Synthesis and characterization of potential and degraded impurities of Regadenoson

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Abstract: ***Background:*** Regadenoson is an A_{2A} adenosine receptor agonist that is a coronary vasodilator and commonly used as a pharmacologic cardiac stressing agents.

Method: HPLC method was used for related substances analysis. The degraded impurities during the process were isolated and characterized by IR, Mass and NMR spectral analysis.

Result: Forced degradation study of regadenoson under conditions of hydrolysis (neutral, acidic and alkaline) and oxidations suggested in the ICH Q1A(R2) was accomplished. The drug showed significant degradation under all the above conditions. On the whole, five novel degradation products were found under diverse conditions along with process related impurities which are not reported earlier.

Conclusion: All the degradation products were well characterized by using advanced spectroscopic analysis like IR, ^{1}H NMR, ^{13}C NMR and Mass. The identification of these impurities will be productive for the quality control during the production and stability behavior of the regadenoson drug substance. Based on the study, regadenoson is sensitive towards acidic, basic and photolytic conditions.

Key words: Regadenoson, Potential impurities, ICH guidelines, NMR

1. INTRODUCTION

Regadenoson (**Fig.1)** is an A_{2A} adenosine receptor agonist that is a coronary vasodilator and commonly used as a pharmacologic cardiac stressing agents. As a stressing agent, regadenoson works by increasing the blood flow in the arteries. Regadenoson systemic is used in the treatment of radionuclide myocardial perfusion study, diagnosis and investigation. It produces hyperemia quickly and maintains for a duration that is useful for the radionuclide myocardial perfusion imaging [1]. Regadenoson has a 2 to 3 minutes biological half-life, as compared with adenosine's 10 seconds half-life. As a result, regadenoson stress protocols used as a single bolus, instead of 4-6 minutes continuous infusion, which is needed with adenosine. In addition adenosine infusion is weight based (140mcg/kg/minute), while with regadenoson 0.4mg/5mL preloaded syringe dose is standard for all weights. Regadenoson stress tests are not affected by the presence of beta blockers, as regadenoson vasodilates via the adenosine pathway without stimulating beta adrenergic receptors.

Based on the high importance of Regadenoson activity, United States Food and Drug Administration (USFDA) was approved as a drug on April 10, 2008 and it is marketed by Astellas Pharma under the trade name Lexiscan. It was approved for medical use in the British Pharmacopoeia under the brand name of Rapiscan. Different regulatory authorities such as USFDA, European Medicine Agency (EMA) and the Canadian Drug and Health Agency (CDHA) are emphasizing on the purity of the drug substance and they are gradually incorporating impurity limits to the allowable levels present in APIs or formulations [2]. This reveals that, the need and scope of impurity profiling of drugs in pharmaceutical research. Thus impurity profiling like identification, isolation and characterization are done and their threshold values comply with the limits set and specified by official bodies. The presence of these impurities even in trace amounts may influence the efficacy of the pharmaceutical product. The control and identification of these impurities are currently critical issue in the pharmaceutical industry. The critical impurity profiling was not studied properly even though regadenoson has high biological importance. To commercialize an active pharma ingredient in regulatory markets, it is mandatory for the manufacturer to identify and characterize the unknown impurities that are present in the API even at a level of below 0.10%.

In this context, a wide spread study has been undertaken to identify, synthesis and characterization of the unknown impurities formed during the process development and forced degradation of regadenoson using NMR and LC-MS. For synthetic chemists it is a challenging task to

recognize the origin of potential impurities during the synthesis of a drug substance and the fate of those impurities in the downstream chemistry. So far there is no literature reports on either degradation study or synthesis of Regadenoson impurities. In continuation of our ongoing studies on synthesis and characterization of unknown impurities formed during the process development as well as stability studies, here we wish to report degradation behavior of Regadenoson as per ICH guidelines. Also, present degradation study would support to understand the formation of these impurities in the regadenoson synthesis and provide the evidence on how to control the formation of these impurities so as to obtain a highly pure compound.

