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Short communication

Isolation and identification of ester impurities in RG7128, an HCV polymerase inhibitor

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ABSTRACT

RG7128 is a di-ester prodrug of a cytidine analog for the treatment of hepatitis C virus (HCV) infection. The structures of nine low level impurities (0.05–0.10%) in RG7128 drug substance were elucidated. The majority of the impurities were formed during the synthesis of the prodrug from the parent drug. Structural elucidations of the impurities were achieved either by enrichment of the impurities using preparative chromatography followed by spectroscopic techniques or by confirmation with a reference sample. Heart-cut and recycle chromatographic techniques were applied to purify closely eluting isomers of RG7128.

1. Introduction

RG7128 (2′-α-fluoro-2′-β-methyl-3′,5′-diisobutyryldeoxycytidine) is a selective inhibitor for HCV polymerase (Fig. 1, **1**), and it is presently in clinical studies for the treatment of HCV infection [1–5]. According to International Conference on Harmonization (ICH) guideline on impurities in new drug substance [6], impurities at or above 0.05% should be identified for drugs with a maximum daily dose of equal or greater than 2 g.

Different approaches in carrying out impurity profiling and identification have been published ranging from enriching the impurities using a preparative high-performance liquid chromatography (HPLC) system followed by mass spectrometry (MS) and nuclear magnetic resonance (NMR), using LC–MS and LC–NMR, and synthesizing the impurities for structural confirmation [7–11]. An essential element to perform structural elucidation of the impurities is to have a relatively pure sample. Impurities that are fully separated on the analytical column can be isolated and enriched on the preparative LC column with little difficulties. However, closely eluting or unresolved impurities on the analytical column represent challenges to obtain pure fractions due to column overloading [12] and lesser resolution of the preparative LC column. Redevelopment of the HPLC method or application of preparative chromatographic techniques has to be considered during purification and enrichment processes in order to obtain a pure fraction for structural determination with spectroscopic techniques.

This paper describes the process of enriching impurities and techniques used to purify closely eluting isomeric impurities from RG7128 as well as identification of the impurities in the drug substance. In addition, an HPLC method was developed to separate the isomeric impurities from RG7128. Nine impurities (**2**–**10**) were revealed in three representative early lots of RG7128 drug substance (Fig. 1).

2. Experimental

2.1. Chemicals and impurity samples

Acetonitrile and methanol (HPLC grade) were purchased from Burdick and Jackson (Muskegon, MI). Formic acid (98% purity) was purchased from EMD Chemicals (Gibbstown, NJ). Ammonium hydroxide (6.0 N volumetric solution) was purchased from VWR International (West Chester, PA). Ultra pure water was obtained using a Barnstead Nanopure Water Purification System (Dubuque, IA).

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Impurities in RG7128 drug substance, Lots 30826-PW-125 and PA31575-102, were isolated using a preparative HPLC system. Impurities **5** and **6** were isolated from 0.25 g of Lot PA31575-102 dissolved in 5 mL of methanol. Separately, **7**–**9** were isolated from 0.5 g of Lot 30826-PW-125 dissolved in 5.0 mL of methanol. Isomers of **1**, **9a**, **9b** and **9c** were purified from a synthetic mixture of 120 mg of Lot PA31575-102-ORG dissolved in 2 mL of methanol.

2.2. Preparative HPLC instrument, conditions and sample isolations

Impurities were isolated using a Waters preparative liquid chromatography system (Milford, MA). The system consists of a 2525 Binary High-Pressure LC Pump, a 2767 One-Bed Autosampler-Collector Sampling Manager, a Column Fluidic Organizer, a 515 Pump (as a make-up pump with 80% methanol in 0.1% formic acid at 1 mL/min), a 2996 Photodiode Array Detector and a Dionex LC Packings Reversed Phase Accurate Splitter at 1:1000 ratio (Amsterdam, Netherlands).

A Waters SunFire C18 column (100 mm × 19 mm, 5 μm) was used to isolate impurities **5**–**9**. The mobile phases consisted of 10 mM formate buffer, pH 3.5, for A and acetonitrile for B at a flow rate of 17 mL/min with UV detection at 276 nm. Impurities **5** and **6** were isolated from seven replicate injections at 12.5 mg with an HPLC condition of 24% B from 0 to 15 min, from 24 to 45% B between 15 and 30 min and followed by 10 min of isocratic condition. For isolation of impurities **7**–**9**, 10 replicate injections at 25 mg each were carried out using an isocratic condition of 27% B for 35 min. Impurities **5**–**8** were collected by their retention times. Impurity **9** was collected by applying a heart-cut collection technique with 15-s intervals during its elution time window.

Separately, RG7128, **9a**, **9b** and **9c** were isolated from a synthetic mixture. Phenomenex, Luna Phenyl-Hexyl analytical column (250 mm × 4.6 mm, 5 μm) at 1 mL/min and preparative column (250 mm × 10 mm, 5 μm) at 5 mL/min were used with an isocratic elution of 25% B. Each isomeric impurity was collected by retention time from four replicate injections at 6 mg each.

