Characterization of Cladribine and Its Related Compounds by High-Performance Liquid Chromatography/Mass Spectrometry

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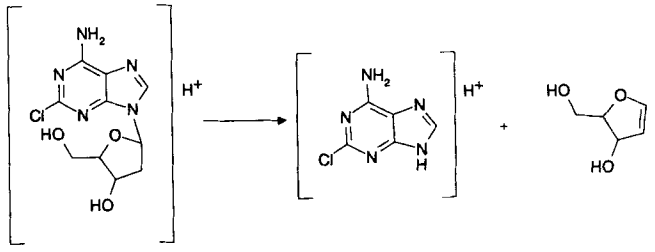
Abstract □ High-performance liquid chromatography/mass spectrometer (HPLC/MS) was used to identify and structurally characterize the modified nucleoside cladribine (2-chloro-2′-deoxy-β-adenosine) and 13 synthesis-related byproducts in bulk drug. Confirmation of compound identity was accomplished by spectral analysis (^1H and ^{13}C NMR spectroscopy, mass spectrometry, and UV absorption spectroscopy) of the related compounds as isolated from crude mixtures of the drug substance and by spiking experiments with authentic standards. The use of on-line mass spectrometric analysis (i.e., LC/MS) to augment UV absorption spectra permitted rapid identification of many of the compounds of interest.

Introduction

Characterization of any active drug substance suitable for use in both research and clinical programs is essential in the pharmaceutical industry. A particularly important aspect of the chemical characterization of bulk drug substance includes the structural identification of various byproducts associated with the therapeutic agent (reactants, reaction intermediates, degradation products, etc.). Typically, the levels of these related compounds present in the drug substance are very low, usually on the order of 0.01–0.5%. Isolation of these minor constituents (<0.1%) in quantities large enough to facilitate structural identification is time consuming and dependent on a sufficient supply of bulk drug. Alternatively, HPLC in combination with thermospray ionization (TSI) mass spectrometry and UV detection can provide important structural information required to identify drug substance components in a timely manner.

Cladribine (1, 2-chloro-2′-deoxy-β-adenosine) is a simple nucleoside whose structure differs from the naturally occurring nucleoside, deoxyadenosine, by virtue of the substitution of chlorine for hydrogen in the 2-position of the purine ring. This substitution renders the molecule highly resistant to degradation by adenosine deaminase.[1] The first reported synthesis of this compound was by Christiansen *et al.*[2] Cladribine has been shown to be an effective agent in the treatment of many types of lymphoid neoplasms.[3,4] In this paper we report on the use of HPLC/MS to augment UV absorption spectroscopy in the structural characterization and identification of various compounds observed in the chromatograms of 14 lots of cladribine drug substance.

Detailed reports of the mass spectrometric behavior of purines, pyrimidines, and nucleosides have appeared in the literature.[5-7] Under classical electron ionization (EI) conditions (vaporization *via* ballistically heated insertion probe), nucleosides are prone to thermal cleavage in addition to the expected ionic decomposition pathways. These reactions result in mass spectra that are characterized by ions corresponding to the purine base ([M − sugar]$^{+\cdot}$), the sugar ([M − purine base]$^{+\cdot}$), and generally weak signals for the intact molecular ion ([M]$^{+\cdot}$, see Scheme 1).



Scheme 1—Mass spectral fragmentation of cladribine

Thermospray ionization (TSI), in contrast to EI, is considered to be a "soft" ionization technique that deposits considerably less energy, producing spectra with few, if any, fragment ions.[8-11] The generally accepted mechanism of ion formation under thermospray conditions proposes that most of the primary species are formed in solution, with the remainder resulting from chemical ionization processes initiated by the auxiliary corona discharge or filament. The net result is that in addition to analyte ions, other ions are also observed in typical TSI mass spectra, due to the various components of the HPLC mobile phase. In the ammonium acetate/acetonitrile solvent system used for these experiments, ionization of the LC effluent produces numerous and extremely abundant "reagent" or "background" ions (comparable to those observed under chemical ionization conditions) such as H_3O^+, NH_4^+, $[CH_3COOH]H^+$, and $[CH_3CN]H^+$, as well as clusters of these ions that form with molecules of water, acetonitrile, ammonia, and acetic acid. In addition to molecular weight information, further insight into the identity of certain compounds can be gained from the isotopic distribution pattern observed in the mass spectrum. This factor is extremely useful for cladribine and its related compounds since ions containing a chlorine atom will display an easily recognized $[M + 2]^+$ signal.

Experimental Section

Materials—Water (Millipore *MILLI-Q UF PLUS* purification system), acetonitrile (*OPTIMA* Fisher Scientific), ammonium acetate (Mallinckrodt), acetic acid (Fisher Scientific), Rainin Microsorb C-18 HPLC column 5 μm, 4.6 mm × 25 cm equipped with a Rainin C-18 5-μm guard column, cladribine drug substance (RWJ 26251-000 Lots B-P), 6-amino-2-chloropurine (4, RWJ 27629-000-B), 2-amino-2′-deoxyadenosine (2, RWJ 46479-000-A), deoxyadenosine (3, Aldrich Chemicals), 2-chloro-2′-deoxy-α-adenosine (10, RWJ 47024-000-A).

Equipment—For TSP/HPLC/MS: Solvent delivery system (Perkin-Elmer Series 410); Waters 994 diode-array detector (Millipore), set to monitor 264 nm; Finnigan MAT TSQ-70 triple quadrupole mass spectrometer using a Finnigan thermospray LC/MS interface; Rheodyne Model 7125 100-μL loop injector. For preparative chromatography: Solvent delivery system (two Rainin HPX or HPXL series pumps (25 mL/min heads)), dispensing pump (Rainin HPX or HPXL series pump (10 mL/min head)) controlled with the Dynamax HPLC Method Manager; dual-wavelength detector, (Gilson Model 116 and Dynamax Model UV-D) set to monitor 264 and 300 nm; fraction collector (Gilson Model 201 and Dynamax Model FC-1); chart recorder (Kipp & Zonen BD41). For quantitative HPLC: Solvent delivery system (Perkin-Elmer Series 410); autosampler (Perkin-Elmer ISS-100); diode-array detector

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(Hewlett-Packard, Model 1040A, set to monitor 264 and 300 nm); laboratory automation system (Hewlett-Packard 3350A, the digital output from the two detector channels were transferred to the LAS for integration). The HPLC–UV spectra were obtained with the same equipment as described above using the Hewlett-Packard Chemstation/HP-1040M detector. ^{1}H NMR and ^{13}C NMR spectra were obtained in CD_3SOCD_3 or CD_3OD or D_2O or CD_3COOD (with tetramethylsilane as an internal standard) on a GE QE-300 instrument at 300 and 75 MHz, respectively, or a Varian XL-400 instrument at 400 and 100 MHz. Desorption chemical ionization (DCI) and desorption electron ionization (DEI) mass spectra were obtained on a Finnigan MAT 8230 or Finnigan INCOS 50 mass spectrometer. Fast atom bombardment (FAB) mass spectra and accurate mass data were obtained on a Finnigan MAT 8230 mass spectrometer.

