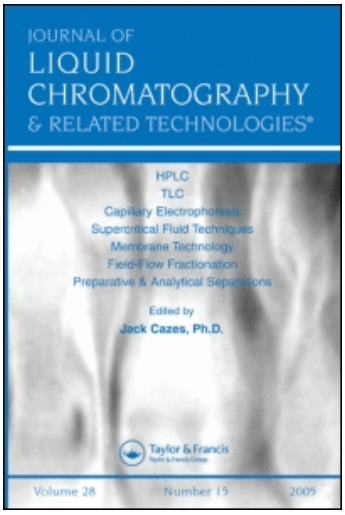

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation, Isolation, and Characterization of Isoform Impurities of Gemcitabine Formed During the Anomerization of Protected α-Gemcitabine to Gemcitabine

S. G. Hiriyanna[a]; K. Basavaiah[a]; Hari N. Pati[b]; Bijay K. Mishra[b]
[a] Department of Chemistry, University of Mysore, Mysore, India [b] Department of Chemistry, Sambalpur University, Jyoti Vihar, India

To cite this Article Hiriyanna, S. G. , Basavaiah, K. , Pati, Hari N. and Mishra, Bijay K.(2007) 'Separation, Isolation, and Characterization of Isoform Impurities of Gemcitabine Formed During the Anomerization of Protected α-Gemcitabine to Gemcitabine', Journal of Liquid Chromatography & Related Technologies, 30: 20, 3093 — 3105
To link to this Article: DOI: 10.1080/10826070701633780
URL: http://dx.doi.org/10.1080/10826070701633780

Journal of Liquid Chromatography & Related Technologies®, 30: 3093–3105, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701633780

Separation, Isolation, and Characterization of Isoform Impurities of Gemcitabine Formed During the Anomerization of Protected α-Gemcitabine to Gemcitabine

S. G. Hiriyanna and K. Basavaiah
Department of Chemistry, University of Mysore, Manasagangothri, Mysore, India

Hari N. Pati and Bijay K. Mishra
Department of Chemistry, Sambalpur University, Jyoti Vihar, India

Abstract: In the impurity profile of the gemcitabine active pharmaceutical ingredient, two process related impurities (0.12%) were detected other than the known α-isomer of gemcitabine impurity in HPLC with respect to gemcitabine. The mass spectral data and UV spectrum of these two impurities were similar to that of gemcitabine. These impurities were referred as isoform impurity I and isoform impurity II. These isoform impurities were isolated from a crude sample of gemcitabine bulk drug using a reverse phase preparative high performance liquid chromatography (prep-HPLC), and were characterized by LC-MS, LC-MS/MS, and FT-NMR experimental techniques. Based on the results obtained from the different spectroscopic experiments, these isoform impurities were characterized as β-anomer of 4-amino-1-(3,3-difluoro-4,5-dihydroxy tetrahydro-2H-pyran-2-yl)pyrimidin-2(1H)-one (isoform impurity I) and α-anomer of 4-amino-1-(3,3-difluoro-4,5-dihydroxy tetrahydro-2H-pyran-2-yl)pyrimidin-2(1H)-one (isoform impurity II), respectively. Formation, separation, isolation, and characterization of these isoform impurities were discussed in detail.

Keywords: Gemcitabine, Isoform impurities, Isolation, Prep-HPLC, Spectroscopy, Characterization

Address correspondence to S. G. Hiriyanna, Department of Chemistry, University of Mysore, Manasagangothri, Mysore 570009, India. E-mail: hiriyannag@gmail.com

INTRODUCTION

Gemcitabine, chemically known as 2′-deoxy-2′,2′-difluorocytidine monohydrochloride (β-isomer), is a potential anticancer agent used in chemotherapy (originally accomplished by Hertel et al.).[1] Gemcitabine is used for various carcinomas, non-small cell lung cancer, pancreatic cancer, and breast cancer. It is being investigated for use in oesophageal cancer, and is used experimentally in lymphomas and various other tumor types. Gemcitabine is found to be beneficial to pancreatic cancer patients who have successful tumor resections.[2–7] The high performance liquid chromatographic (HPLC) method is cited in the literature for the assay of gemcitabine from biological fluids.[8–11] The degradation of the gemcitabine in an acid aqueous solution at pH 3.2 and identification of degradation products was reported in the literature.[12] Gemcitabine is officially mentioned in the USP.[13]

In this paper, we describe the identification, separation, isolation, characterization, and formation of the two process related isoform impurities that were found in the gemcitabine bulk drug (API). As per the stringent regulatory requirements, the impurity profile study has to be carried out for any drug substance to identify and characterize all the unknown impurities that are present at a level of $>0.1\%$. The synthetic process involved for making of gemcitabine API was anomerization/conversion of protected α-gemcitabine to gemcitabine.[14] These impurities were formed during the base catalysed anomerization/conversion to gemcitabine. To the best of our knowledge, these isoform impurities are novel and unknown.