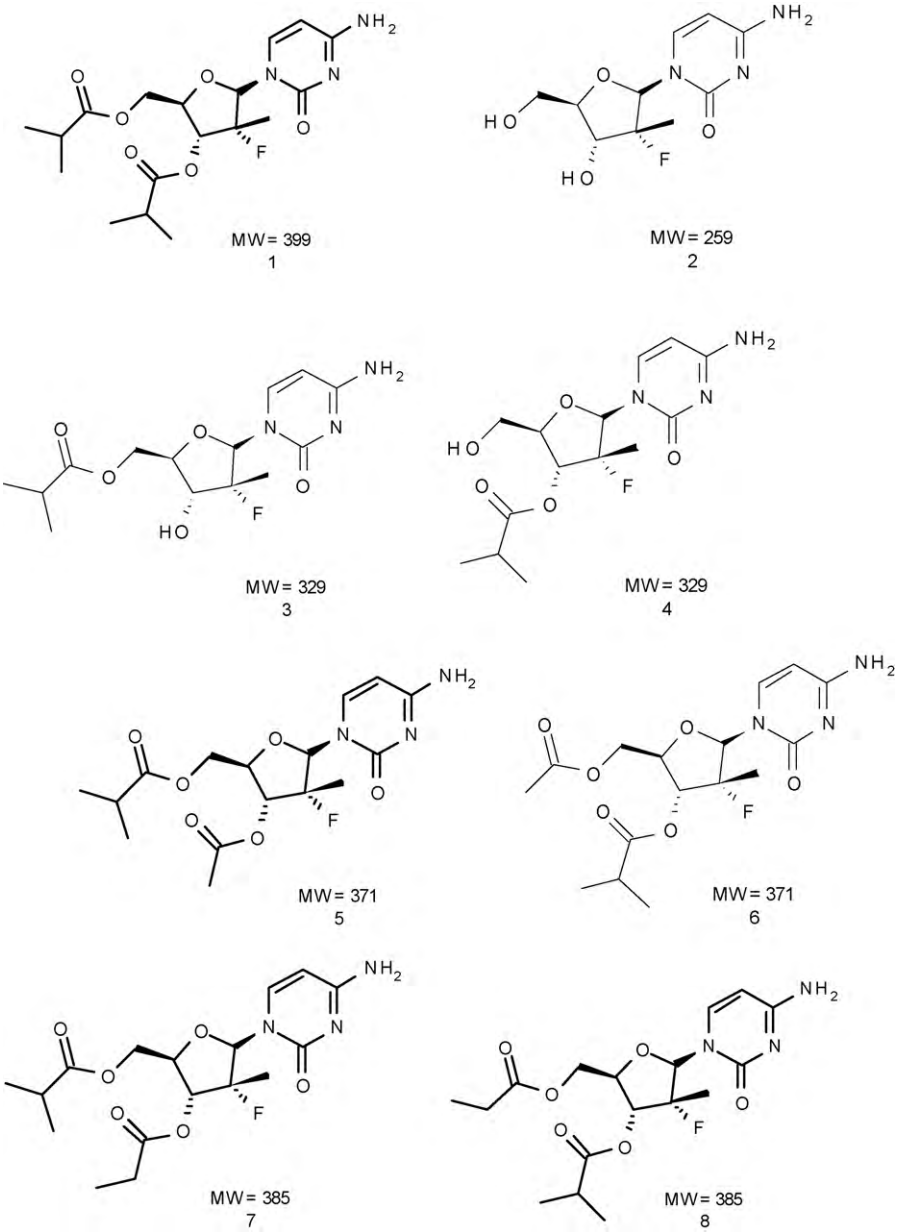

Fig. 1. Structure of RG7128 (**1**) and impurities **2**–**10**.