Methods for TSP/HPLC/MS. Method A: The method was a binary gradient reversed-phase HPLC procedure. Mobile phase A was a 0.1 M solution of ammonium acetate in water. Mobile phase A was prepared by adding 4 L of Milli-Q water to 30.83 g of ammonium acetate. The final pH of the solution was adjusted to pH 6.55 ± 0.1 with dilute acetic acid. This solution was filtered through a 0.45-μm filter. Mobile phase B was acetonitrile. Cladribine drug substance solutions were typically prepared in water at concentrations of 4.0 mg/mL for analysis. Samples were manually injected (50–100 μL) followed by a 22-min linear gradient from an initial eluent composition of 96% A/4% B to 80% A/20% B at a flow rate of 1.2 mL/min, followed by a 12-min hold. Method B: For analysis of late-eluting impurities, a second method was employed that used a linear gradient of 21 min from an initial eluent concentration of 89% A/11% B to 50% A/50% B at a flow rate of 1.2 mL/min, followed by a 5-min hold. Ionization mode: thermospray ionization, vaporizer temperature: 85 °C. Ion source temperature: 250 °C. Discharge voltage: 2000 V. Scan time: 2 s. Scanned mass range: 145–850 u.

Method for Quantitative Impurity Profile of Cladribine—The method was a binary gradient reversed-phase HPLC procedure. Mobile phase A was a 0.1 M solution of ammonium acetate in water. Mobile phase A was prepared by adding 4 L of Milli-Q water to 30.83 g of ammonium acetate. The final pH of the solution was adjusted to pH 6.55 ± 0.1 with dilute acetic acid. This solution was filtered through a 0.45-μm filter. Mobile phase B was acetonitrile. Cladribine drug substance solutions were prepared in water at concentrations of 0.5 mg/mL for analysis. Samples were automatically injected (triplicate runs, 10-μL injections) followed by a 22-min linear gradient from an initial eluent composition of 96% A/4% B to 80% A/20% B at a flow rate of 1 mL/min, followed by a 1-min hold and a linear gradient from 80% A/20% B to 50% A/50% B over 10 min followed by a 20-min hold.

Method for Preparative Chromatography—The method was a binary gradient reversed-phase HPLC procedure. Mobile phase A was either Milli-Q water or a 0.1 M solution of ammonium acetate in water. Mobile phase A was prepared by adding 4 L of Milli-Q water to 30.83 g of ammonium acetate. The final pH of the solution was adjusted to pH 6.5 ± 0.1 with dilute acetic acid. This solution was filtered through a 0.45-μm filter. Mobile phase B was acetonitrile. The separation was carried out on a C-18 reversed-phase HPLC column (Rainin Dynamax-60A C18 HPLC column 8 μm, 41.4 mm × 25 cm equipped with a Dynamax-60A C-18 8-μm guard column or Rainin Dynamax-60A C18 HPLC column 8 μm, 21.4 mm × 25 cm equipped with a Dynamax-60A C-18 8-μm guard column or Rainin Microsorb C-18 HPLC column 5 μm, 10 mm × 25 cm equipped with a Rainin C-18 5 μm (guard column) at ambient temperature. Typically cladribine was dissolved in water and pumped onto the column with the dispensing pump. A 15-min linear gradient was then initiated from an initial eluent composition of 70% A/30% B to 60% A/40% B at a flow rate of 24 mL/min. A second linear gradient followed immediately, which changed the eluent composition from 60% A/40% B to 50% A/50% B over 5 min. The column effluent was collected by the fraction collector, and appropriate fractions were combined and rechromatographed as required using variations of this method and the 21.4- and 10-mm HPLC columns until pure compounds were obtained. Acetonitrile was removed by rotary evaporation, and the residue was diluted with water and lyophilized.

Cladribine (1)—^{1}H NMR (300 MHz, d_6-DMSO): δ 2.30 (m, J = 3.3, 6.2, −13.3 Hz, 1H), 2.67 (m, J = 5.9, 7.5, −13.4 Hz, 1H), 3.54 (m, J = 4.4, −11.7, 5.7 Hz, 1H), 3.62 (m, J = 4.6, −11.8, 5.7 Hz, 1H), 3.88 (m, J = 2.8, 4.5, 4.5 Hz, 1H), 4.41 (m, J = 2.9, 3.1, 5.6, 4.2 Hz, 1H), 4.99 (d, J = 5.7 Hz, 3′-OH, 1H), 5.34 (d, J = 4.2 Hz, 5′-OH, 1H), 6.28 (dd, J = 6.2, 7.4 Hz, 1H), 7.84 (br s, NH_2, 2H), 8.38 (s, 1H). ^{13}C NMR (100 MHz, d_6-DMSO): δ 39.39, 61.66, 70.71, 83.58, 87.94, 118.17, 139.81, 150.06, 152.96, 156.78. UV max: 264 nm.

2,6-Diamino-2′-deoxyadenosine (2)—Supplied by Raylo Chem. Edmonton, Alberta, Canada. ^{1}H NMR (300 MHz, d_6-DMSO): δ 2.17 (m, J = 2.7, 6.0, −13.2 Hz, 1H), 2.60 (m, J = 5.7, 8.4, −13.5 Hz, 1H), 3.52 (m, 1H), 3.59 (m, 1H), 3.84 (m, 1H), 4.35 (m, 1H), 5.26 (m, 5′-OH, 1H), 5.29 (m, 3′-OH, 1H), 5.76 (br s, 2 NH_2, 2H), 6.17 (dd, J = 6.0, 8.1 Hz, 1H), 6.76 (br s, 6 NH_2, 2H), 7.92 (s, 1H), ^{13}C NMR (75 MHz, d_6-DMSO): δ 39.31, 61.91, 70.94, 83.03, 87.57, 113.39, 135.69, 151.12, 156.08, 159.94. MS: m/e 267 (M + 1)$^+$. UV max: 255, 280 nm.