EXPERIMENTAL

Materials

The samples of gemcitabine, α-isomer of gemcitabine, and crude gemcitabine were received from Cipla Ltd., India. HPLC grade methanol and acetonitrile were obtained from Spectrochem (India). A.R. grade ammonium acetate and potassium dihydrogen orthophosphate were obtained from Rankem, India. A.R. grade trifluoro acetic acid and ortho phosphoric acid were procured from Spectrochem, India. MilliQ water was obtained from Elix Millipore water purification system (Millipore, India).

HPLC (Analytical)

Chromatographic separation was performed on a Waters HPLC system equipped with 2695 low pressure quaternary gradient pump along with pulse dampener/degasser, photo diode array detector, and auto sampler. The data was collected and processed using Empower 2 version 6.00.00.00

software. An Inertsil C18 (150 * 4.6 mm, 5 μ, GL Sciences, Japan) column was employed for the separation of isoform impurities from gemcitabine. A linear gradient programme was optimized for the separation of isoform impurities from gemcitabine, where the initial mobile phase consisted of mobile phase A (Mix 970 mL of 10 mM potassium dihydrogenorthophosphate and 30 mL of acetonitrile, the pH adjusted to 3.0 with ortho phosphoric acid and degassed) and mobile phase B (acetonitrile, degassed) in a ratio of 100:0 (v/v) for 3 min. Subsequently, the percentage of mobile phase B was increased from 0 to 60, up to 13 min. The same ratio (mobile phase A and B in the ratio of 40:60 (v/v)) was held for 7 min, and brought back to initial condition within 5 min. The column was allowed to get equilibrated for 10 min before performing the next injection. Chromatography was performed at room temperature using a flow rate of 1.2 mL min^{-1}. The column eluent was monitored at 275 nm. Sample concentration was about 1.0 mg mL^{-1} prepared in mobile phase A.

HPLC (Preparative)

An Agilent preparative HPLC system equipped with 1200 series pump, photo diode array detector, auto sampler, and 1200 series preparative fraction collector were used. The data was collected and processed using Agilent "Chemstation" 1200 series software. A 250 * 20 mm i.d. column packed with 5 μ particle size Inertsil C18 (GL Sciences, Japan) was employed for loading the sample. An analytical method was modified to resolve these isoform impurities, followed by scaling up the same method for prep HPLC to collect the required isoform impurity fractions. A linear gradient programme was optimized for the separation of isoform impurities from gemcitabine, where the initial mobile phase ratio was a mixture of mobile phase A (10 mM ammonium acetate, degassed) and mobile phase B (methanol, degassed) in the ratio of 96:4 (v/v) for about 17 min. Subsequently, the percentage of mobile phase B in the mobile phase ratio was increased from 4 to 80 in a 5 min duration. The same ratio (mobile phase A and B in the ratio of 20:80 (v/v)) was held for 6 min and brought back to initial condition within 4 min. The flow rate was set at 20 mL min^{-1}. The UV detection wavelength was 254 nm. Approximately 200 mg mL^{-1}crude gemcitabine sample was prepared in the mixture of methanol-water (50:50 (v/v)). From the above sample preparation, 900 μL was injected into the prep HPLC system.

Mass Spectroscopy (LC-MS/MS)

LC-MS/MS analysis was performed on API 2000 (Applied Biosystems) coupled with an HPLC system consisting of Agilent 1100 series low