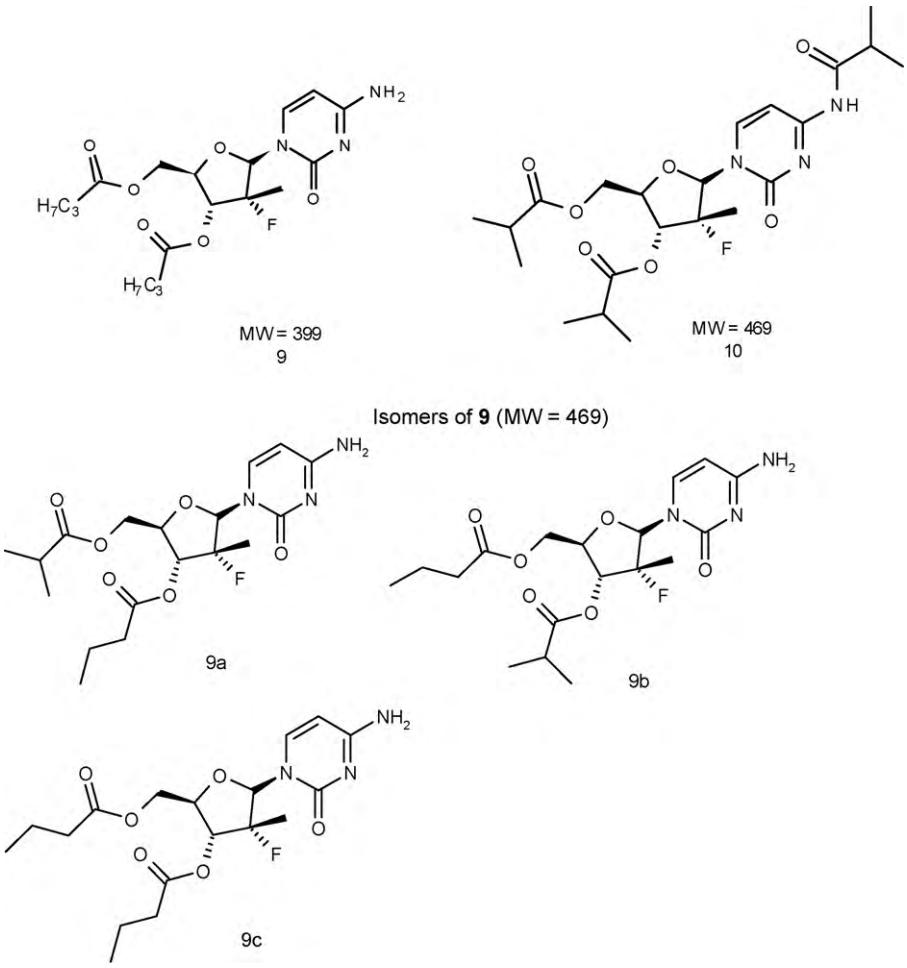

Fig. 1. (*Continued*).

Each impurity fraction was frozen inside a round bottom flask under a slush of acetone and dry ice and then lyophilized on an FTS Systems lyophilizer (Stoneridge, NY) for NMR and MS experiments.

2.3. Analytical liquid chromatography system

The purity of each impurity fraction from the preparative HPLC collection was confirmed by the validated analytical method for RG7128 using an Agilent Technologies 1100 liquid chromatography system (Wilmington, DE). An Ace C18 column (250 mm × 4.6 mm, 5 μm) (Mac Mod Analytical, Chadds Ford, PA) was used with mobile phases consisting of 10 mM formate buffer, pH 3.5, for A and acetonitrile for B at a flow rate of 1.0 mL/min with UV detection at 276 nm. Elution was achieved with a gradient of 1–35% B between 0 and 15 min, followed by an isocratic condition for 15 min, then from 35 to 60% B between 30 and 40 min, and isocratic condition at 60% B for 9 min. A 0.3 mg/mL RG7128 solution in 90% A and 10% B was prepared from Lot PA31575-103-REF for retention time reference of the impurity fractions collected.

2.4. Analytical method to separate isomeric impurities

To separate RG7128, **9a**, **9b** and **9c**, two coupled Waters X-Bridge C18 columns (150 mm × 4.6 mm, 3.5 μm) were used with mobile phases consisting of 10 mM formate buffer, pH 3.5, for A and acetonitrile for B at a flow rate of 1.0 mL/min with UV detection at 276 nm. Elution was achieved with an isocratic condition of 75% A and 25% B for 60 min, followed by a gradient from 25 to 30% B between 60 and 80 min, and then an isocratic condition at 30% B for 15 min.

2.5. LC–MS

Each lyophilized impurity fraction was analyzed on an Agilent 1100 HPLC coupled with a Thermo-Finnigan LTQ Linear Ion Trap mass spectrometer (San Jose, CA) operating in ESI positive source mode. The LC–MS adopted the same analytical HPLC method from Section 2.3 and the mass spectrometer was programmed to scan from 104 to 1000 *m*/*z*. The instrument was tuned with a spray voltage of 5.2 kV and capillary heated to 350 °C. Xcalibur was used to control the instrument, acquire and analyze the data.

2.6. NMR

All proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded at ambient temperature (300 K) and 350 K on a Bruker Avance DRX-500 Ultrashield NMR spectrometer using 5 mm BBI probe with Z-gradient operating at a ^{1}H frequency of 500.13 MHz and ^{13}C frequency of 125.76 MHz. Each NMR sample was dissolved in 180 μl of dimethylsulfoxide-*d*6 with tetramethylsilane (TMS) as an internal reference standard in a 3 mm NMR tube. All assignments were proven by conventional NMR experiments including the two-dimensional correlation spectroscopy (COSY), heteronuclear multiple-bond correlation spectroscopy (HMBC) and heteronuclear multiple quantum coherence spectroscopy (HMQC).