2′-Deoxyadenosine (3)—Obtained from Aldrich Chem. ^{1}H NMR (300 MHz, d_6-DMSO): δ 2.27 (m, J = 2.9, 6.1, −13.2 Hz, 1H), 2.74 (m, J = 5.7, 7.9, −13.4, 1H), 3.55 (m, J = 4.3, 6.7, −11.2 Hz, 1H), 3.63 (m, J = 4.6, 4.6, −11.8 Hz, 1H) 3.89 (m, 1H), 4.43 (m, 1H), 5.26 (dd, J = 5.1, 6.6 Hz, 5′-OH, 1H), 5.32 (d, J = 3.9 Hz, 3′-OH, 1H), 6.36 (dd J = 6.0, 7.8 Hz, 1H), 7.32 (br s, NH_2, 2H), 8.15 (s, 1H), 8.34 (s, 1H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 39.43, 61.88, 70.95, 83.92, 87.97, 119.25, 139.50, 148.87, 152.33, 156.07. MS: m/e 252 (M + 1)$^+$.

2-Methoxy-2′-deoxy-α-adenosine (7)—To a stirring solution of **10** (99.8 mg, 0.3 mmol) was added 0.3 mL of NaOMe solution (25 wt % in methanol). The resulting solution was refluxed overnight. The solution was allowed to cool to room temperature. This was purified by preparative HPLC to yield 7 (36.4 mg, 37%). ^{1}H NMR (300 MHz, d_6-DMSO): δ 2.321 (dt, J = 3.1, 3.1, −14.1 Hz, 1H), 2.743 (m, J = 7.7, 7.7, −13.9 Hz), 3.451 (m, J = 4.6, 11.2 Hz, 1H), 3.469 (m, J = 4.4, 11.8 Hz, 1H), 3.824 (s, 1H), 4.114 (q, J = 3.4, 4.3, 4.4 Hz 1H), 4.292 (m, J = 3.2, 3.2, 6.9 Hz 1H), 4.87 (br s, 5′-OH, 1 H), 5.69 (br s, 3′-OH, 1H), 6.235 (dd, J = 3.2, 7.9 Hz), 7.282 (s, 2 NH_2, 2H), 8.214 (s, 1H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 39.71, 53.92, 61.60, 70.68, 83.05, 88.23, 115.27, 138.39, 150.62, 156.74, 161.67. MS: m/e 282 (M + 1)$^+$. UV max: 253, 267 nm.

2-Methoxy-2′-deoxy-β-adenosine (8)—To a refluxing suspension of 1 (1 g, 3.5 mmol) in MeOH (50 mL) was added 1 mL of NaOMe solution (25 wt % in methanol). The resulting suspension was kept at 70 °C for 1.5 h. The resulting solution was then cooled to room temperature and stirred overnight. A white precipitate had formed overnight. The temperature was raised to 70 °C, and an additional equivalent of NaOMe was added. After 42 h the reaction was complete by HPLC. Then 5 mL of H_2O was added and the mixture evaporated under reduced pressure to remove MeOH. The resulting mixture was allowed to cool to room temperature during which a white precipitate formed. This was filtered and washed with H_2O to yield 8 (630 mg, 64%). ^{1}H NMR (300 MHz, d_6-DMSO): δ 2.235 (m, J = 2.9, 6.2, 13.2 Hz, 1H), 2.749 (m, J = 5.8, 7.9, 13.4 Hz, 1H), 3.530 (m, J = 4.0, 11.4 Hz, 1H), 3.615 (m, J = 4.7, 11.6 Hz, 1H), 3.824 (s, 3H), 3.866 (m, J = 2.6, 4.6, 4.6 Hz, 1H), 4.422 (m, 1H), 5.060 (br s, 5′-OH, 1H), 5.320 (br s, 3′-OH, 1H), 6.258 (t, J = 6.2, 7.8 Hz, 1H), 7.312 (s, NH_2, 2H), 8.144 (s, 1H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 38.95, 53.98, 61.94, 70.99, 83.50, 87.78, 115.67, 138.27, 150.67, 156.80, 161.68. UV max: 253, 267 nm.

2-Chloroadenosine (9)—To a stirring suspension of guanosine (11.0 g, 38.8 mmol, dried over P_2O_5) in 15 mL of pyridine and 40 mL of DMF was added 30 mL of acetic anhydride. The suspension was then heated to 75 °C for 4.5 h. The resulting solution was evaporated in vacuo to a slurry and filtered. The filtercake was then washed with 2-propanol. Second and third crops were recovered, filtered, and washed with 2-propanol. These were combined to yield 12.93 g of crude triacetylguanosine (81.44% yield) which was recrystallized from 2-propanol. To a solution of recrystallized triacetylguanosine (9.1 g, 22.3 mmol, dried over P_2O_5) and Et_4NCl (8.3 g, 50.09 mmol, dried over P_2O_5) in 50 mL of CH_3CN were added N,N'-dimethylaniline (3.2 mL, 25.25 mmol) and $POCl_3$ (13.7 mL, 147 mmol). The resulting solution was refluxed for 10 min. This was evaporated in vacuo to a thick red oil. The oil was dissolved in 150 mL of $CHCl_3$ and stirred with crushed ice for 15 min. The layers were separated, and the aqueous layer was extracted with $CHCl_3$ (5 × 50 mL). The combined organic layers were washed with water (6 × 30 mL) and 5% $NaHCO_3$ (3 × 30 mL). The organic layer was dried over $MgSO_4$ and filtered. Next, 60 mL of 2-propanol was added and the resulting solution evaporated in vacuo to a thick orange oil. A 100-mL aliquot of 2-propanol was added. The resulting solid was filtered and washed with 2-propanol. The solid was recrystallized from 2-propanol to yield 6.74 g of 6-chloro-2-aminotriacetyladenosine (70.85% yield). To a solution of 6-chloro-2-aminotriacetyladenosine (6.5 g, 15.2 mmol), 9.3 g of triphenylmethyl chloride (9.3 g, 33.4 mmol), and 1.2 g of K_2CO_3 in CH_2Cl_2 (50 mL) was added isoamyl nitrite (1.7 mL in 5 mL of CH_2Cl_2, 12.7 mmol). The resulting solution was refluxed for 30 min, cooled to room temperature, and filtered. The solution was evaporated in vacuo to a thick yellow oil which partially solidified upon standing. This was dissolved in 400 mL of 80:20 hexane/ethyl acetate and evaporated to yield 5.36 g of crude 2,6-dichlorotriacetyladenosine (78.9% yield). To