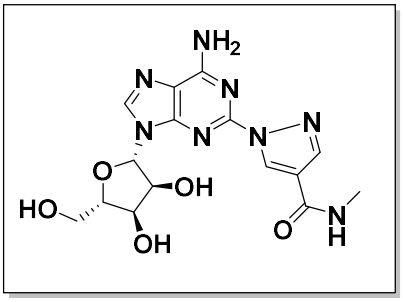


Fig. 1 Regadenoson

2. EXPERIMENTAL

2.1. Chemicals and reagents

Regadenoson drug substance was obtained from Synthetic group of Gland Pharma Limited, Hyderabad, India. HPLC grade methanol, acetonitrile (Merck, Darmstadt, Germany). Sodium hydroxide (NaOH) (Extrapure), Hydrochloric acid (HCl), Potassium phosphate and Sodium carbonate (AR grade) were purchased from Lobachemie (Mumbai, India). Ammonium formate and formic acid (MS grade) was purchased from Biosolve Chemie (Valkenswaard, The Netherlands). Ultra-pure water was obtained from Milli-Q water system (Millipore integral water purification system, Darmstadt, Germany). Orthophosphoric acid and Triethylamine were purchased from S.D. Fine-Chem Ltd. (Mumbai, India).

2.2 Instrumentation

The analysis was carried out using Shimadzu 2010 integrated HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with binary pump, auto liquid sampler, column compartment and the UV

detector set at 246 nm. Chromatographic separation was achieved on Symmetry C8 (250 x 4.6mm, 5.0 µm) column (Waters Corporation, Milford, Massachusetts, United States). Mobile phase was degassed using transonic Sonicator bath (PCI analytics, Mumbai, India).Shimadzu FTIR spectrometer IR Prestige-21(Shimadzu Corporation, Kyoto, Japan) at a resolution of 2 cm^{-1}, in 400-4000 cm^{-1} frequency range. ^{1}H NMR, ^{13}C NMR spectra were recorded using Bruker instrument operating at 400MHz Advance- III HD (Bruker, Billerica, Massachusetts, United States).The samples were dissolved in DMSO-d_6 by using tetramethylsilane (TMS) as an internal standard. All spectra were acquired at room temperature. The ^{1}H and ^{13}C chemical shift values were reported in ppm on δ scale. The mass measurements were performed on an Agilent Infinity 1200 series instrument (Agilent Technologies, Santa Clara, California, USA) coupled to Agilent 6310 ion trap was controlled using Chemstation software.

2.3. HPLC method and sample preparation

The reverse phase HPLC method was developed to determine the level of process and degradation impurities (impurity A to impurity G) and tigecycline drug substance. A prominence photodiode array detector (SPD-M20A) was used for peak purity and spectral analyses. Data acquisition, analyses, and reporting were performed by Lab Solutions software (Version 6.4). The chromatographic separation was achieved on resolved on Symmetry C8 (250 x 4.6mm, 5.0 µm) column and eluted with binary gradient using solution A: 0.1% ortho phosphoric acid (Aq) (pH 4.0 with triethylamine) and solution B: (Acetonitrile: MeOH: 1:1) with 50 min run time. The gradient program is as follows: (T_{min}/%B): 0.01/5.0, 20/17, 35/45, 40/50, 42.0/5, 50.0/5.0. The mobile phase flow rate, sample injection volume, auto sampler, and column oven temperature were set at 1.0 mL/min, 50µL, 10°C and 35°C respectively. Regadenoson, process and the degradation impurities were determined at 246 nm. All the process and the degradation impurities were diluted with the buffer prior to the injection, to obtain a final concentration of 0.2 mg/mL.