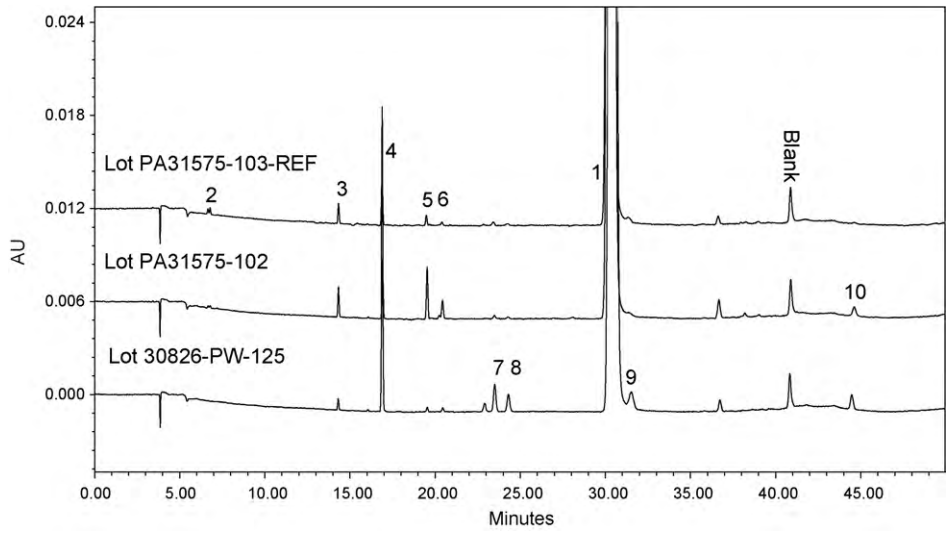

Fig. 2. Chromatograms of RG7128 drug substance lots showing impurities at UV 276 nm.

3. Results and discussion

3.1. Impurities detected in drug substance lots

An HPLC method was developed and validated for purity determination of RG7128 drug substance (**1**) and to quantitate its related impurities resulting from chemical synthesis and degradation. Nine impurities (**2**–**10**) at 0.05% or greater by area normalization were detected in three representative lots of the drug substance as shown in Fig. 2. The molecular masses of these impurities were determined by LC–MS. Structural elucidation of the impurities was carried out by spiking reference samples into RG7128 drug substance or enriching via preparative LC purification followed by LC–MS and NMR.