Table 1—Quantitative Impurity Profile of Impurities in Cladribine Drug Substance Lots B–P

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MW	285	266	251	169	283	280	281	281	301	285	299	300	403	403
						Approximate Area %[a]								
Lot														
B	98.8	tr	tr	0.2	tr	tr	tr	tr	0.3	tr	tr	tr	nd	tr
C	99.6	tr	nd	0.3	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
D	99.4	0.1	nd	tr	tr	tr	tr	0.1	tr	0.3	tr	tr	tr	tr
E	96.5	tr	nd	1.7	tr	tr	tr	0.4	tr	1.2	tr	tr	tr	tr
F	99.4	0.3	nd	tr	tr	tr	tr	0.1	tr	0.1	tr	tr	tr	tr
G	99.7	0.1	nd	tr	tr	tr	tr	0.1	tr	tr	tr	tr	tr	tr
H	99.7	tr	nd	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.1	tr
I	99.7	0.1	nd	tr	tr	tr	tr	0.1	tr	tr	tr	tr	tr	tr
J	99.7	tr	nd	tr	tr	tr	nd	tr	tr	0.1	tr	tr	tr	tr
K	99.5	tr	tr	tr	tr	tr	nd	tr	tr	tr	0.3	0.2	nd	nd
L	99.4	0.1	nd	tr	tr	tr	tr	0.1	tr	0.2	tr	tr	tr	tr
N	99.4	0.2	nd	tr	tr	nd	tr	0.1	nd	tr	tr	tr	tr	tr
O	99.5	0.2	nd	tr	tr	nd	tr	0.1	nd	tr	tr	tr	tr	tr
P	99.2	0.1	nd	tr	tr	nd	tr	0.1	tr	tr	tr	tr	0.2	0.1

[a] tr = peak seen by HPLC or HPLC/MS but area is too small for quantitation (<0.1%). nd = not detected.

a solution of crude 2,6-dichlorotriacetyladenosine (300 mg, 0.67 mmol) in 1 mL of THF was added 3 mL of concentrated NH_4OH. The resulting solution was stirred for 3 days at room temperature. The solvent was evaporated, and the residue was triturated twice with absolute ethanol and filtered to yield 50.0 mg of crude 2,6-dichloroadenosine (24.8% yield). This was used without further purification. ^1H NMR (300 MHz, d_6-DMSO): δ 3.55 (m, 2H), 3.65 (m, 1H), 4.09 (m, 1H), 4.52 (m, 1H), 5.01–5.58 (br m, 2′-, 3′-, and 5′-OH's 3H), 5.56 (m, 1H), 7.46 (br s, NH_2), 8.39 (s, 1H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 51.71, 73.68, 73.86, 85.72, 87.30, 119.52, 140.03, 148.25, 152.81, 156.36. MS: m/e 302 $(M+1)^+$. UV max: 264 nm.

2-Chloro-2′-deoxy-α-adenosine (10)—Supplied by Raylo Chem., Edmonton, Alberta, Canada. ^1H NMR (300 MHz, d_6-DMSO): δ 2.31 (m, J = 2.6, 2.6, −14.2 Hz, 1H), 2.74 (m, J = 7.5, 7.5, −14.4 Hz, 1H), 3.45 (m, J = 5.0, 5.0, 2H), 4.12 (m, J = 2.7, 4.5, 4.5 Hz, 1H), 4.32 (m, 1H), 4.86 (t, J = 5.6 Hz, 5′-OH, 1H), 5.52 (d, J = 3.8 Hz, 3′-OH, 1H), 6.27 (dd, J = 2.7, 7.8 Hz, 1H), 7.81 (br s, NH_2, 2H), 8.40 (s, 1H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 39.70, 61.59, 70.61, 83.49, 88.69, 117.78, 139.97, 150.09, 152.91, 156.71. MS: m/e 286 $(M+1)^+$. UV max: 264 nm.

2-Chloro-6-methoxy-2′-deoxy-β-adenosine (12)—Isolated from acetonitrile wash of crude cladribine supplied by Raylo Chem., Edmonton, Alberta, Canada. ^1H NMR (400 MHz, d_6-DMSO): δ 2.349 (m, J = 3.7, 6.4, 13.3 Hz, 1H), 2.680 (m, J = 5.9, 7.0, 13.2 Hz, 1H), 3.533 (m, J = 4.6, 5.5, 11.7 Hz, 1H), 3.607 (m, J = 4.8, 5.3, 11.8 Hz, 1H), 3.882 (q, J = 3.2, 4.6, 4.6 Hz), 4.112 (s, 3H), 4.417 (m, J = 3.7, 3.7, 3.7, 6.0 Hz, 1H), 4.925 (dd, J = 5.5, 5.5 Hz, 5′-OH, 1H), 5.344 (d, J = 4.4 Hz, 3′-OH, 1H), 6.358 (t, J = 6.7, 6.7 Hz, 1H), 8.610 (s, 1H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 39.38, 54.91, 61.38, 70.43, 83.81, 88.00, 120.24, 151.36, 152.58, 160.78. MS: m/e 301 $(M+1)^+$. UV max: 258 nm.

2-Chloro-5′-(p-toluoyl)-2′-deoxy-β-adenosine (13)—Isolated from crude cladribine supplied by Raylo Chem., Edmonton, Alberta, Canada. ^1H NMR (400 MHz, d_6-DMSO–$CDCl_3$, 8:5 v/v): δ 2.397 (s, 3H), 2.428 (m, J = 4.4, 6.8, 13.5 Hz, 1H), 2.795 (m, J = 6.3, 6.3, 13.6 Hz), 4.172 (m, J = 4.3, 4.3, 5.6 Hz, 1H), 4.423 (dd, J = 5.8, 11.8 Hz, 1H), 4,536 (dd, J = 4.4, 11.8 Hz, 1H), 4.601 (br s, 1H), 5.473 (br s, 1H), 6.339 (t, J = 6.5, 6.5 Hz, 1H), 7.266 (d J = 8.5 Hz, 2H), 7.60 (br s, NH_2, 2H), 7.820 (d, J = 8.2 Hz, 2H), 8.157 (s, 1H). MS: m/e 404 $(M+1)^+$. UV max: 246, 266 nm.