2.4. Preparation and isolation of impurities

2.4.1. Preparation of 2-hydrazineyl-9H-purin-6-amine (Impurity A)

2-Chloroadenine (2.0 g, 12.0 mmoles) was added to 30 ml of 50% hydrazine hydrate. Then the reaction mass was heated to 50-55°C. After completion of the starting material monitored by TLC, the reaction mass was evaporated and kept for drying under high vacuum at 50-55°C to yield a white color solid of 2-hydrazinyl-9H-purin-6-amine (Yield: 95%).

2.7.2. Preparation of Methyl 1-(6-amino-9H-purin-2-yl)-1H pyrazole-4-carboxylate (Impurity B)

Compound **3** (350 mg, 0.9 mmoles) was dissolved in 30ml of 0.1N HCl solution and heated to 100-105^{o}C. The reaction mixture become clear and after some time white color solid was thrown out from the reaction mass. The stirring was continued for 4 hours at 100-105^{o}C. The reaction mass was cooled to room temperature and formed thick white solid was filtered off. The filtered solid was washed with water. The solid was dried under high vacuum to yield methyl 1-(6-amino-9H-purin-2-yl)-1H pyrazole-4-carboxylate (Yield: 85%).

2.7.3. Preparation of N'-(6-amino-9-((2S, 3S, 4R, 5S)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)acetohydrazide (Impurity C)

2-Hydrazino adenosine (100 mg, 3.36 mmoles) is heated in a mixture of acetic acid (7.5 ml) and methanol (7.5 ml) at 80-85°C for 8 hrs. The reaction mixture becomes clear. After completion of reaction, distill out the solvent mixture completely under vacuum. The obtained semi solid was dried under high vacuum yield the title compound (Yield: 90%).

2.7.4. Preparation of 1-(6-Amino-9H-purin-2-yl)-*N*-methyl-1H-pyrazole-4-carboxamide (Impurity D)

Regadenoson **4** (500 mg, 1.28 mmoles) was dissolved in 50ml of 0.1N HCl solution and heated to 100-105^{o}C. The reaction mixture become clear and after some time white color solid was thrown out from the reaction mass. The stirring was continued for 4 hours at the same temperature, then the reaction mass was cooled to room temperature and white solid formed was filtered off and washed with water followed by dried under vacuum gave the title compound (Yield: 90%).

2.7.4. Preparation of 1-(6-amino-9H-purin-2-yl)-N,N-dimethyl-1H-pyrazole-4-carboxamide (Impurity E)

2-{4-[(Dimethylamino) carbonyl]-1*H*- pyrazol-1-yl} adenosine (500 mg, 1.28 mmoles) was dissolved in 50 ml of 0.1N HCl solution and heated to 100-105^{o}C. The reaction mixture become clear and after some time white color solid was thrown out from the reaction mass. The stirring was continued for 4 hours at 100-105^{o}C. The reaction mass was cooled to room temperature and a thick white solid formed was filtered off. The filtered solid was washed with water and dried under high vacuum resulted the impurity E (Yield: 90%).

2.7.4. Preparation of 1-(6-amino-9-((2S, 3S, 4R, 5S)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)-1H-pyrazole-4-carbaldehyde (Impurity F)

To a suspension of 2-Hydrazinoadenosine (0.53 g, 1.8mmols) in a mixture of MeOH-acetic acid (8.0 ml, 1:1ratio), trialdehyde (0.6g, 6.0mmol) was added under nitrogen at room temperature. The resultant reaction mixture was stirred at 25-30^{o}C for 2h. After completion of the reaction by HPLC, the reaction mixture was filtered and the solid washed with 5 ml of ethanol and 2.5ml of MTBE. The obtained solid was stirred with 5.3ml of MTBE at room temperature for 15 minutes, filtered and dried the material under high vacuum for 1h at 40°C to gave pale yellow colour solid (Yield: 0.74g). The obtained crude compound was stirred with 5% methanol/MDC (15ml) at 25-30°C for 25minutes filtered the solid (yield: 0.46g). The crude compound obtained was purified by column chromatography using silica gel and 5% ethyl acetate/hexane used an eluent. Collect the pure fractions and solvent was evaporated and dried under high vacuum resulted pale yellow colour solid compound (Yield: 45%).