pressure quaternary gradient pump along with dampener/degasser, auto sampler, and the column oven. The analysis was done in a positive ionization mode (ESI) with turbo ion spray interface under the following conditions. Ion source voltage IS = 5500 V; declustering potential, DP = 70 V; focusing potential, FP = 400 V; capillary temperature = 350°C; entrance potential, EP = 10 V with nebuliser gas as nitrogen at 40 psi and curtain gas as nitrogen at 25 psi. An Inertsil C18 (250 * 4.6 mm, 5 μ, GL Sciences, Japan) column was used for the separation. A linear gradient programme was optimized for the separation of isoform impurities from gemcitabine, where the initial mobile phase ratio was a mixture of mobile phase A (10 mM ammonium acetate, degassed) and mobile phase B (methanol, degassed) in the ratio of 96:4 (v/v) for about 17 min. Subsequently, the percentage of mobile phase B in the mobile phase ratio was increased from 4 to 80 in the 5 min duration. The same ratio (mobile phase A and B in the ratio of 20:80 (v/v)) was held for 6 min and brought back to initial condition within 4 min. The flow rate was set at 1.2 mL min^{-1}. The UV detection wavelength was set as 254 nm. The column eluent was introduced into the electron spray ionization (ESI) chamber of the mass spectrometer with the split ratio of 3:7. Mass fragmentation studies were carried out by maintaining normalized collision energy at 35 eV with the range of m/z 50–300 amu.

NMR Spectroscopy

The ^{1}H NMR and ^{13}C NMR studies of the isoform impurities (I and II) were carried out at precessional frequencies of 399.939 MHz and 100.574 MHz, respectively. The solvent methanol-d_4 was used at 25°C on a Varian AS-400 FT NMR spectrometer. The ^{1}H and ^{13}C chemical shifts are reported on the δ scale in ppm, relative to tetramethyl silane (TMS) (δ 0.00) present in methanol-d_4 solvent. An H/D exchange experiment was performed to confirm the exchangeable protons.

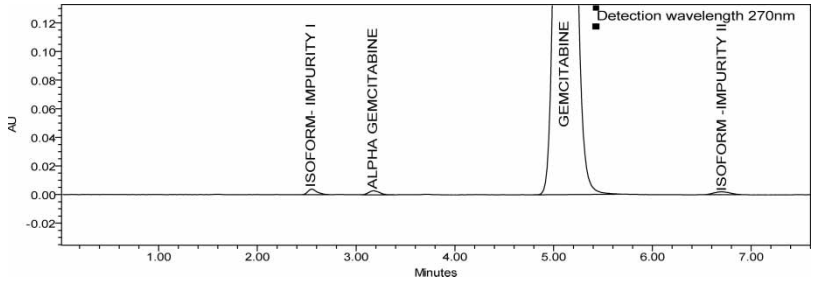

Figure 1. Analytical HPLC chromatogram of gemcitabine bulk drug sample (API).

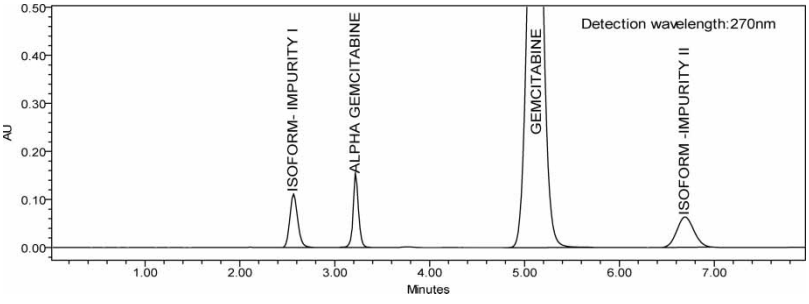

Figure 2. Analytical HPLC chromatogram of gemcitabine bulk drug sample spiked with impurities.

RESULTS AND DISCUSSION

Detection of Isoform Impurities by HPLC

A typical analytical HPLC chromatogram (Fig. 1) of α-isomer of gemcitabine, gemcitabine, isoform impurity I and II, obtained by using the HPLC method is discussed in the Experimental section. The targeted isoform impurities under study, marked as I and II, eluted at retention times of about 2.4 min and 6.6 min, respectively; while α-isomer of gemcitabine and gemcitabine eluted at about 3.2 min and 5.2 min, respectively (Fig. 2). UV spectra of α-isomer of gemcitabine, gemcitabine, isoform impurity I, and isoform impurity II were recorded in the UV detector (PDA). The UV spectra of all the four peaks were comparable and are shown in Fig. 3. The impurity enriched crude gemcitabine bulk drug sample was also injected to check the