2-Chloro-3′-(p-toluoyl)-2-deoxy-β-adenosine (14)—Isolated from crude cladribine supplied by Raylo Chem., Edmonton, Alberta, Canada. ^1H NMR (400 MHz, d_6-DMSO–$CDCl_3$, 8:5 v/v): δ 2.429 (s, 3H), 2.636 (m, J = 1.4, 5.6, 13.9 Hz, 1H), 2.974 (m, J = 6.0, 9.0, 14.5 Hz, 1H), 3.784 (dd, J = 3.4, 5.4 Hz, 2H), 4.279 (m, J = 1.3, 2.7 Hz, 1H), 5.282 (t, J = 5.9, 5.9 Hz, 1H), 5.636 (d, J = 5.9 Hz, 1H), 6.448 (dd, J = 5.8, 8.8 Hz, 1H), 7.321 (d, J = 8.1 Hz, 1H), 7.681 (br s, NH_2, 2H), 7.946 (d, J = 8.2 Hz, 1H), 8.339 (s, 1H). MS: m/e 404 $(M+1)^+$. UV max: 247, 266 nm.

Results and Discussion

Related Substance Profiling of Cladribine Drug Substance—The HPLC gradient method described above as method A separated 12 of the impurities detected in 14 samples of the drug substance. The estimated levels of the detected components found in cladribine drug substance are shown in Table 1.

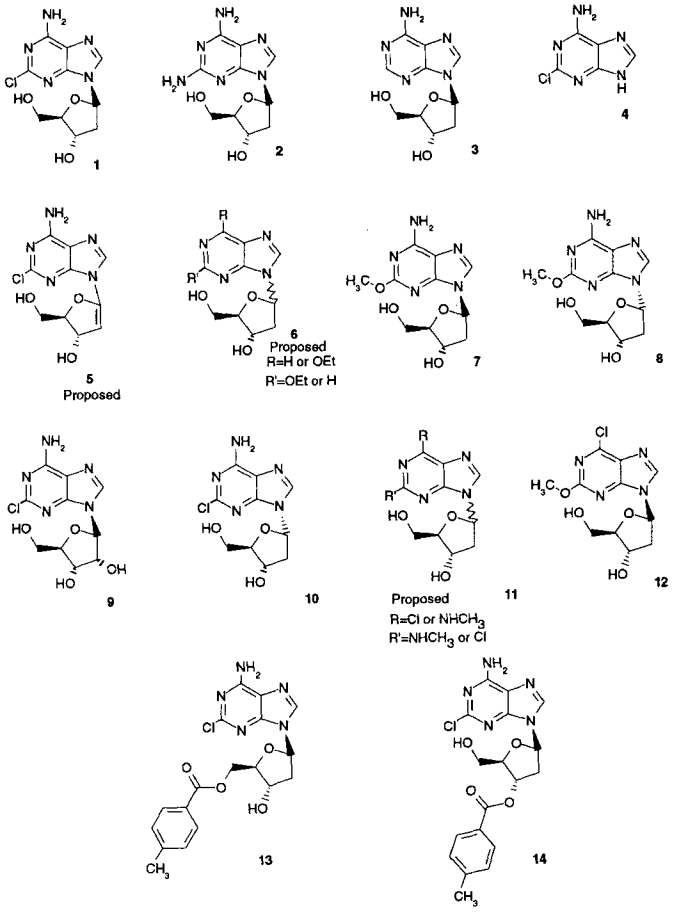
Table 2—Mass Spectral Data for Cladribine and Related Compounds

Compd	MW	$[MH]^+$	$[M+2]^+$	$[BH]^+$	$[BH + CH_3CN]^+$
1	285	286	288	170	211
2	266	267		151	
3	251	252			177
4	169	170	172		211
5	283	284	286	170	
6	280	281			206
7	281	282		166	207
8	281	282		166	207
9	301	302	304	170	211
10	285	286	288	170	211
11	299	300	302	184	225
12	300	301	303	185	226
13	403	404	406	170	211
14	403	404	406	170	211

The gradient method described as method B eluted two additional impurities that were detected at low levels in several of the samples (toluoyl-blocked derivatives of cladribine). During the course of this investigation, 13 compounds were identified by HPLC/MS (Scheme 2). Each of the compounds detected by UV detection (solution concentrations: 4 mg/mL of cladribine) was also observed by HPLC/MS using either full scan or selected ion monitoring detection.

Structural Assignments for Cladribine and Related Substances—Figures 1 and 2 show representative HPLC chromatograms for a typical aqueous solution of cladribine (solution concentration: 2 mg/mL, 50-μL injection). In addition to the parent compound (1; $C_{10}H_{12}N_5O_3Cl$), 13 additional compounds also are detected. These compounds are present at levels ranging from 0.1 to <0.01%.

The structures of the various compounds were deduced using a combination of data, including mass spectra and UV absorption spectra. Confirmation of assigned structures was accomplished by isolating (compounds **2**, **12**, **13**, **14**) or synthesizing the compound (compounds **3**, **4**, **7**, **8**, **9**, **10**) followed by full spectral analysis with both proton and carbon NMR spectra. Mass spectral analysis of an isolated sample of compound 1 yielded



Scheme 2—Assigned structures for cladribine (**1**) and related compounds

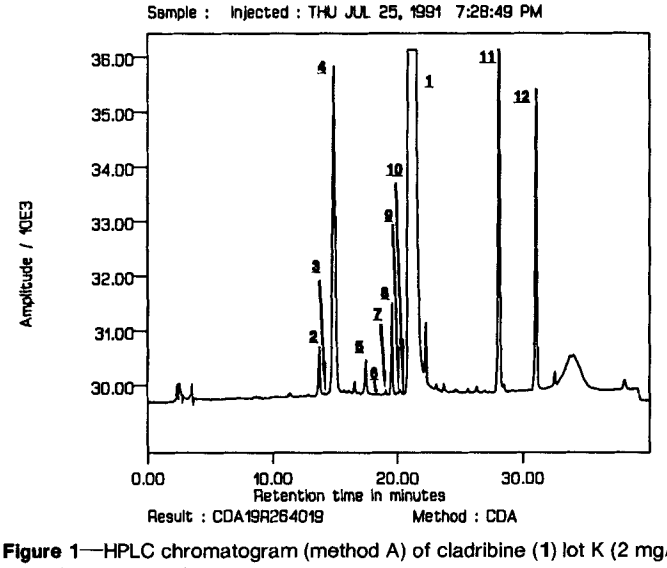


Figure 1—HPLC chromatogram (method A) of cladribine (**1**) lot K (2 mg/mL, 50-μL injection).