2.7.4. Preparation of 2-(6-amino-2-(2-((1-(6-amino-9-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)-1H-pyrazol-4-yl)methylene)hydrazineyl)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (Impurity G)

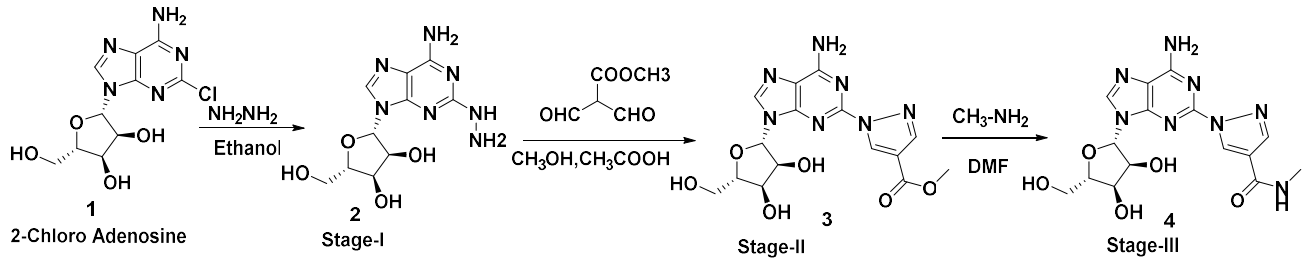
To a suspension of 2-Hydrazinoadenosine (0.5 g, 1.68mmols) in a mixture of MeOH-acetic acid (7.5 ml, 1:1ratio), trialdehyde (0.18g, 1.85mmols) was added under nitrogen at room temperature. The resultant reaction mixture was stirred at 80-85^{o}C for 4h. After completion of the reaction, reaction mixture was cooled to room temperature and stirred for 30minutes. The solid formed was filtered and washed with 5 ml of ethanol and 2.5ml of MTBE. The obtained solid compound was stirred with 5.0ml of MTBE at room temperature and the resultant solid obtained was filtered and dried under high gave pale yellow colour solid compound (Yield: 90%).

3. RESULTS AND DISCUSSION

3.1. Chemistry Discussion

An improved process for the commercial synthesis of regadenoson was well known in the literature and starting from 2-chloroadenosine (**1**) which upon coupling with hydrazine hydrate formed 2-hydrazino adenosine (**2**) [3, 4]. The resulted hydrazide derivative (**2**) is subjected to cyclocondensation with methyl diformylacetate produced 2-(4-methxoycarbonylpyrazyl-1-yl) adenosine (**3**). The resulting

ester was further reacted with methylamine to give regadenoson (**4**). The complete synthetic scheme is shown below (**Scheme 1**).



Scheme 1. Preparation of Regadenoson (**4**) from 2-chloro adenosine (**1**)

In pharmaceutical industry, during the process development of a drug substance, it is necessary to identify all the possible process, degradation and by-product impurities in each stage and develop analytical methods to monitor all the impurities and control those impurities to be well within the ICH limit. Based on the route of synthesis in scheme 1, the possible impurities are presented in (**Table 1**). All the impurities are well characterized and are already reported in the literature. If methylamine contained dimethylamine as an impurity, the formation of impurity E is possible. The impurity-E can be prepared easily by the direct amidation of 2-(4-methxoycarbonylpyrazol-1-yl) adenosine with dimethylamine. This impurity is also well characterized and reported in the literature.

In addition to the above impurities, several process related and degradation impurities are possible in each stage, which have not been reported elsewhere to the best of our knowledge. To identify those impurities, forced degradation study is requisite for an active pharma ingredient to develop a good analytical method in a paradigm. Forced degradation studies will give useful information on the possible degradation pathways and help in controlling those impurities. It facilitates to establish the specific stability method and to envisage potential degradation impurities that could form through the stability studies. Knowledge of chemical behavior of drug substances under stress conditions will provide precious information relating to the choice of the excipients for formulation development. Forced degradation studies can help pharmaceutical development as well in areas such as manufacturing, formulation development, packaging and transportation, where the knowledge of chemical behavior can be used to get a better drug product.