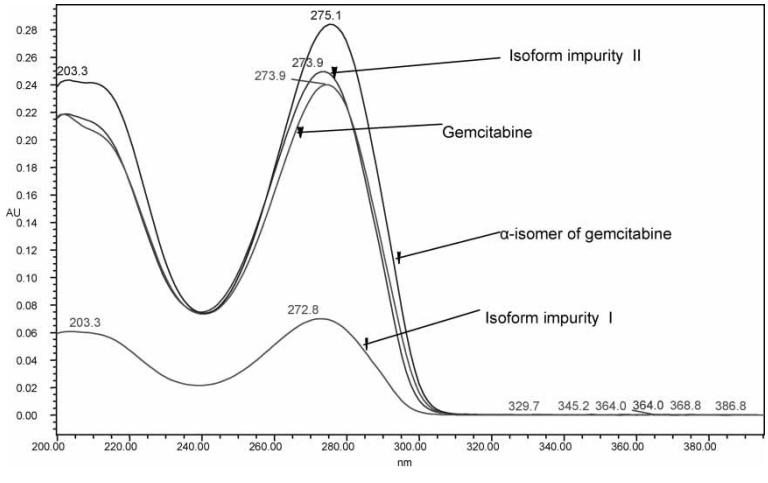

Figure 3. UV spectra of gemcitabine and its impurities.

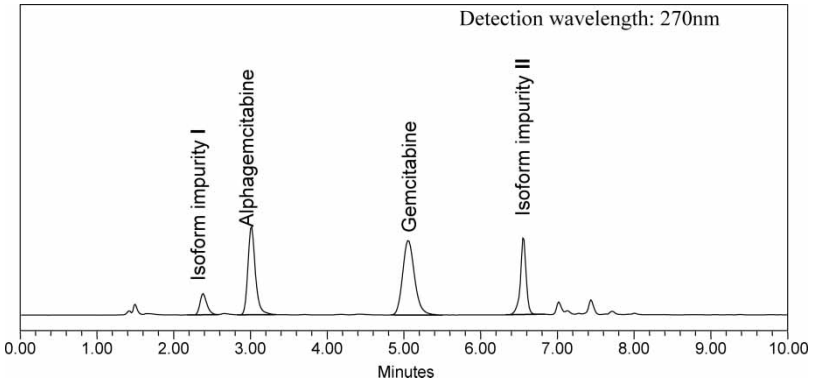

Figure 4. Analytical HPLC chromatogram of gemcitabine bulk drug crude sample.

levels of isoform impurities. The crude sample contained about 3.0 and 8.0% by area percentage of isoform impurity I and II, respectively (Fig. 4).

LC-MS/MS Analysis

LC-MS/MS analysis of gemcitabine bulk drug sample was performed using the solvent system as described in the Experimental section. Results of LC-MS/MS analysis revealed that the isoform impurities I and II exhibited the molecular ion at m/z (M + 1) 264.1 *amu*, which is the same as that of the gemcitabine molecular ion. The fragmentation pattern of these impurities was similar to that of gemcitabine. Based on the results obtained from LC-MS/MS, it was assumed that these impurities were very similar to that of gemcitabine and, possibly, they are the structural isomers.

Isolation of Isoform Impurities by Preparative HPLC

A simple reverse phase system discussed in the Experimental section was used for isolating the isoform impurity I and II. In this solvent system, the α-isomer

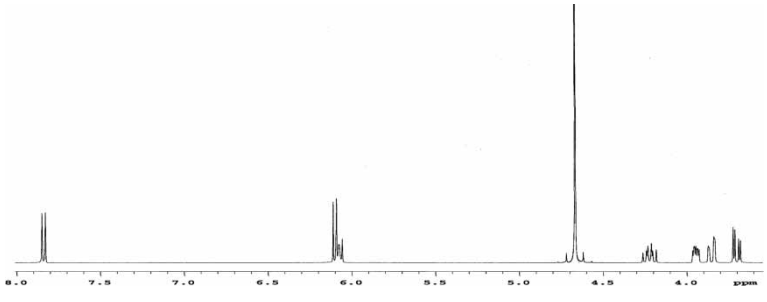

Figure 5. ^{1}H NMR spectrum of gemcitabine.