an intense signal for the molecular ion (m/z 285) under EI conditions. Accompanying this signal in the spectrum were the characteristic chlorine isotope peak (m/z 287) and a major fragment ion at m/z 169. This fragment ion corresponds to the intact purine base (easily recognized by the presence of the $[M + 2]^+$ isotope peak) and is generated from either thermal scission of the purine–saccharide bond (with subsequent ionization of the neutral purine) or gas-phase ionic decomposition of the

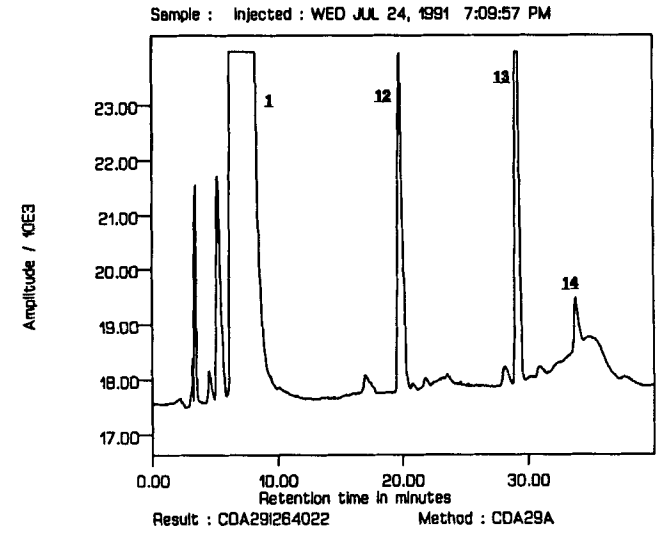


Figure 2—HPLC chromatogram (method B) of cladribine (**1**) lot H (2 mg/mL, 100-μL injection, peak splitting due to column overload).

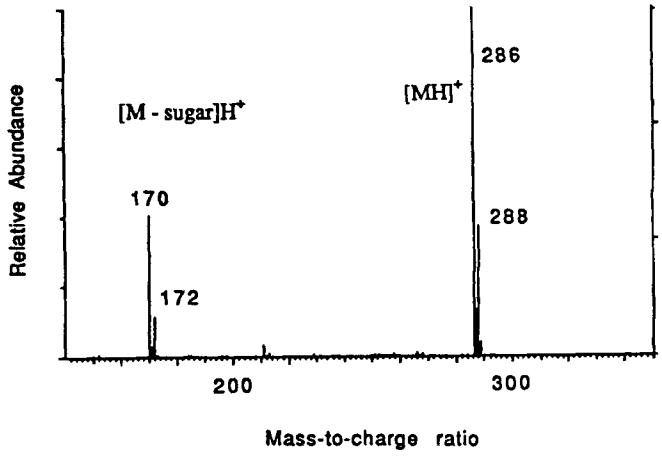


Figure 3—Thermospray ionization mass spectrum of cladribine (**1**) lot H (4 mg/mL, 100-μL injection).

molecular ion ($[M - C_5H_8O_3]^{\cdot+}$). The chemical ionization mass spectrum was obtained using isobutane as reagent gas and confirms the molecular weight by virtue of the appearance of an intense signal at m/z 286 ($[MH]^+$), with its accompanying isotope peak at m/z 288. The thermospray ionization mass spectrum of compound 1, obtained during on-line LC/MS analysis of actual drug substance, is shown in Figure 3. The spectrum exhibits a signal for the protonated molecule (m/z 286), a fragment ion representing the protonated purine, $[BH]^+$ (*via* thermal or ionic decomposition, m/z 170), and a gas-phase cluster ion of the purine base with acetonitrile (m/z 211).

As is often the case, the thermospray spectrum is very similar (with the exception of the acetonitrile cluster) to the CI mass spectrum. In addition to the mass spectral data, both the ^1H and ^{13}C NMR spectra and the UV absorption spectrum of the compound were found to support the structure of cladribine. This type of information, coupled with knowledge of the synthetic reaction scheme, provided the basis for structural assignments of many of the related substances detected in various samples of cladribine drug substance.

Compound 2 was detected in most of the samples and was present at levels of 0.1–0.2%. The thermospray mass spectrum of this compound (Figure 4) displays a signal for the protonated molecule at m/z 267. The only other signal of significance in the spectrum was observed at m/z 151. Knowledge of the general fragmentation behavior of nucleosides allows assignment of this

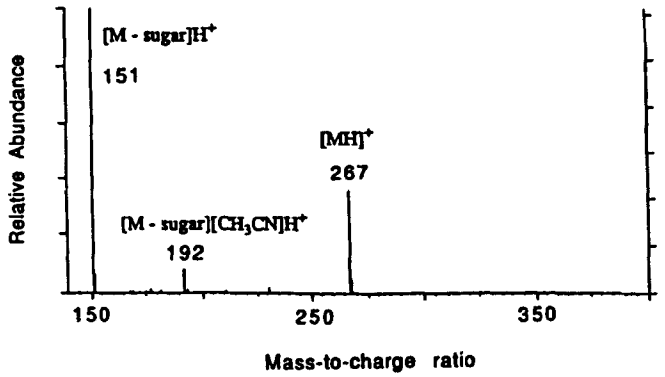


Figure 4—Thermospray ionization mass spectrum of 2-amino-2′-deoxyadenosine (2) lot F (4 mg/mL, 100-μL injection).

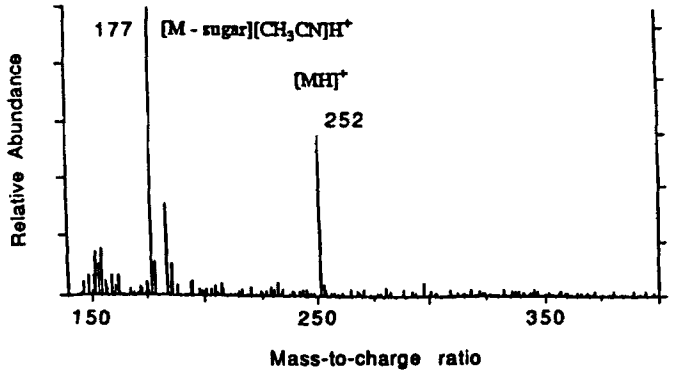


Figure 5—Thermospray ionization mass spectrum of deoxyadenosine (3) lot K (4 mg/mL, 100-μL injection).

ion as the protonated purine ($[BH]^+$) portion of the molecule (same loss of 116 u observed in the spectrum of cladribine, 1). The absence of the chlorine isotope pattern combined with the mass shift of 19 u (with respect to the parent compound, cladribine), and an even-numbered molecular weight (i.e., even number of nitrogen atoms in the molecule) led to a proposed identity for compound 2 of 2-amino-2′-deoxyadenosine ($C_{10}H_{14}N_6O_3$).