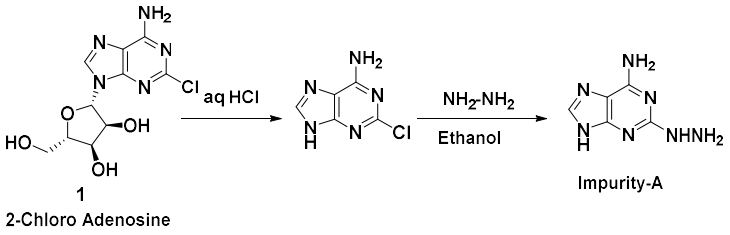
The forced degradation on regadenoson was performed as follows [5]. Weighed accurately 25 mg of highly pure Regadenoson (99.9% HPLC purity) drug substance and subjected to normal stress with 25 mL of 5N HCl at 50°C for 20 min. The results showed an unknown impurity at 0.89 RRT

about 11% with respect to regadenoson as shown in (**Figure 2**) and it is not matching with the specified impurity (**Table 1**). The same impurity was observed in normal peroxide test (The test was conducted by dissolving 50 mg of regadenoson in 10 mL of methanol then 10% hydrogen peroxide solution was added and stirred at room temperature for 3 hours) in about 0.2% and the results are presented in (**Table 2**). To identify the structure of the unknown impurity at RRT 0.89, LC-MS study was performed and the mass results showed at m/z 259.1 in positive mode which is matched with de-ribose regadenoson. The de-glycosylation of Regadenoson and its intermediates were not studied properly till date and a probable mechanism proposed is shown in (**Scheme 2**). The major unknown peaks at different RRT's other than 0.89 in peroxide stress shows 16 mass units greater than the parent ion due to *N*-oxide formation on one of the nitrogen present in purine ring.

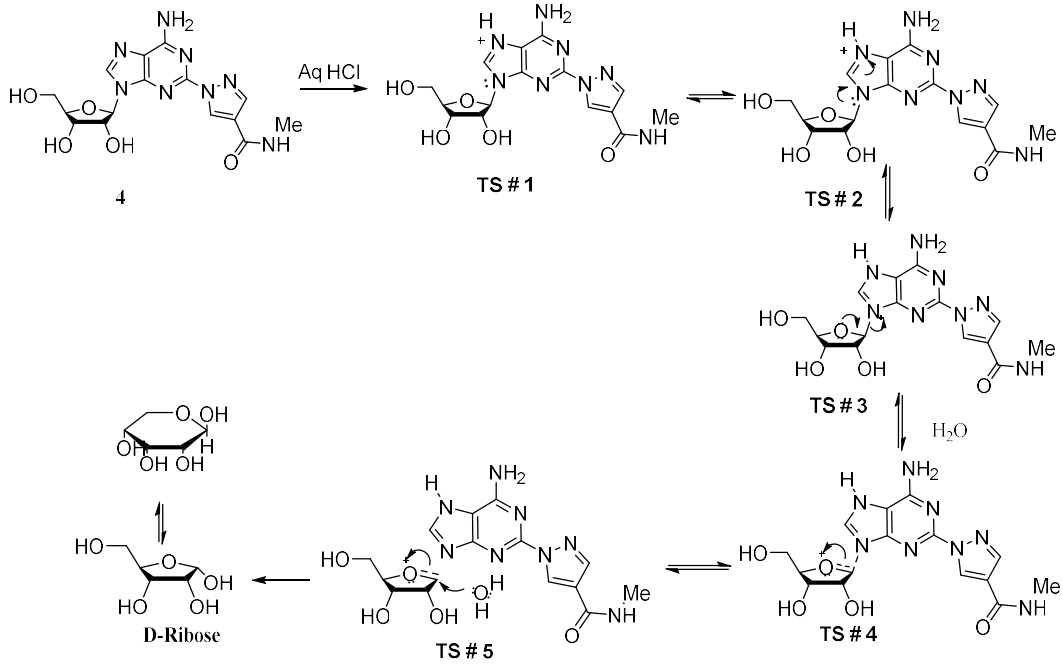
According to the literature [6-9] hydrolytic stability of the *N*-glycosidic bond is very weak and cleaves spontaneously as C-2 carbon is attached to highly electronegative oxygen and nitrogen atoms. According to the literature de-glycosylation is also possible at neutral pH, then the deribose-regadenoson impurity has to be controlled as a part of related substances specification. During the process development of regadenoson, which involves condensation of methyl diformylacetate with 2-(hydrazino) adenosine under acidic medium at, the formation of the Impurity A and impurity B is highly possible. If impurity B carry over further, it generates impurity C. Hence, the possible carry over impurities from adenosine were synthesized and checked in the next stages. And those identified structures were incorporated in the below table and were denoted as impurity D and impurity E (**Table 3**). In addition, during the reaction of 2-hydrazino adenosine (**2**) with diformylacetate, the presence of trialdehyde impurity in diformylacetate generated impurity F and impurity G.