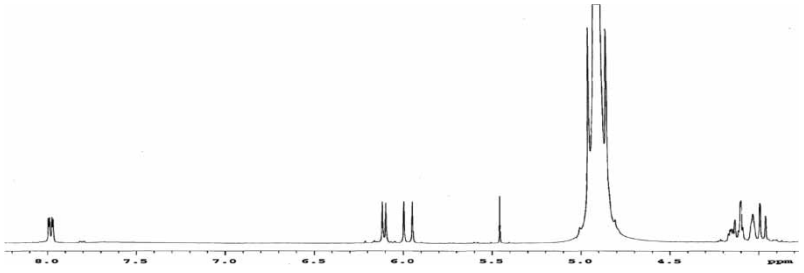

Figure 6. [1]H NMR spectrum of isoform impurity I isolated sample.

of gemcitabine eluted at about 7.9 min, whereas the isoform impurity I and II were eluted at about 5.6 min and 15.2 min, respectively. Approximately, about 5.0 g of impurity enriched gemcitabine bulk drug crude sample was loaded onto the preparative HPLC and these isoform impurity fractions were collected separately and concentrated at room temperature under high vacuum on a Buchii Rotavapour Model R124. The solids thus obtained were re-analyzed for purity on an analytical HPLC. The purity of isolated isoform impurity I and II were found to be in excess of 96%, and these impurity samples were used for further spectroscopic experiments .

Structural Elucidation of Isoform Impurity I

The NMR and mass spectrometry analysis data of the isoform impurity I was compared with that of gemcitabine (Figs. 5, 6, and 9). LC-MS/MS analysis data of gemcitabine and the isoform impurity I exhibited the molecular ion peak at m/z 264.1 $[M + H]^+$. The primary fragment of the m/z 264 product ion was found at m/z 112, which corresponds to the loss of a ribose unit. All other fragments are very less intense as compared to the 264.1 m/z and 112 m/z.[15] The similar mass fragmentation pattern was also observed with isoform impurity II and α-isomer of gemcitabine.

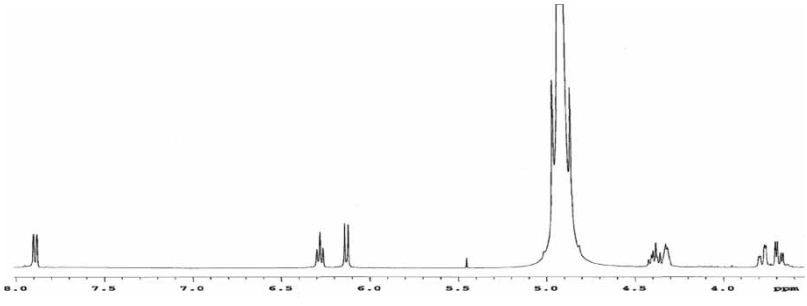

Figure 7. [1]H NMR spectrum of α-isomer of gemcitabine.

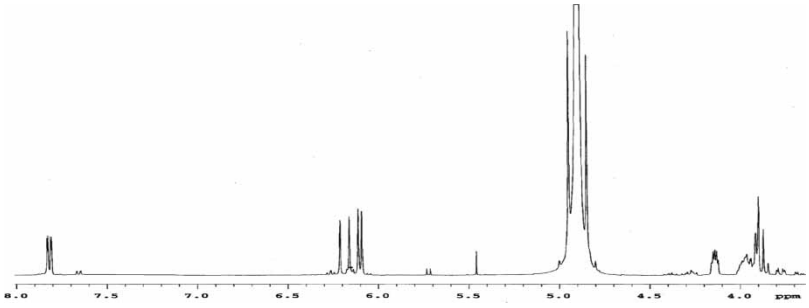

Figure 8. ^{1}H NMR spectrum of isoform impurity II isolated sample.

The ^{1}H NMR spectrum of gemcitabine showed two multiplicities at 6.10 ppm and 7.84 ppm,[16] respectively, which corresponds to the 5, 6 positions of pyrimidine moiety (Table 1). The signals corresponding to the 2′, 4′, 5′, and 6′ positions appeared at about 6.08, 4.18, 3.93, and 3.68 ppm, respectively. Similarly, the ^{13}C NMR spectrum of gemcitabine showed signals corresponding to the 2, 4, 5, and 6 positions at 165.2, 176.3, 112.4, and 160.8 ppm, respectively. The signals corresponding to the 2′, 3′, 4′, 5′, and 6′ positions of sugar moiety appeared at 102.0, 140.0, 86.1, 97.8, and 76.1 ppm, respectively.

The ^{1}H NMR spectrum of isoform impurity I showed two multiplicities, which corresponds to the 5 and 6 positions of pyrimidine moiety at 6.10 and 7.95 ppm, respectively. The signals corresponding to the 2′, 4′, 5′, and 6′ positions appeared at about 5.95, 4.13, 4.09, and 3.96 ppm, respectively.

