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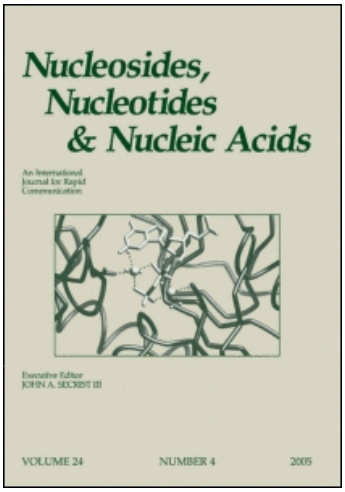

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ANALYSIS OF ANTIRETROVIRAL NUCLEOSIDES BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY AND COLLISION INDUCED DISSOCIATION

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Abstract

Antiretroviral nucleoside drugs used against the human immunodeficiency virus (HIV) infection have been analyzed using negative ion electrospray ionization (ESI) mass spectrometry and collision-induced dissociation (CID-MS/MS). Mass fragmentation of azidothymidine (AZT), didanosine (ddI), dideoxycytidine (ddC) and dideoxythiacytidine (3TC) were obtained at different cone voltages and collision energies. Fragmentation of purines and pyrimidines occurred by different pathways. For purines (ddI), the fragmentation was similar to those found in endogenous nucleosides; mainly the pseudo molecular ion is present $(M-H)^-$ and a cleavage through the glycosidic bond forming $(B)^-$ was observed. For pyrimidines (AZT, ddC, 3TC), the fragmentation pathways were different from endogenous nucleosides; for AZT, the fragmentation occurred primarily through the elimination of the azido group in the 3'-position $(M-H_2-N_3)^-$, whereas ddC and 3TC presented more complex fragmentation patterns. For ddC, fragmentation appeared to be dominated by a retro Diels-Alder mechanism $(M-CONH)^-$. For 3TC, the sulfur atom in the sugar moiety provided greater stability to the charge, producing fragments where the charge resided initially in the dideoxyribose $(M-C_2O_2H_6)^-$.

Introduction

Nucleoside analogues have been shown to be an excellent therapy as antiretroviral agents against the replication of the Human Immunodeficiency Virus (HIV) infection.[1] These deoxynucleoside analogues are a family of compounds lacking the 3'-hydroxyl group in the sugar moiety: azidothymidine (3'-azido-2',3'-dideoxythymidine, AZT), lamivudine ((–)-2'-deoxy-3'-thiacytidine, 3TC), and zalcitabine (2',3'-dideoxycytidine, ddC) represent the pyrimidine analogues; whereas didanosine (2',3'-dideoxyinosine, ddI) represents the purines (FIGURE 1). The intracellular characterization and quantitation of these compounds and their metabolites is a major priority in the clinical setting since drug plasma levels have not correlated with efficacy or toxicity.[2-4] Cellular levels of these drugs could provide a better marker for their efficacy; however, this is a difficult task due to the

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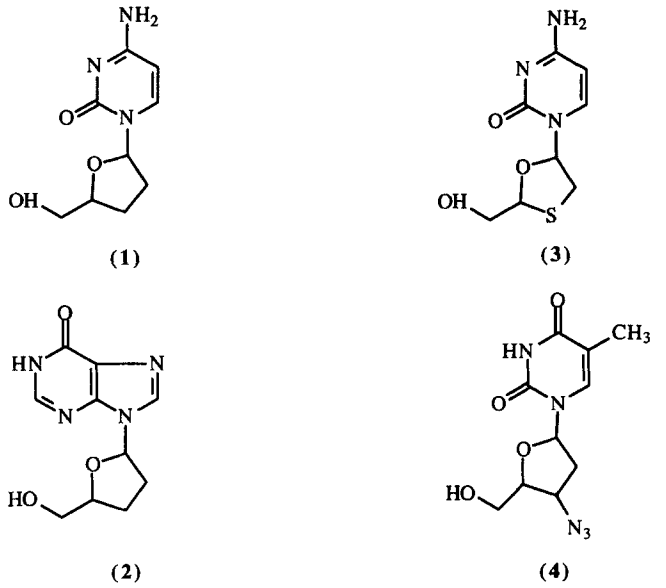

FIGURE 1. Chemical structures of antiretroviral nucleosides: (1) ddC, (2) ddI, (3) 3TC, and (4) AZT.