Impurity A: Next our attention is moved towards synthesizing those impurities and checking in each stage in the manufacturing process of regadenoson. Acetic acid is being used as a solvent during the cyclocondensation reaction with methyl diformylacetate. It is well known that acid medium is enough to break the glycoside bond. Hence, the formation of 2-hydrazinoadenine is possible. In order to check the formation of this impurity the same expected deglycoylated impurity was synthesized by the hydrolysis of 2-chloroadenosine (**2**) in the presence of diluted HCl to give 2-chloroadenine, which further reacts with hydrazine hydrate to give the impurity A. The obtained product was checked in the HPLC method to identify the RRT of the impurity with respect to the stage-I intermediate. The RRT of synthesized compound is perfectly matched with unknown impurity. The ^{1}H NMR spectrum of the compound showed absence of peaks around 3.50-5.50 ppm this confirms that, the compound is not

having ribose moiety and the presence of singlets at 8.14, 8.40 and 7.69 ppm due to the imidazole CH and NH and NH_2 protons. The structure of the compound was further confirmed by ^{13}C NMR and HRMS analysis. The synthetic scheme is shown in (**Scheme 2**). The de-glycosylation of ribonucleoside proceeds through S_N1 type mechanism involving the expulsion of the nucleobase leaving group followed by hydrolysis of the ribose oxocarbenium intermediate [10]. The same de-glycosylation mechanism is applicable to all impurities.