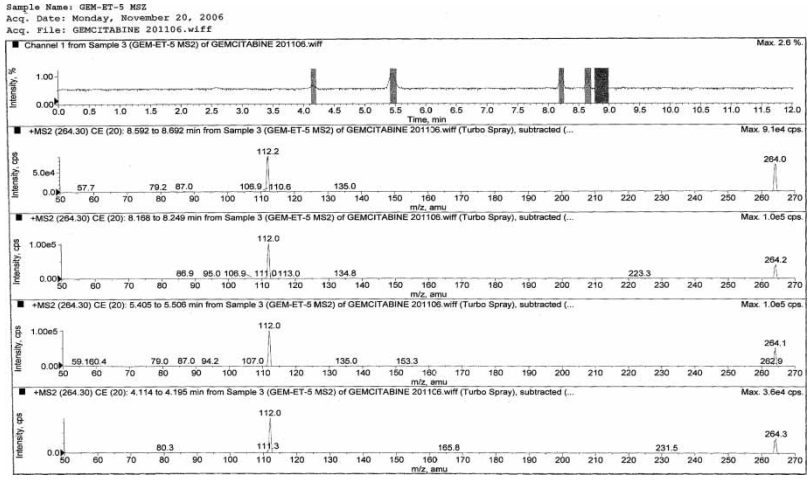

Figure 9. LC-MS/MS spectrum of isoform impurity I, isoform impurity II, α-isomer of gemcitabine, and gemcitabine.

Table 1. Comparative ^{1}H NMR and ^{13}C NMR assignments for gemcitabine and isoform impurity I

	Gemcitabine			Isoform impurity I		
Position	ppm, ^{1}H NMR	^{13}C ppm	DEPT	ppm, ^{1}H NMR	^{13}C ppm	DEPT
6′	3.68–3.87 (m, 2H)	76.1	76.1	3.96–4.03 (m, 2H)	70.2	70.2
5′	3.93–3.97 (m, 1H)	97.8	97.9	4.09–4.11 (m, 1H)	68.6	68.6
4′	4.18–4.26 (m, 1H)	86.1	86.2	4.13–4.17 (m, 1H)	69.4	69.4
3′	—	140	—	—	115.0	—
2′	6.06–6.08 (t, 1H, JH21, F = 7.93 HZ)	102	101.5	5.95–5.99 (d, 1H, JH21, F = 19.61 HZ)	79.9	80.2
2	—	165.2	—	—	161	—
4	—	176.3	—	—	163	—
5	6.09–6.11 (d, 1H)	112.4	112.5	6.09–6.12 (d, 1H)	94.7	95.1
6	7.84 (d, 1H, JH5, H6 = 8.02 HZ)	160.8	160.8	7.95 (dd, 1H, JH5, H6 = 8.0 HZ, JH5, F = 3.0 HZ)	145.0	144.2

t-Triplet, s-singlet, m-multiplet and d-doublet. For numbering refer Fig: 10.

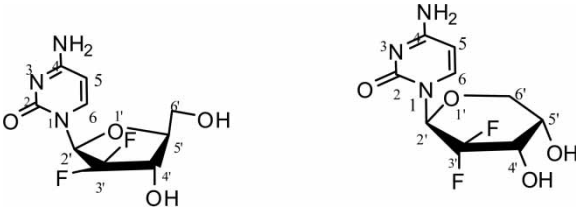

Figure 10. The chemical structures of isoform impurity I and gemcitabine.

Similarly, the ^{13}C NMR spectrum of isoform impurity I showed signals, which corresponds to the 2, 4, 5, and 6 positions at 161.0, 163.0, 94.7, and 145.0 ppm, respectively. The signals corresponding to the 2′, 3′, 4′, 5′, and 6′ positions of sugar moiety appeared at 80.2, 115.0, 69.4, 68.6, and 70.2 ppm, respectively.

The coupling constant (J) values for protons at 2′ position of gemcitabine and the isoform impurity I were 7.93 Hz, and 19.61 Hz, respectively, which suggests that there was a change in the dihedral angle between the 2′ proton and the adjacent fluorine atoms. Similarly, the coupling constant values of proton located on position 6 of gemcitabine and the isoform impurity I were 8.02 Hz and 3.05 Hz, respectively. Based on the above data, it is concluded that the structure of the isoform impurity I has been characterized as β-anomer of 4-amino-1-(3,3-difluoro-4,5-dihydroxytetrahydro-2H-pyran-2-yl) pyrimidin-2(1H)-one.