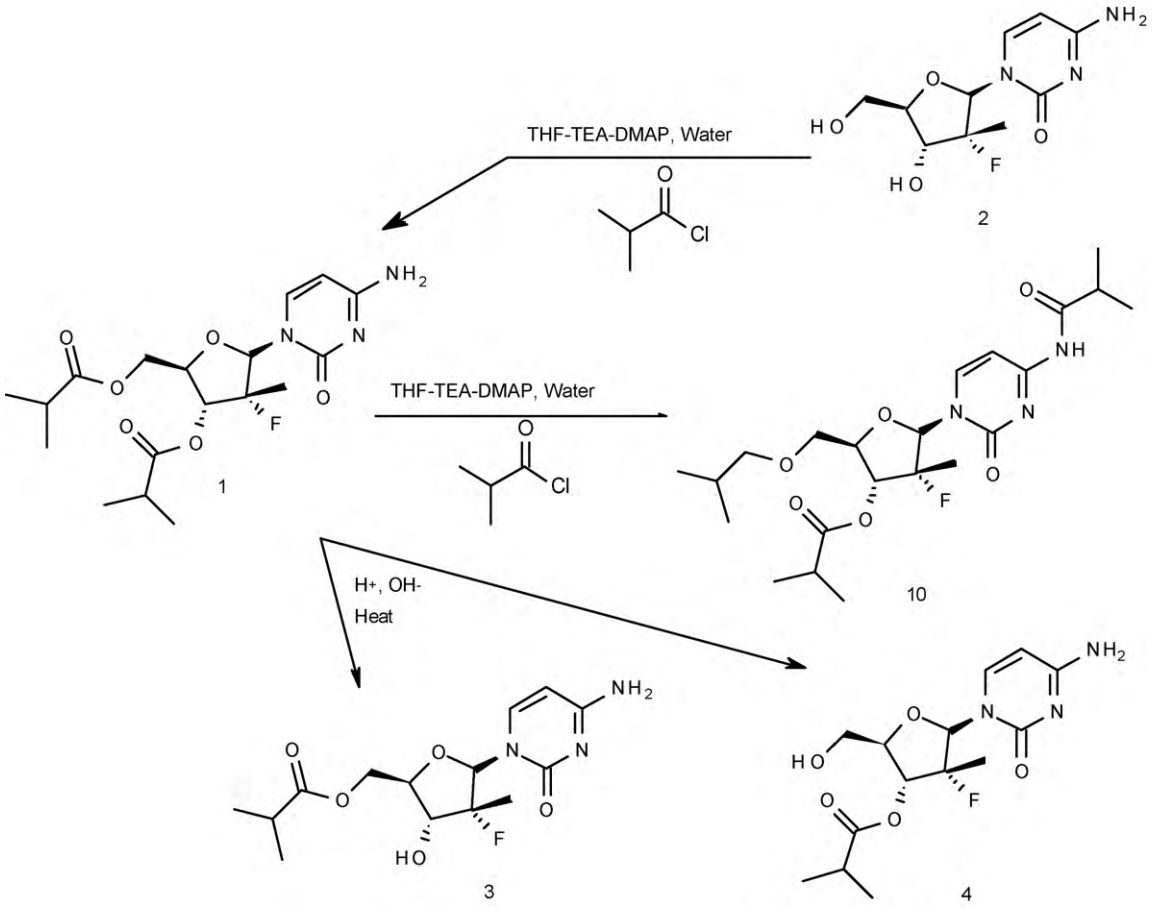

Fig. 3. Synthesis of **1** and proposed formation of impurities **3**, **4** and **10**.

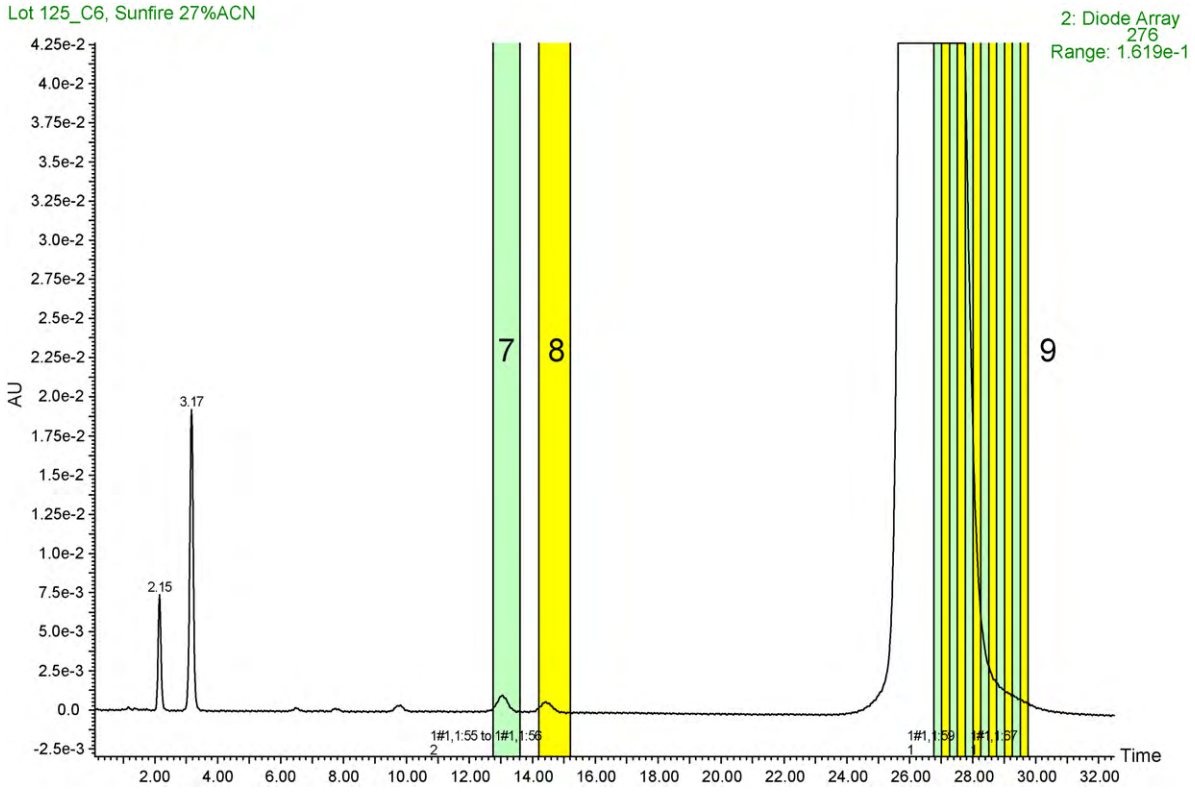

Fig. 4. Preparative chromatogram of impurities **7**–**9**. Heart-cut chromatography was employed to isolate crude fractions of impurity **9**.

3.2. Reference samples

RG7128 is a prodrug of a cytidine nucleoside analog (parent drug or impurity **2**) prepared by esterification with isobutyryl chloride (Fig. 3). The presence of impurity **2** was confirmed by spiking **2** into drug substance lots. Reference samples for impurities **3**, **4** and **10** were synthesized based on the knowledge of the chemical synthesis and degradation pathway of RG7128 shown in Fig. 3. Each reference sample was correlated to the impurities in drug substance by HPLC retention time with structure confirmation by mass spectral analysis.