Analysis of the UV absorption spectrum of compound 2 reveals a bathochromic shift with respect to cladribine, which is consistent with substitution of an amino substituent for chlorine on the purine ring. Independent analysis of authentic 2-amino-2′-deoxyadenosine reveals chromatographic retention time, a UV absorption spectrum, and a TSI mass spectrum that are all indistinguishable from those of compound 2, confirming the proposed identity. The proposed identity of this major reaction byproduct is also completely consistent with the synthetic scheme, resulting from excessive amination in the final step of the synthesis. Compound 2 was subsequently isolated from a crude reaction mixture and characterized by ultraviolet (UV), mass spectral (MS), and proton and carbon magnetic resonance data (^1H and ^{13}C NMR).

Compound 3 is a minor component that was detected only in a few of the samples, and then, only at trace levels (<0.05%). The TSI mass spectrum of this compound displays signals for the protonated molecule ($[MH]^+$) at m/z 252 and a major fragmentation product at m/z 177 (Figure 5). Because the mass difference between these two prominent ions is not the expected 116 u (elimination of deoxyribose), the ion at m/z 177 is recognized to be something other than the protonated purine portion of the molecule. If the ion at m/z 252 is assumed to be a nucleoside of similar structure to cladribine, the signal due to the protonated purine portion of the molecule would be detected at m/z 136. Because of the presence of extremely intense "reagent ion" signals

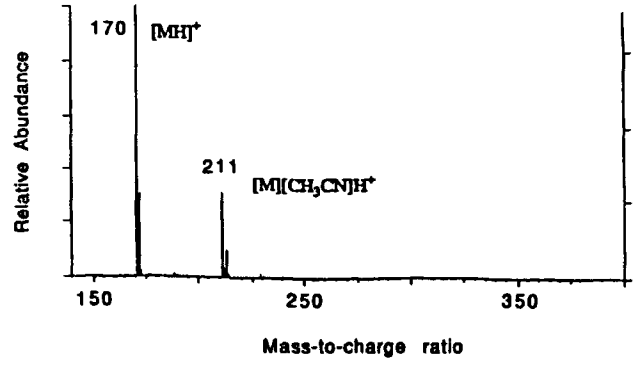


Figure 6—Thermospray ionization mass spectrum of 6-amino-2-chloropurine (4) lot E (4 mg/mL, 100-μL injection).

(resulting from the ionization and clustering reactions of the mobile phase constituents), the scanned mass range is adjusted so as to exclude these ions from detection. Unfortunately, one of the undesirable outcomes of restricting the mass range (to avoid saturation of the detection circuitry) is the omission of structurally diagnostic ions from the mass spectrum. For all of the experiments described here, the lower limit of the scanned mass range was set at 145 u. While this setting will obviously cause the expected ion at m/z 136 to be excluded from the data, its presence can be inferred from the ion at m/z 177, which is its acetonitrile adduct ($[BH + CH_3CN]^+$). Thus, the odd-numbered mass of the purine (135 u as inferred from the 177^+ cluster ion), the absence of the chlorine isotope pattern in the signal of the protonated molecule, and the mass shift relative to the parent compound all indicate the identity of compound 3 to be deoxyadenosine ($C_{10}H_{13}N_5O_3$). Further confirmation of the proposed identity of compound 3 was accomplished through mass spectral and chromatographic analysis of authentic deoxyadenosine. Both the TSI mass spectrum and the chromatographic retention time of compound 3 were identical to those of the authentic standard.

Compound 4, while a minor constituent, was detected in a majority of the samples that were examined. The TSI mass spectrum of the compound displays an abundant ion at m/z 170 (with associated $[M + 2]^+$ signal) with an apparent acetonitrile adduct at m/z 211 (Figure 6). Since there are no ions of higher mass in the spectrum, and the mass difference between the prominent ion in this spectrum (probable $[MH]^+$) and that of the parent compound is 116 u corresponding to the loss of deoxyribose), the logical conclusion is that compound 4 is 2-chloroadenine (6-amino-2-chloropurine; $C_5H_4N_5Cl$). Again, comparison of spectral data (both TSI mass spectrum and UV spectrum) and the chromatographic retention times of compound 4 and authentic 2-chloroadenine revealed complete agreement.

Compound 5 appears at very low levels (<0.1%) in all of the drug substance lots that were examined. The TSI mass spectrum exhibits a significant $[MH]^+$ signal at m/z 284, with a corresponding isotope peak ($[M + 2]^+$ ion at m/z 286). The fragment ion at m/z 170 ($[BH]^+$; also with accompanying isotope peak) indicates that the purine base is intact, containing both the amino and chlorine substituents. The 2-u mass differential between compound 5 and cladribine, and its location in the saccharide portion of the molecule, point to dehydrodeoxyribose as the source of structural variation. Given the odd-numbered molecular weight, the presence of chlorine, and the mass differential with respect to cladribine, a reasonable proposal for the identity of compound 5 is 2-chloro-2′,3′-didehydrodeoxyadenosine ($C_{10}H_{10}N_5O_3Cl$).

Compound 6 also appears at very low levels (<0.1%). The TSI mass spectrum exhibits a significant $[MH]^+$ signal at m/z 281, with no corresponding isotope peak (no $[M + 2]^+$ ion at m/z 283). The acetonitrile adduct of the purine portion of the

molecule infers the molecular weight of base to be 165 u. Given the odd-numbered molecular weight, the absence of chlorine, and the mass differential with respect to cladribine, a reasonable proposal for the identity of compound **6** is either 2-ethoxypurine or 6-ethoxypurine ($C_{12}H_{16}N_4O_4$).

Compounds **7** and **8** appear to be major byproducts of the synthesis as they are detected in nearly all of the samples and are present at levels on the order of 0.1%. The TSI mass spectra obtained for the two chromatographic peaks are virtually identical, suggesting that the compounds are structural isomers. The spectra display signals for the protonated molecule ($[MH]^+$) at m/z 282, the protonated purine base ($[BH]^+$) at m/z 166, and the acetonitrile adduct of the base at m/z 207. The mass difference between the $[MH]^+$ ion and the signal for the protonated base is indicative of deoxyribose as the sugar moiety in this nucleoside (loss of 116 u). Once again, there is no characteristic chlorine isotope pattern in the spectrum indicating substitution of the halogen atom by some other substituent. The mass differential between these compounds and cladribine is indicative of a methoxy functional group, and this proposal is completely consistent with the slight bathochromic shift that is observed in the UV spectra of compounds **7** and **8** ($C_{11}H_{15}N_5O_4$). Compound **7** was synthesized and characterized by UV, MS, and NMR spectroscopy. The data supported the assignment of **7** as 2-methoxydeoxy-α-adenosine. Compound **8** was also synthesized and characterized by UV, MS, and NMR spectroscopy. The data supported the assignment of **8** as 2-methoxydeoxy-β-adenosine.