Structural Elucidation of Isoform Impurity II

The ^{1}H NMR spectrum of α-isomer of gemcitabine shows two signals corresponding to the 5 and 6 positions of pyrimidine moiety at 6.07 ppm and 7.85 ppm,[16] respectively (Table 2). The signals corresponding to the 2′, 4′, 5′, and 6′ positions appeared at about 6.08, 4.37, 4.29, and 3.65 ppm,

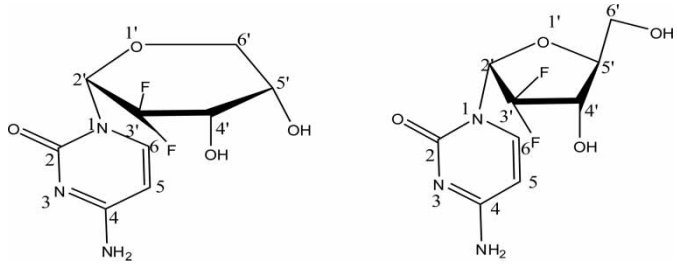

Figure 11. The chemical structures of isoform impurity II and α−isomer of gemcitabine.

Table 2. Comparative ^{1}H NMR and ^{13}C NMR assignments for α-isomer of gemcitabine and Isoform impurity II

	α – isomer of gemcitabine			Isoform impurity II		
Position	ppm, ^{1}H NMR	^{13}C ppm	DEPT	ppm, ^{1}H NMR	^{13}C ppm	DEPT
6′	3.65–3.81 (m, 2H)	60.2	60.2	3.85–3.92 (m, 2H)	65.8	65.8
5′	4.29–4.33 (m, 1H)	85.0	85	3.94–4.00 (m, 1H)	65.3	65.3
4′	4.37–4.436 (m, 1H)	70.2	70.3	4.12–4.16 (m, 1H)	69.8	69.8
3′	—	116.5	—	—	118	—
2′	6.06–6.08 (t, 1H, JH21, F = 7.90 HZ)	85.5	85.6	6.16-6.21 (d, 1H, JH21, F = 19.61 HZ)	76.8	76.9
2	—	161.4	—	—	160	—
4	—	163.7	—	—	163	—
5	6.07–6.09 (d, 1H)	94.7	94.7	6.09–6.12 (d, 1H)	95.0	95.1
6	7.85 (d, 1H, JH5, H6 = 8.02 HZ)	142.9	143	7.82 (dd, 1H, JH5, H6 = 7.95HZ, JH6, F = 2.62 HZ)	144.5	144.4

t-Triplet, s-singlet, m-multiplet and d-doublet. For numbering refer Fig: 11.

respectively. Similarly, the ^{13}C NMR spectrum of α-gemcitabine shows signals corresponding to the 2, 4, 5, and 6 positions at 161.4, 163.7, 94.7, and 142.9 ppm, respectively. The signals corresponding to the 2′, 3′, 4′, 5′, and 6′ positions of sugar moiety appeared at 85.5,116.5, 70.2, 85.0, and 60.2 ppm, respectively.

The ^{1}H NMR spectrum of isoform impurity II shows two signals corresponding to the 5, 6 positions of pyrimidine moiety at 6.09 and 7.80 ppm, respectively. The signals corresponding to the 2′, 4′, 5′, and 6′ positions appeared at about 6.16, 4.12, 3.94, and 3.85 ppm, respectively. Similarly, the ^{13}C NMR spectrum of isoform impurity II shows signals corresponding to the 2, 4, 5, and 6 positions at 160.0, 163.0, 95.0, and 144.5 ppm, respectively. The signals corresponding to the 2′, 3′, 4′, 5′, and 6′ positions of sugar moiety appeared at 76.8, 118.0, 69.8, 65.3, and 65.8 ppm, respectively.

The coupling constant (J) values for protons located at 2′ position of α-isomer of gemcitabine and the isoform impurity II are 7.90 Hz and 19.61 Hz, respectively, which suggests that there was a change in dihedral angle between the 2′ proton and the adjacent fluorine atoms. Similarly, the coupling constant values of proton located on position 6 of α-isomer of gemcitabine and the isoform impurity II are 8.02 Hz and 2.62 Hz, respectively. Based on the above data, the structure of the isoform impurity II has been characterized as α-anomer of 4-amino-1-(3,3-difluoro-4,5-dihydroxytetrahydro -2H-pyran-2-yl)pyrimidin-2(1H)-one.