3.3. Impurities **5**–**9**

Impurities **5** and **6** were collected by preparative HPLC and confirmed for purity and retention time by the analytical HPLC. Greater than 100 μg of each impurity was obtained after lyophilization with a purity of greater than 90%. Impurities **5** and **6** have the same mass with a loss of ethyl compared to the parent compound **1**. Comparison of the NMR and MS data between these impurities and the parent compound indicates that each impurity has only one isobutyryl group, the second isobutyryl ester has been replaced with an acetyl ester, and that impurities **5** and **6** are structural isomers. For impurity **5**, a key correlation was observed in the HMBC NMR spectrum between the carbonyl carbon of isobutyrate and the 5′ CH_2 protons. This led to the assignment of the isobutyryl to the 5′ position and acetyl to the 3′ position for impurity **5**. The NMR results for impurity **6** were inconclusive due to a lack of purified material needed to collect the requisite NMR data. Since impurity **6** is an isomer of impurity **5**, the isobutyryl was assigned to the 3′ position and acetyl to the 5′ position. Impurities **7** and **8** fractions were collected from the preparative HPLC and analyzed using the analytical HPLC to confirm purity and retention time. Greater than 100 μg with acceptable purity was obtained for each impurity. The LC–MS detected a loss of 14 mass units for each and inferred a methyl displacement. There was not sufficient material collected for 2D NMR to differentiate the isomers. Based on the HPLC elution pattern of isomer pair **3** and **4** and pair **5** and **6**, the 5′ position with the larger component elutes first. The isobutyrate was thus assigned to impurity **7** at 5′ and impurity **8** at 3′.

Impurity **9** was not fully separated from RG7128 by the analytical HPLC method and represented a challenge to isolate by preparative HPLC. Therefore, a heart-cut technique was employed in collecting this impurity. Impurity **9** was predicted to elute at the latter half of RG7128 peak. The preparative HPLC was programmed to collect 15-s interval fractions starting at the mid-point of RG7128 peak to isolate crude fractions of impurity **9** (Fig. 4). Each 15-s fraction was analyzed by the analytical HPLC method. Crude fractions that contained significant amount of pure impurity **9** were pooled and lyophilized. Crude fractions that did not possess the desired purity were pooled, lyophilized and re-injected onto the preparative HPLC for re-purification by heart-cut technique with 15-s intervals. Re-injection of a collected fraction is also known as recycle chromatography. After two cycles of heart-cut purification process, a small quantity of impurity **9** was obtained.

3.4. LC–MS and ^{1}H NMR of impurity **9**

Impurity **9** isolated by heart-cut preparative chromatography was subjected to LC–MS and NMR. LC–MS determined the molecular mass of impurity **9** to be *m*/*z* 400 $(M+H)^+$, which has the same molecular weight as **1**. It was suspected that impurities **9** and **1** are isomers. Therefore, three possible isomers (Fig 1, **9a**, **9b** and **9c**) were proposed based on the synthetic scheme of RG7128. Isomer **9c** is not expected to be present. Statistically, the percentage of esterifying two *n*-butyryl chlorides onto its parent drug is minimal because the esterifying agent is mainly isobutyryl chloride. Based on the ^{1}H NMR spectrum of impurity **9** (Fig. 5), structural