Compound **9** was detected in the majority of the lots of drug substance that were analyzed. The TSI mass spectrum shows a prominent $[MH]^+$ ion at m/z 302, complete with chlorine isotope pattern. Also present in the spectrum are significant signals for both the protonated purine base (m/z 170, with chlorine isotope pattern) and its acetonitrile adduct (m/z 211). Interestingly, unlike all of the previous compounds, the mass shift between the protonated molecule and the protonated purine is not 116 u but rather 132 u. This addition of 16 u in the molecular weight of the compound (with respect to cladribine) and its location in the saccharide portion of the molecule suggests strongly that this nucleoside contains an additional oxygen atom and is therefore 2-chloroadenosine ($C_{10}H_{12}N_5O_4Cl$; containing ribose instead of deoxyribose). The UV spectrum of compound **9** is extremely similar to that of cladribine, supporting the hypothesis that the structural modification is not located in the chromophore (purine ring). Further confirmation of the proposed identity of compound **9** was accomplished through spectral and chromatographic analysis of a synthesized standard.

Compound **10** is a significant constituent of all 14 of the samples that were examined, being detected at levels near 0.1%. The TSI mass spectrum of this component exhibits an $[MH]^+$ ion at m/z 286 with the diagnostic $[M + 2]^+$ signal at m/z 288 (chlorine containing species). Also present in the spectrum were the now familiar signals for the protonated purine and its acetonitrile adduct (m/z 170 and 211, respectively). The many similarities between the spectral information (UV absorption and mass spectra) obtained for this compound and that of the parent compound lead to the conclusion that compound **10** is a structural isomer of cladribine. Given the information available from the synthetic scheme, the identity of compound **10** was proposed to be 2-chloro-2′-deoxy-α-adenosine ($C_{10}H_{12}N_5O_3Cl$). Chromatographic analysis of a sample of the authentic α anomer yielded a retention time that was identical to that observed for compound **10**.

Compound **11** appears in all but one of the samples and is generally found to be present at levels below 0.1%. The protonated molecule is observed in the TSI mass spectrum at m/z 300 and is accompanied by an easily recognized chlorine isotope (m/z 302). Like many of the other compounds, this molecule also produces ions in the spectrum that represent the protonated purine portion of the molecule and its associated acetonitrile adduct (m/z 184 and 225, respectively). The mass difference between the $[MH]^+$ ion and the characteristic purine fragment immediately identifies the saccharide moiety as deoxyribose (loss of 116 u). The 14-u increase in the mass of the purine base suggests the addition of a methyl group. Since the bathochromic shift in the absorption maximum of the UV spectrum of compound **11** is consistent with N-methylation, and the amino substituent represents the most likely reactive site, the structure assigned to compound **11** is 2-chloro-*N*-methyldeoxyadenosine ($C_{11}H_{14}N_5O_3Cl$).

Compound **12** produces a TSI mass spectrum that is dominated by the three characteristic ions observed for all of the previous compounds: $[MH]^+$ at m/z 301 (with $[M + 2]^+$ isotope peak), $[BH]^+$ at m/z 185, and the CH_3CN adduct of the purine at m/z 226. The even-numbered molecular weight indicates that there are an even number of nitrogen atoms in this molecule. Taken together with the chlorine isotope pattern, it is very likely that the 6-amino substituent is no longer present in the molecule. The observed mass of the protonated molecule (15 u higher than cladribine) together with the likely absence of the amino substituent suggests that the 6-position of the purine ring is now occupied by a methoxy group. This supposition is completely consistent with the mass exhibited by the $[BH]^+$ ion at m/z 185 and the slight hypsochromic shift observed in the UV spectrum of compound **12**. Compound **12** was successfully isolated from a crude reaction mixture wash. The ^1H and ^{13}C NMR spectra obtained for the isolated material allowed for assignment of the structure as 2-methoxy-6-chloro-2′β-deoxyadenosine.

Compounds **13** and **14** yielded nearly identical TSI mass spectra, suggesting that the compounds are structural isomers. In addition to the signal for the protonated molecule at m/z 404 (with associated chlorine isotope peak), the characteristic and familiar fragment and adduct ions were also noted at m/z 170 and 211, respectively. The mass of the $[BH]^+$ ion (same as that observed for cladribine) is an immediate indication that the structural variation in this molecule is located in the saccharide moiety. Simple arithmetic reveals the mass of the sugar moiety to differ from that of deoxyribose by the weight of a toluoyl substituent (a blocking reagent used in an intermediate step of the synthesis). Since these two compounds elute with a retention time significantly longer than cladribine or any other of the previously detected compounds, the presence of some bulky, nonpolar substituent is highly probable. The high degree of similarity in the UV spectra and the retention times of these compounds fully supports the proposal of structural isomerism based on the mass spectral information. Characterization (^1H and ^{13}C NMR spectroscopy) of the two compounds upon isolation from an acetonitrile wash of crude cladribine identifies them as 2-chloro-6-amino-9β-(2′-deoxy-5-*p*-toluoylribofuranosyl)purine (**13**) and 2-chloro-6-amino-9β-(2′-deoxy-3-*p*-toluoylribofuranosyl)purine (**14**).

Conclusion

HPLC/MS analysis, using thermospray ionization, provided molecular weight and structurally diagnostic fragmentation information for the modified nucleoside cladribine and its related substances. Because the analysis of synthetic therapeutic agents and their associated byproducts (reactants, reaction intermediates, degradation products, etc.) is crucial to the complete characterization of any bulk drug substance, techniques that can provide information "on-line" (i.e., without prior isolation) are particularly valuable. Since the typical levels of related compounds present in drug substance can be very low (0.01–0.5%), isolation of these minor constituents is often time consuming and dependent on a sufficient supply of bulk drug.

Thermospray ionization (TSI) mass spectrometry provided important structural information required to identify drug substance components in a timely manner. This information was useful in assigning structures and confirming identities of compounds observed by HPLC.

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