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small intracellular drug concentrations found in peripheral lymphoid cells.[5,6] Different analytical methodologies have been developed for the quantitation of these nucleosides, but the amount of patient's blood required for these assays is enormous.[7-9] This is an important aspect particularly if the studies are to be conducted in a pediatric population. An approach that has not been explored in this context is the use of mass spectrometry.

Different liquid interfaces for mass spectrometry have been used for the study of nucleosides and nucleotides. Fast atom bombardment (FAB) [10-13] and Electrospray Ionization (ESI) [14-16] are the most common interfaces used to obtain structural information and fragmentation patterns of nucleosides. The FAB mass spectra of nucleosides in the positive ion mode presents a major $(M+H)^+$ ion and several matrix adducts. The major fragmentation of $(M+H)^+$ ion is by glycosidic bond cleavage producing $(BH_2)^+$ ion. This $(BH_2)^+$ ion becomes the base ion in the Collision Activated Dissociation (CAD-MS/MS) spectrum. Two other characteristic ions [$(B+44)^+$ and $(B+30)^+$], corresponding to the ribose cleavage have been characterized. In the negative ion mode, two major ions have been observed corresponding to $(M-H)^-$ and $(B)^-$ ions.[10-13] In addition, a $(B+42)^-$ ion, analogous to $(B+44)^+$ ion in the positive mode is present in the

negative ion spectra providing information with respect to modifications in the 1', 2' or 4' positions of the ribose.[10,11]

Compared to FAB, ESI is a softer ionization technique forming mainly molecular ions.[17,18] A previous study with ESI and modified deoxynucleosides showed the presence of their pseudo molecular ions.[14] Protonated molecular ions $(M+H)^+$ were abundant in the positive ion spectrum, while $(M-H)^-$ appeared in the negative ion mode. Fragmentation was usually low in both spectra, but it was enhanced by increasing the skimmer cone voltage. For quantitation purposes, limits of detection around 3 pmol were reported with a linearity of two orders of magnitude.[14]

ESI-MS is very efficient for characterization and quantitation of exogenous compounds in biological samples, since the mass spectrum provides specific responses to particular mass ions.[17] In addition, CID-MS/MS provides even better specificity and selectivity when performing daugther ion experiments, providing specific fragmentation and quantitation for a particular ion.[17,18]

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We present the fragmentation pattern of AZT, ddI, ddC and 3TC using ESI-MS and CID-MS/MS in the negative ion mode. To our knowledge, this is the first time that such mass spectra are presented for these antiretroviral drugs. The absence of hydroxyl groups in the sugar moiety of these drugs hinders some of the fragmentation pathways observed with the endogenous analogues. In addition, CID-MS/MS provided the opportunity to describe the fragmentation pathways of these antiretroviral agents.

EXPERIMENTAL:

Materials: 2',3'-dideoxycytidine, 2',3'-dideoxyinosine, 2'-deoxy-3'-thiacytidine were obtained from Sigma Chemical Co. (St. Louis, MO). 3'-azido-2',3'-dideoxythymidine was obtained from Moravek Biochemicals (Brea, CA). Water and acetonitrile were of high performance liquid chromatography (HPLC) grade quality obtained from Fisher Scientific (Fairlawn, NJ) and J.T. Baker Inc. (Phillisburg, NJ), respectively. Ammonium hydroxide was obtained from Fisher Scientific (Fairlawn, NJ) and ammonium acetate from Aldrich Co. (Milwaukee, WI).

Sample Preparation: A solution 50:50 acetonitrile:ammonium acetate buffer (20 mM, pH = 8) was used as mobile phase for the flow injection analysis and also to dissolve antiretroviral drugs to a final concentration of 5 ng/μL. An aliquot of 10 μL of each sample was injected.