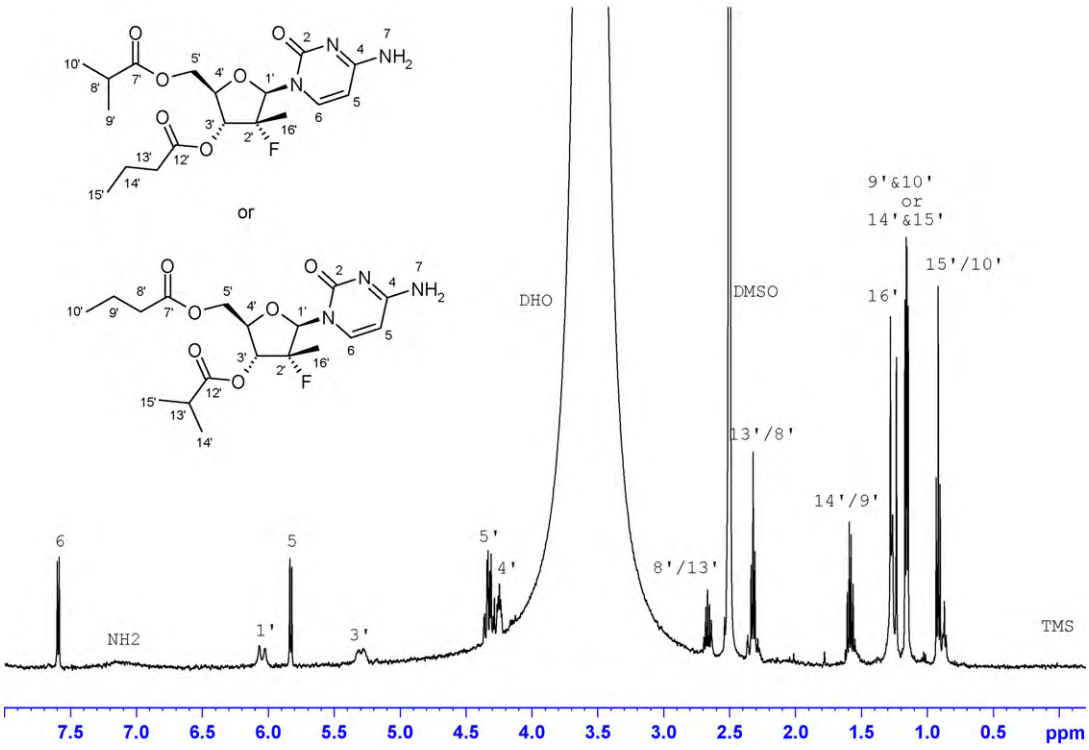

Fig. 5. ^{1}H NMR spectrum of impurity **9** in DMSO at 350 K.

assignment was inconclusive as either **9a** or **9b** due to insufficient material for further NMR experiments.

3.5. Purification of impurity 9 isomers

A synthetic mixture of RG7128, **9a**, **9b** and **9c** was prepared to assist in structural elucidation of impurity **9** as either **9a** or **9b** and to efficiently isolate sufficient quantity of these impurities. Different stationary phase analytical HPLC columns were evaluated to separate the isomers from RG7128. Phenyl phase column provided the best separation, but **9a** and **9b** were not completely resolved. The initial purification of the synthetic mixture on the preparative HPLC did not provide pure fractions for the co-eluting peaks **9a** and **9b**. Further purification with recycle chromatography was carried out after lyophilization of the initial collections to enhance their purity.

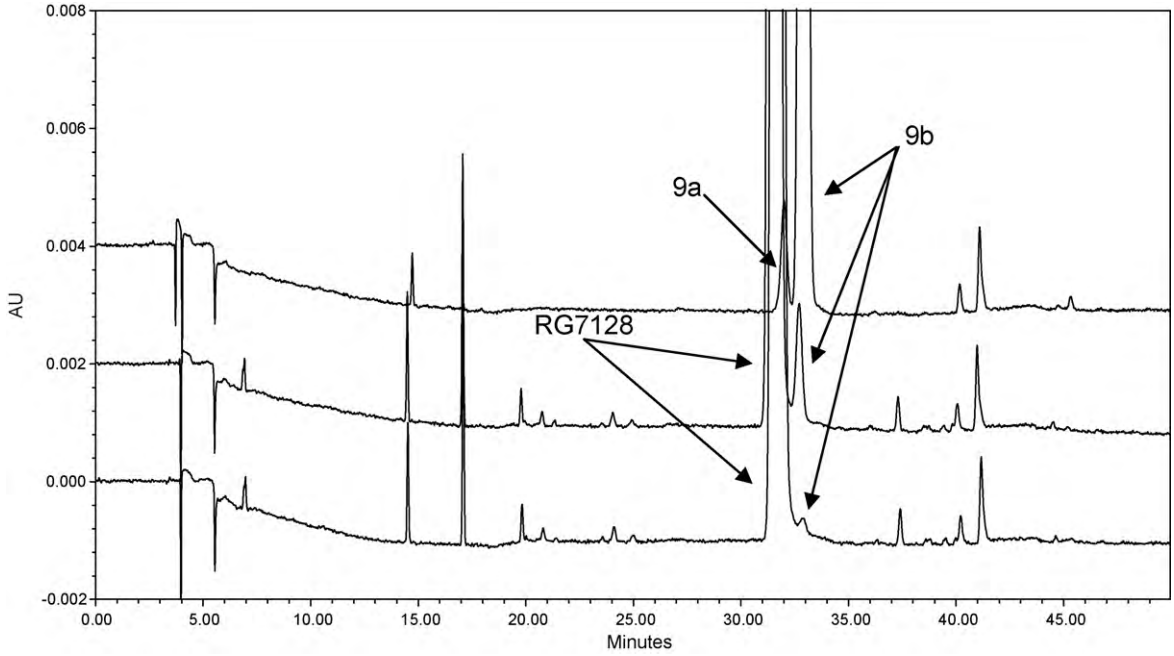

Fig. 6. Chromatograms of **9b** spiked into RG7128 drug substance for confirmation of its presence (top: purified **9b**, middle: drug substance spiked with **9b**, bottom: drug substance).

