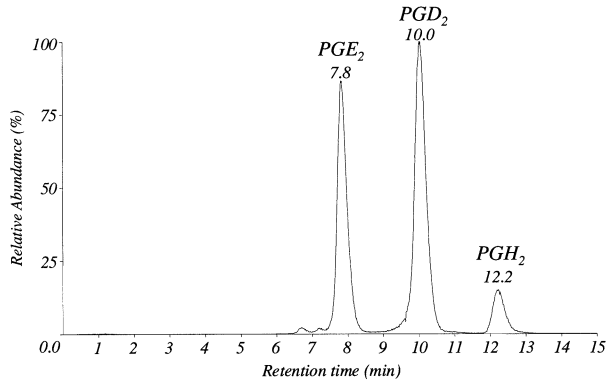


Figure 6. Isocratic RP-HPLC separation and LC/MS/MS analysis of PGE_2, PGD_2, and PGH_2 (2.5 ng each) as detected by monitoring the transition m/z 351 → 189, a reaction common to all three prostanoids.

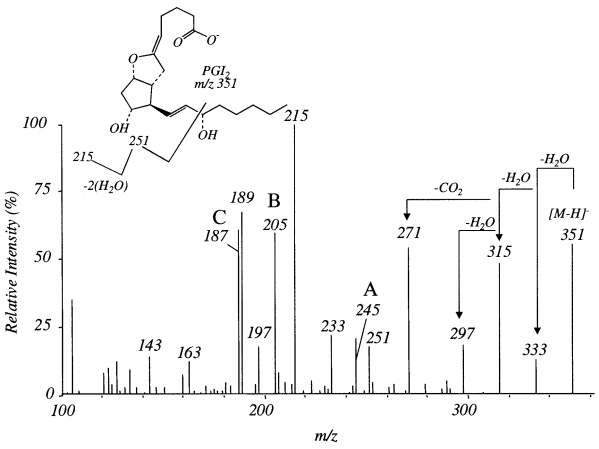


Figure 7. Negative ion electrospray and tandem mass spectrometry following collisional activation of the carboxylate anion [M − H]$^-$ of PGI_2 (m/z 351). Sample was introduced by infusion.

eicosanoids (PGH_2, PGD_2, and PGE_2) did not permit differentiation of each of these structures, it was possible to separate these three eicosanoids by using reversed phase HPLC with a basic mobile phase. This has been a difficult task to achieve under more traditional reversed phase HPLC separation with acidic or neutral solvent systems. Isocratic LC/MS afforded excellent separation of these three compounds with PGH_2 eluting as the most lipophilic component as expected (Figure 6). The mobile phase used for isocratic reversed phase HPLC was 10 mM triethylamine in 25% acetonitrile:methanol (65:35) and 75% water. PGE_2 was the least lipophilic compound under these conditions and eluted with a retention time of approximately 7.8 min and PGD_2 had intermediate lipophilicity with a retention time of 10.0 min.

Prostaglandin I_2

Analysis of prostaglandin I_2 (PGI_2), is typically carried out by analyzing the corresponding chemically stable metabolite, 6-keto-$PGF_{1\alpha}$ [22]. However, the use of alkaline solvents facilitated analysis of intact PGI_2 with very little hydrolysis into 6-keto-$PGF_{1\alpha}$. When PGI_2 was analyzed by LC/MS, the carboxylate anion was observed at m/z 351. Collisional activation of this [M − H]$^-$ ion generated a large number of product ions (Figure 7) which had the elemental compositions listed in Table 1. The highest mass ions observed were derived from the sequential losses of the elements of water observed at m/z 333, two losses of water at m/z 315, and three losses of water at m/z 297. The elemental composition of the ion at m/z 271 supported the loss of two neutral molecules of water as well as carbon dioxide.