Instrumentation: Electrospray MS and MS/MS spectra were acquired using a VG Quattro Mass Spectrometer (Fison Instruments, UK) triple quadrupole mass spectrometer in the negative ion mode. Sample solutions were introduced into the 75 μm i.d. stainless

steel capillary of the electrospray source by a constant flow of 6 mL/hr of mobile phase using a Scientific syringe pump, model 100. The electrospray capillary potential was set to 3 kV. Nitrogen (200 L/hr) was used as drying gas for solvent evaporation and as the nebulizer gas (20 L/hr). The source temperature was set to 150°C. For each sample, different cone voltage values were evaluated from 15 to 50 V. For the MS/MS spectra of AZT, 3TC and ddC a cone voltage of 20 V was used; whereas for ddI 30 V was selected. Argon was used as the collision gas for the CID experiments with a collision cell pressure at 1.0 $x10^{-3}$ mbar and collision energies between 5 to 40V. Tuning was performed by continuous injection of drug solutions. The centroid data was processed using MassLynx software, version 2.00 from Fison Instruments, UK.

RESULTS AND DISCUSSION

Mass spectra from four antiretroviral nucleosides drugs (ddI, AZT, ddC and 3TC) currently used against HIV infection were obtained using ESI-MS and CID-MS/MS in the negative ion mode. The relative intensities of all ions obtained from the antiretroviral nucleoside drugs are presented in TABLE 1. Mainly, the pseudo molecular ions were the most abundant ions in ESI-MS and were used as the precursor ions for the CID-MS/MS determinations. All four nucleosides revealed different fragments in ESI-MS; attributed to the formation of adducts, or the cleavage of the base and sugar moieties. In addition, the relative intensities of the fragment ions were dependent on the experimental conditions. As the cone voltage increased more fragmentation from the nucleosides was observed. For the CID-MS/MS spectra, the intensities of the fragment ions increased with collision energy and correspondingly decreased in the $(M-H)^-$ relative intensities.

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FIGURE 2 shows the typical ESI-mass spectra recorded for ddI and AZT. Two ions are observed for ddI at m/z 235 and 135 corresponding to the pseudo molecular ion $(M-H)^-$ and the electrophilic base $(B)^-$, respectively. The cleavage of the glycosidic bond in ddI to form $(B)^-$ is favored by the extensive charge delocalization in the resonance structures present in the purine base. However, this cleavage was only observed when the skimmer cone voltage was increased to values higher than 25 V. The AZT spectrum shows the pseudo molecular $(M-H)^-$ ion at m/z 266, and the elimination of the azido group in the 3'-position $(M-H_2-N_3)^-$ at m/z 223. The ion fragment at m/z 302 appears to come from an adduct formation with the solvent, but at the present time the identification is still uncertain. It is important to point out that thymidine (AZT endogenous analogue) did not follow this fragmentation pathway when ESI-MS or FAB were used.[14] On the other hand, extensive fragmentation of thymidine was observed with FAB and CAD, including the formation of $(B)^-$ and the sugar fragments [$(B+44)^+$ and $(B+30)^+$].[10] These two fragments were not observed in our experiments with AZT.

TABLE 1. Pseudo molecular ions and significant fragment ions with their respective relative intensities in ESI-MS and CID-MS/MS of antiretroviral drugs in the negative ion mode.

Purines	$(M-H)^-$	B^-	$(M-N_3)^-$	Others
Didanosine-ddI				
(M=236, B=135)				
ESI-MS	235(100)	135(11)		
ESI-CID	235(P)	135(100)		
Pyrimidines				
Azidothymidine-AZT				
(M=267, B=124)				
ESI-MS	266(100)		223(9)	302(27)
ESI-CID	266(P)		223(46)	
Zalcitabine-ddC				
(M=211, B=110)				
ESI-MS	210(100)			
ESI-CID	210(P)			167(100), 107(43)
Lamivudine-3TC				
(M=229, B=119)				
ESI-MS	228(100)			288(14)
ESI-CID	228(P)			168(21),135(100)

P= Precursor ion of the spectra

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The ESI mass spectra for ddC and 3TC were relatively simple (TABLE 1). They showed their pseudo molecular $(M-H)^-$ ion and some solvent adduct formation. Fragmentation of these cytidine nucleoside analogues was almost non-existent under the conditions used in these experiments, even at the higher skimmer voltages (30-40 V).