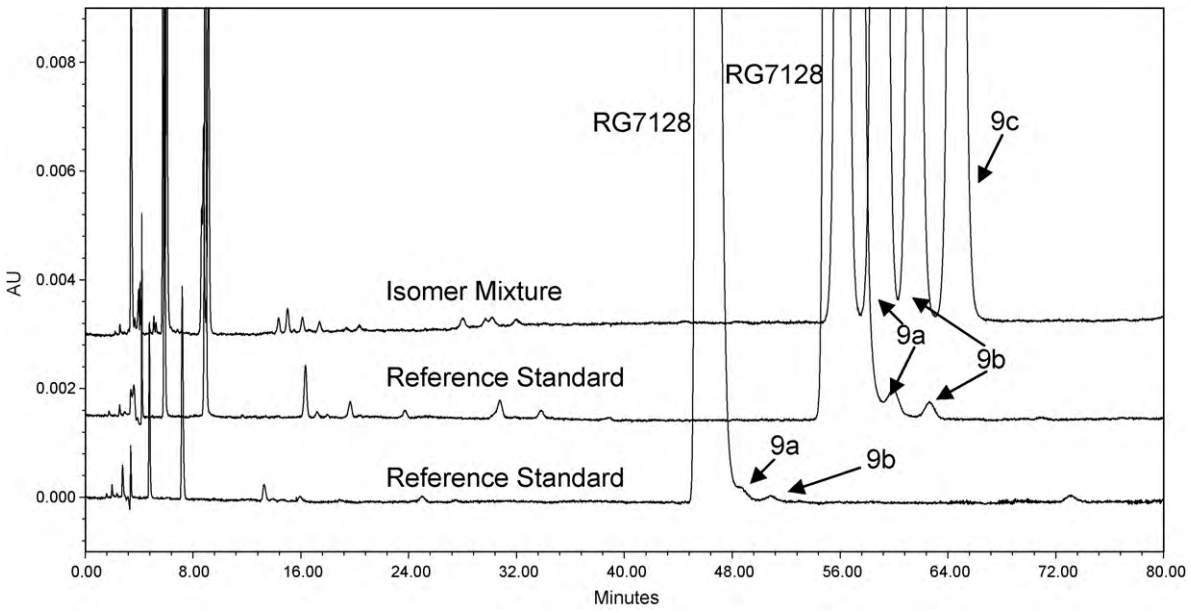

Fig. 7. Chromatograms of RG7128 and its isomeric impurities. Top and middle: Waters X-Bridge Phenyl column, 2× (150 mm × 4.6 mm, 3.5 μm column coupling). Bottom: Waters X-Bridge Phenyl column, (250 mm × 4.6 mm, 3.5 μm).

*3.6. Confirmation of impurity **9** in RG7128 drug substance*

The confirmation of impurity **9** was achieved by spiking each of the purified fractions of isomers **9a**, **9b** and **9c** into RG7128 drug substance. The spiked experiment confirmed that impurity **9** was **9b** (Fig. 6). The structure of impurity **9b** was elucidated by proton NMR. However, the spiked experiment also showed that **9a** co-eluted with RG7128 using the analytical HPLC method for RG7128. An alternative HPLC method dedicated for the separation of RG7128, **9a**, **9b** and **9c** was investigated.

Since RG7128 and its impurities are unstable at high temperature and neutral or basic pH, modification of column temperature and mobile phase pH was not considered during the method development to resolve impurities **9a**, **9b** and **9c** in RG7128 drug substance. The option of increasing column plate count was investigated. Based on previous column evaluations, a phenyl HPLC column provided a good separation of the isomers. Consequently, Waters X-Bridge phenyl HPLC columns were used to separate **9a**, **9b**, **9c** and RG7128. As shown in Fig. 7, a Waters X-Bridge phenyl column (250 mm × 4.6 mm, 3.5 μm) did not provide adequate efficiency to resolve the impurities from RG7128. Further increase in plate count to obtain higher efficiency was achieved by coupling two 150 mm × 4.6 mm columns to provide a total length of 300 mm. RG7128, **9a**, **9b** and **9c** were well resolved and impurities **9a** and **9b** were detected in RG7128 drug substance. However, the back pressure from column coupling reached 4500 psi, which is not desirable in routine analytical testing. A more suitable separation method needs to be developed.

4. Conclusions

The di-ester prodrug, RG7128, was synthesized via a one-step process by esterification of the cytidine analog with isobutyryl chloride. Nine impurities were detected in the representative early lots of RG7128 drug substance. A preparative chromatography system was employed to isolate and enrich the impurities for structural elucidation by LC–MS and NMR. Of all the impurities elucidated, impurity **9** was very challenging to purify and heart-cut preparative chromatographic technique was used to combat overloading and masking by the main peak. Furthermore, a column coupling analytical method was developed to separate the isomeric impurities from RG7128 and detected the presence of **9a** and **9b** in the drug substance.

All nine impurities were identified by either LC–MS and NMR or confirmation with reference samples. Impure starting materials, synthetic byproducts and degradation were the main sources for the formation of these impurities.

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