A common fragment lost during collisional activation of all primary prostaglandin species has been previously shown to correspond to the loss of hexanal (100 u) from the ω-end of the molecule [21]. This loss from PGI_2 was also observed at m/z 251 which could also lose two molecules of water to form the most abundant product ion at m/z 215. Several product ions (m/z 233, 197, and 189) corresponded to the loss of multiple water molecules and carbon dioxide from this ion at m/z 251.

One of the more interesting ions in the spectrum of PGI_2 was observed at m/z 245 and appeared at first glance to fit into the series of ions corresponding to the loss of small neutral species such as CO_2 and water from the carboxylate anion since it corresponded to the loss of two 18 u and two 44 u neutral species. The elemental composition of this ion was found to be $C_{17}H_{25}O$ (Table 1) and therefore corresponded to an ion structure that had only one of the original oxygen atoms as well as 5-rings or double bonds. The collisional activation of the carboxylate anion derived from 6-keto-$PGF_{1\alpha}$ (m/z 369) and deuterium labeled 6-keto-$PGF_{1\alpha}$ (m/z 373) resulted in formation of a similar species observed at m/z 245 and 249, respectively (Table 2). One of the carbon atoms lost in the formation of these latter

Table 1. Elemental composition of product ions formed following the collisional activation of the carboxylate anion [M − H]$^-$ of PGI_2.[a]

m/z	Elemental composition	Error (mDa)
351.2171	$C_{20}H_{31}O_5$	0.0
333.2067	$C_{20}H_{29}O_4$	0.1
315.1973	$C_{20}H_{27}O_3$	1.3
297.1863	$C_{20}H_{25}O_2$	0.8
271.2058	$C_{19}H_{27}O$	−0.4
251.1296	$C_{14}H_{19}O_4$	1.3
245.1899 (A)	$C_{17}H_{25}O$	−0.6
233.1180	$C_{14}H_{17}O_3$	0.2
215.1075	$C_{14}H_{15}O_2$	0.3
207.1039	$C_{12}H_{15}O_3$	1.8
205.1586 (B)	$C_{14}H_{21}O$	−0.6
197.0964	$C_{14}H_{13}O$	−0.2
189.1291	$C_{13}H_{17}O$	1.2
187.1494 (C)	$C_{14}H_{19}$	0.7

[a]High resolution data was obtained using a Q-TOF mass spectrometer.

Table 2. Production ions from the carboxylate anion $[M - H]^-$ of PGI_2, its stable metabolite 6-keto-$PGF_{1\alpha}$, and related compounds following collision induced decomposition and tandem mass spectrometry

	$[M - H]^-$	$-H_2O$	$-2H_2O$	$-3H_2O$ m/z (abundance)[b]	$-H_2O - CO_2$	A[a]	B[b]	C[a]
PGI_2	351 (53)	333 (12)	315 (48)	297 (18)	nd[c]	245 (2)	205 (60)	187 (65)
6-keto-$PGF_{1\alpha}$	369 (100)	351 (15)	333 (15)	315 (15)	307 (10)	245 (14)	205 (14)	187 (8)
D_4-6-keto-$PGF_{1\alpha}$	373 (100)	355 (19)	337 (21)	319 (25)	311 (11)	249 (42)	205 (17)	187 (13)

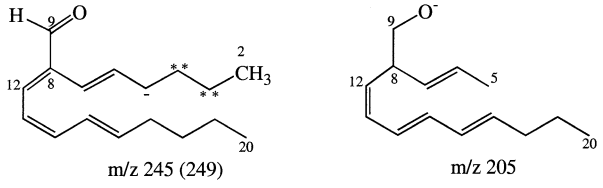
[a]Lettered designations correspond to product ions shown in Figure 7.
[b]Relative abundance in parentheses.
[c]Not detectable.

two ions was likely derived from the carboxylate moiety based on the number of oxygen atoms retained, but the other two carbon atoms lost during the fragmentation mechanism did not come from either carbon-3 or carbon-4 since no deuterium atoms were lost in the product ion derived from D_4-6-keto-$PGF_{1\alpha}$. The carbon atoms most likely eliminated in the mechanism of formation of this ion were derived from the cyclopentane ring in the form of C_2H_4O, as has been previously described for the collisional activation of the carboxylate anions from prostaglandin $F_{2\alpha}$ and the isomeric isoprostanes [23]. A structure consistent with the elemental composition and deuterium labeled species of related structure corresponded to a highly conjugated carbon-centered anion (Scheme **3**).