Two major differences in the fragmentation patterns were observed for the antiretroviral drugs when using CID-MS/MS compared to FAB-MS/MS studies of endogenous nucleosides. First, the CID-MS/MS spectra showed $(M-H)^-$ ion and $(B)^-$ ion for purine analogues, but $(B)^-$ ion was not observed for pyrimidines. In FAB-MS/MS studies, $(M-H)^-$ and $(B)^-$ ions were observed in all nucleosides with a proton transfer mechanism from the 2'- or 3'-position hydroxyl group, initiating the glycosidic bond cleavage and providing the residual charge needed for sugar decomposition.[10,13] The absence of hydroxyl groups in the ribose moiety in the drugs studied eliminates this charge transfer mechanism. Thus, the presence of the $(B)^-$ for ddI is apparently due to an excess of energy aided by the extensive charge delocalization provided by the base. Second, the CID-MS/MS determinations did not show the same sugar fragment ions observed with FAB-MS/MS $[(B+44)^+$ and

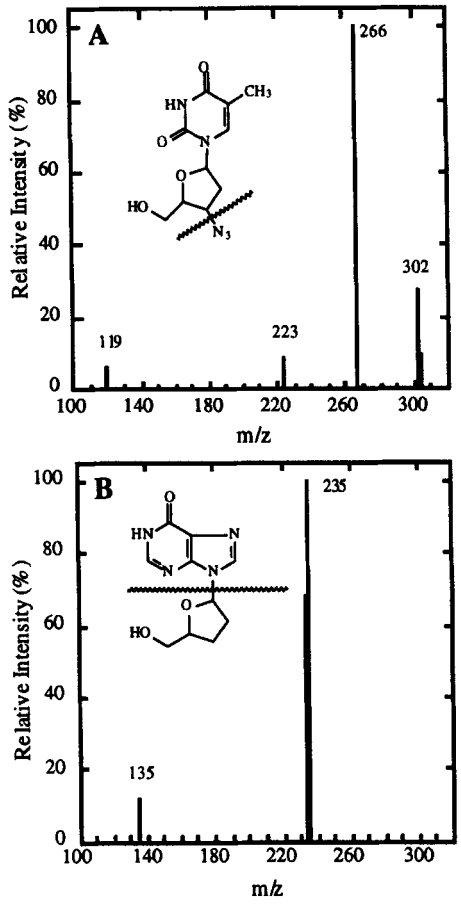

FIGURE 2. Electrospray single mass spectra for (A) AZT and (B) ddI. Experimental conditions as described in the experimental section with Skimmer Cone Voltage for A = 20V and B = 30V. The pseudo molecular ion for AZT appears at mass 266 and the ion mass at 223 is due to the loss of N_3 of AZT. The ion at m/z 235 is due to the pseudo molecular ions of ddI. Ion fragment at m/z 135 is assigned to B^-.

$(B+30)^+$].[10] Only 3TC showed fragmentation from the sugar moiety, but the pathway is different from FAB-MS/MS.

For ddC, the CID-MS/MS produced the pseudo molecular ion (m/z 210) with an additional ion at m/z 167 (FIGURE 3). This fragment ion could be assigned to a tautomeric sturcture of cytidine, where the imino form acts as the precursor for a retro Diels-Alder reaction as shown in FIGURE 4. The same mechanism was observed for uridine and cytidine using FAB,[10,13] but not for 2'-deoxycytidine using ESI-MS.[14] This mechanism is aided by the lack of a proton in the 3-position of the base. It was previously shown that