Formation of the Isoform Impurities

During the process of anomerization,[14] the protected α-isomer of gemcitabine (with base labile protected group) in the presence of a base catalyst anomerizes to gemcitabine along with the formation of these structural isoform impurities I and II, as shown in the scheme (Fig. 12). The process of anomerization of protected α- isomer of gemcitabine under basic condition proceeds through

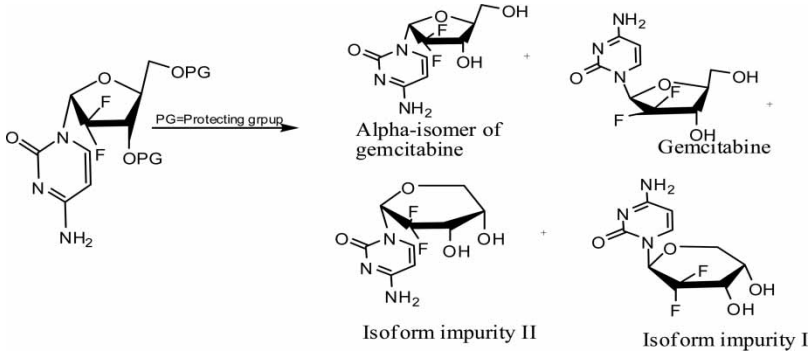

Figure 12. Scheme depicting the formation of isoform impurities.

acyclic intermediate. The cyclisation of this acyclic intermediate produces the required β-anomer (gemcitabine). However, a competitive involvement of C-6′ primary alcohol in the cyclisation process forms isoform impurities I and II.

CONCLUSION

Two isoform impurities (0.12%) were detected other than the α-isomer of gemcitabine in gemcitabine bulk drug, which is produced by anomerization/conversion of protected α-isomer of gemcitabine. A HPLC analytical method was developed to separate two isoform impurities and also α-isomer of gemcitabine impurity from the gemcitabine bulk drug. Identification, isolation, characterization, and formation of the two isoform impurities were discussed in detail.

REFERENCES

1. Hertel, L.; Kroin, S.J.; Misner, J.W.; Tustin, J.M. J. Org. Chem. **1988**, *53*, 2406.
2. De Clercq, E. Nuclosides and Nucleotides **1994**, *13*, 1271.
3. Hvi, Y.F.; Reitz, J. Am. J. Hlth. Syst. Pharm. **1997**, *54*, 12.
4. Noble, S.; Goa, K.L. Drugs **1997**, *54*, 447.
5. Bunn, P.A.; Kelly, K. Cancer Res. **1998**, *4*, 1087.
6. Fugedi, P.; Liplak, A.; Nanasi, P.; Szejtli, J. Carbohydr. Res. **1982**, *104*, 55.
7. Abratt, R.P.; Bezwoda, W.R.; Falkson, G.; Goedhals, L.; Hacking, D.; Pugg, T.A. J. Clin. Oncl. **1994**, *12*, 1535.
8. Losa, R.; Sierra, M.I.; Gion, M.O.; Esterban, E.; Ruera, J.M. J. Chromatogr. B. **2004**, *840*, 44.
9. Bilal, Y.; Yucel, K.; Yilmaz, A. Anal. Biochem. **2004**, *332*, 234.
10. Bilal, Y.; Yucel, K. II Farmaco **2004**, *59*, 425.
11. Bilal, Y.; Yucel, K.; Yilmaz, A. J. Chromatogr. B **2003**, *791*, 103.
12. Patrick, J.J.; Michael, J.A.; Robert, M.A.; Steven, W.B.; Gary, G.C.; Douglas, E.D.; Craig, A.J.K.; Steven, R.M.; Karn, A.M. J. Pharm. Sci. **2000**, *89*, 885.
13. *The United States Pharmacopeia, USP29 NF24*, Asian Edition; 990.
14. Chou, T.S.; Heath, P.C.; Patterson, L.M.; Poteet, L.M.; Lakin, R.E.; Hunt, A.H. Sytnhesi. **1992**, 565.
15. Honeywell, R.; Laan, A.C.; Van, G.C.J.; Strocchi, E.; Ruiter, R.; Giaccone, G.; Peters, G.J. J. Chromatogr. B **2007**, 142.
16. Raul, F.; Sergio, C.L.E. Tetrahedron **1999**, *55*, 8497.

Received May 30, 2007
Accepted July 10, 2007
Manuscript 6157