The abundant ion at *m/z* 205 formed following collisional activation of the PGI_2 carboxylate anion was also observed in the mass spectrum of D_4-6-keto-$PGF_{1\alpha}$, but the latter molecule had lost all four deuterium atoms consistent only with both carbon-3 and carbon-4 lost during ion formation (Table 2). This implied that an additional two carbon atoms were lost from the carboxylate terminus of the molecule and the single remaining oxygen atom was most likely a hydroxyl group attached to carbon-5 (Scheme **3**). This fragmentation process could also lead to the ion observed at *m/z* 187 which would correspond to the simple loss of water to extend conjugation and further delocalize the electrons in the resultant anion.

The facile hydrolysis of PGI_2 in aqueous solution leads to the product 6-keto-$PGF_{1\alpha}$ which chromatographs as a fairly broad peak under reversed phase conditions. The extent of PGI_2 hydrolysis during reversed phase HPLC was assessed using the transition *m/z* 351 → 215 for PGI_2 and the transition *m/z* 369 → 207 for 6-keto-$PGF_{1\alpha}$ (Figure 8) during LC/MS/MS analysis. At pH 5.7, virtually no PGI_2 could be detected eluting from the HPLC column, rather only the internal standard and 6-keto-$PGF_{1\alpha}$ were observed (Figure 8a). However, when the pH of the mobile phase was altered to pH 11, the retention time of the internal standard shifted by 4 min and only a small amount of 6-keto-$PGF_{1\alpha}$ was observed eluting from the column at 6.0 min. PGI_2 was observed and had a retention time of 8.3 min (Figure 8b). Flow injection analysis prior to HPLC revealed that the 6-keto-$PGF_{1\alpha}$ seen at pH 11 was present in the sample and did not result from hydrolysis during the course of HPLC analysis.

In conclusion, the mass spectrometric analysis of three highly reactive eicosanoids by electrospray ionization and tandem mass spectrometry has been carried out. Structurally significant fragment ions were obtained for LTA_4 and PGI_2 following collisional activation of the $[M - H]^-$ anions. PGH_2 yielded a collision spectrum similar to PGE_2 and PGD_2, but using an HPLC column stable to high pH, it was possible to separate and analyze all three prostanoids for the first time.



Scheme 3

Acknowledgments

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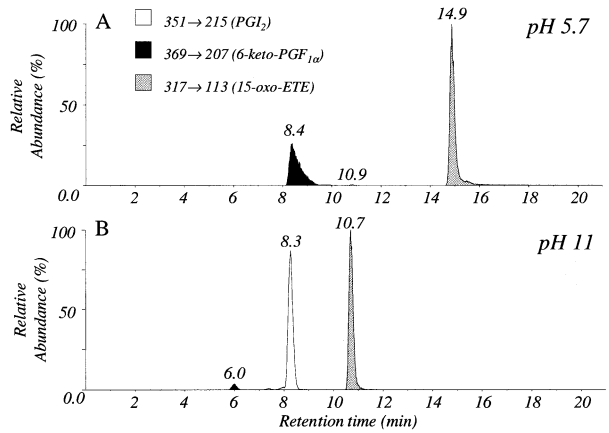

Figure 8. Gradient elution RP-HPLC separation and LC/MS/MS analysis of PGI_2 (8 ng) as detected by multiple reaction monitoring (*m/z* 351 → 215), the stable hydrolysis product, 6-keto-$PGF_{1\alpha}$ (*m/z* 369 → 207), and internal standard 15-oxo-ETE (10 ng) (*m/z* 317 → 113), with mobile phase held at (**a**) pH 5.7 and (**b**) pH 11.