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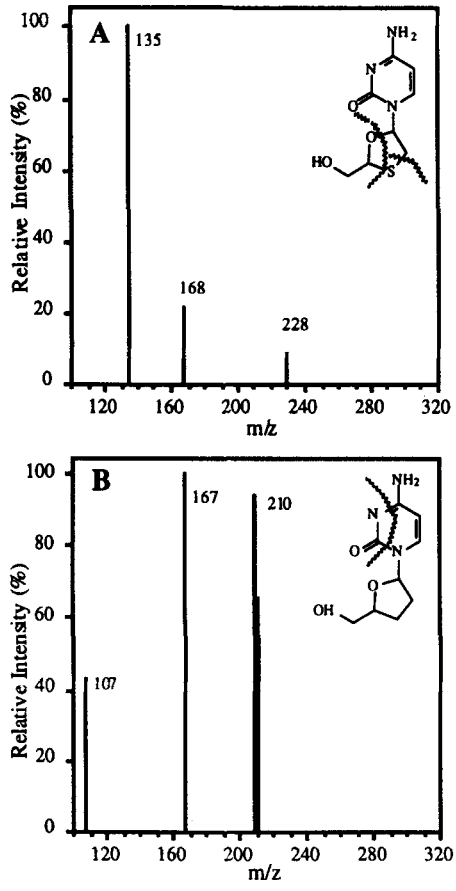

FIGURE 3. Negative ion CID MS/MS of (A) 3TC and (B) ddC. The pseudo molecular ions were selected as the precursor ion. The ions at m/z 168 and 135 in (A) are from sugar cleavage. The ions at m/z 167 and 107 in (B) are fragment ions of the ring cleavages of the base.

the abstraction of this proton produced the $(M-H)^-$ ion of nucleosides with charge retention in the heterocyclic base as observed for AZT.

3TC showed its pseudo molecular ion with CID-MS/MS at m/z 228. In addition, ions with at m/z 168 and 135 were also observed. Unlike ddC, the ion at m/z 168 of 3TC did not correspond to a retro Diels-Alder mechanism, mainly because of the presence of the thiol group in the 3' position. The sulfur atom stabilized the negative charge in the sugar moiety, inducing the rupture of the sugar to produce a fragment ion that contains the base and part of the sugar structure $(B+49)^-$ (Figure 5). The loss of a SH group produces the fragment ion at m/z 135 $(B+16)^-$. Similar results were obtained with 2 and 4 thio-

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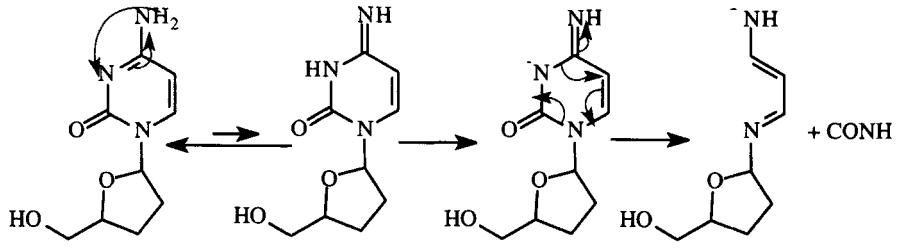

FIGURE 4. ddC negative ion fragmentation pathway for the formation of ion at 167.

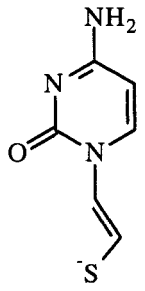

FIGURE 5. Structure assigned to the negative ion fragment at m/z 168 from the CID-MS/MS spectrum of 3TC.

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substituted uridines where the sulfur atom residing in the base moiety stabilized the negative charge. This stabilization produced more molecular ions $(M)^-$ rather than the expected pseudo molecular ion $(M-H)^-$.[10] In addition, the sugar fragmentation diminished significantly when compared with uridine.[10]

We have shown for the first time the ESI-MS and CID-MS/MS of antiretroviral drugs used for the treatment of HIV infection. The fragmentation of ddI was similar to that found in endogenous nucleosides forming the pseudo molecular ion $(M-H)^-$ and the base ion $(B)^-$. The fragmentation pathway for AZT was primarily through the elimination of the azido group in the 3'-position $(M-44)^-$. ddC's fragmentation is dominated by a retro Diels-Alder mechanism $(M-43)^-$. For 3TC, the sulfur atom in the sugar moiety provided greater stability to the charge, producing fragments where the charge resided initially in the dideoxyribose $(M-61)^-$. These studies will be expanded for the characterization of these drugs and their metabolites in biological samples.

Acknowledgments

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