References

1. Smith, W. L.; DeWitt, D. L.; Garavito, R. M. *Annu. Rev. Biochem.* **2000**, *69,* 145–182.
2. Funk, C. D. *Science* **2002**, *294,* 1871–1875.
3. Breyer, R. M.; Bagdassarian, C. K.; Myers, S. A.; Breyer, M. D. *Annu. Rev. Pharmacol. Toxicol.* **2001**, *41,* 661–690.
4. Figueroa, D.; Breyer, R. M.; Defoe, S. K.; Kargman, S.; Daugherty, B. L.; Waldburger, K.; Liu, Q.; Clements, M.; Zeng, Z.; O'Neill, G. P.; Jones, T. R.; Lynch, K. R.; Austin, C. P.; Evans, J. F. *Am. J. Respir. Crit. Care Med.* **2001**, *163,* 226–233.
5. Williams, C. S.; DuBois, R. N. *Am. J. Physiol.* **1996**, *270,* G393–G400.
6. Kurumbail, R. G.; Kiefer, J. R.; Marnett, L. J. *Curr. Opin. Struct. Biol.* **2001**, *11,* 752–760.
7. Ford-Hutchinson, A. W.; Gresser, M.; Young, R. N. *Annu. Rev. Biochem.* **1994**, *63,* 383–417.
8. Yokomizo, T.; Izumi, T.; Shimizu, T. *Life Sci.* **2001**, *68,* 2207–2212.
9. Henderson, W. R., Jr. *Ann. Allergy* **1994**, *72,* 272–278.
10. Fitzpatrick, F. A.; Morton, D. R.; Wynalda, M. A. *J. Biol. Chem.* **1982**, *257,* 4680–4683.
11. Maclouf, J.; Kindahl, H.; Granstrom, E.; Samuelsson, B. *Eur. J. Biochem.* **1980**, *109,* 561–666.
12. Stehle, R. G. *Methods Enzymol.* **1982**, *86,* 436–464.
13. Neue, D. U.; Walter, T. H.; Alden, B. A.; Jiang, Z.; Fisk, R. P.; Cook, J. T.; Glose, K. H.; Carmo, J. L.; Grassi, J. M.; Cheng, Y.-F.; Lu, Z.; Crowle, R. J. *Am. Lab.* **1999**, *31,* 36–39.
14. Dunlap, C. J.; McNeff, C. V.; Stoll, D.; Carr, P. W. *Anal. Chem.* **2001**, *73,* 598A–607A.
15. Carrier, D. J.; Bogri, T.; Cosentino, G. P.; Guse, I.; Rakhit, S.; Singh, K. *Prostaglandins, Leukot. Essent. Fatty Acids* **1988**, *34,* 27–30.
16. Wheelan, P.; Zirrolli, J. A.; Murphy, R. C. *Biol. Mass Spectrom.* **1993**, *22,* 465–473.
17. MacMillan, D. K.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1995**, *6,* 1190–1201.
18. Wheelan, P.; Zirrolli, J. A.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1996**, *7,* 140–149.
19. Zirrolli, J. A.; Davoli, E.; Bettazzoli, L.; Gross, M. L.; Murphy, R. C. *J. Am. Soc. Mass. Spectrom.* **1990**, *1,* 325–335.
20. Margalit, A.; Duffin, K. L.; Isakson, P. C. *Anal. Biochem.* **1996**, *235,* 73–81.
21. Hankin, J. A.; Wheelan, P.; Murphy, R. C. *Arch. Biochem. Biophys.* **1997**, *340,* 317–330.
22. Blair, I. A. *Methods Enzymol.* **1990**, *187,* 13–23.
23. Waugh, R. J.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1996**, *7,* 490–499.