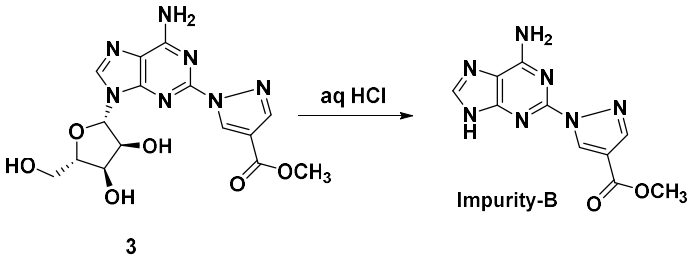
Scheme 2. Preparation of Impurity A



Scheme 3. Mechanism for Deglycosylation

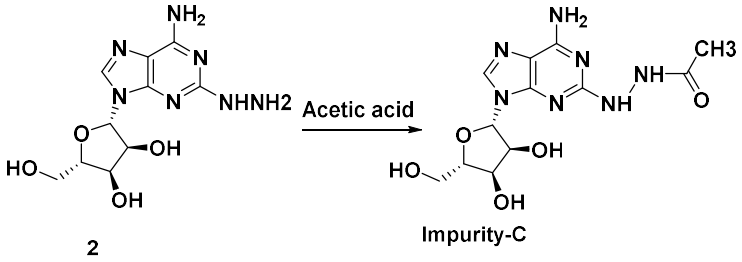
Impurity B*:* Impurity B named as methyl-1-(6-amino-9H-purin-2-yl)-1*H*-pyrazole-4- carboxylate is also one of the potential impurities during the formation of stage-II of regadenoson and hence attempts were made to synthesize the impurity B by acid hydrolysis of 1-[6-amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-9*H*-purin-2-yl]-1*H*-pyrazole-4-carboxylicacid methyl ester in the presence of aq. HCl. The compound (impurity-B) obtained from the above process

was characterized by spectral analysis. The ^{1}H NMR spectrum of the compound showed a singlet at δ 3.81 ppm due to methyl protons of carboxymethyl group. In addition, other signals which were observed in the aromatic region were due to imidazole and pyrazole rings. Apart from these, absence of signals around 3.50-5.55 ppm confirms that ribose cleavage has taken place in the reaction. The structure of the compound was further confirmed by ^{13}C NMR. The synthetic scheme is shown in **Scheme** 4.



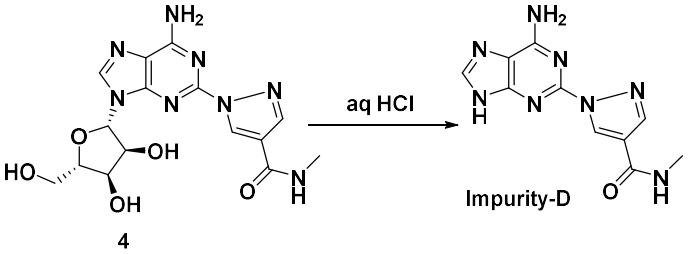
Scheme 4. Preparation of Impurity B

Impurity C: Next our attention is moved to identification and synthesis of impurity C, which is formed during stage-II of regadenoson. The origin of the formation of this impurity is expected to be from the reaction of 2-hydrazino adenosine with acetic acid, since acetic acid is used as the solvent in the reaction. To confirm the same stage-I intermediate of regadenoson was subjected to heating in the presence of glacial acetic acid, which produced the *N*-acetyl derivative. The synthesized impurity structure was confirmed by IR, mass and NMR analysis. The IR spectrum of *N*-acetyl hydrazide impurity showed a band at 3271 cm^{-1} for NH and 1645 cm^{-1} for (CO of amide) confirming the presence of amide functional group. The ^{1}H NMR spectrum of the compound shows a singlet at δ 1.87 ppm confirming the methyl protons of acetyl group, in addition to other signals due to ribose and adenine ring. The structure of the compound was further confirmed by ^{13}C NMR and mass analysis. The synthetic scheme for the preparation of the same is depicted in **Scheme** 5.



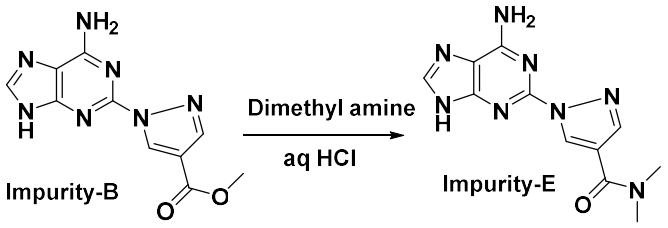
Scheme 5. Preparation of Impurity C

Impurity D: the impurity-D named as 1-(6-amino-9H-purin-2-yl)-N-methyl-1H-pyrazole-4-carboxamide was observed in the final stage at RRT 0.89 in HPLC with respect to regadenoson. The source of the impurity is carryover of impurity-B from Stage-II, which further condensed with methylamine. To synthesize and confirm the impurity, two routes were chosen. The first route of synthesis is the treatment of impurity-B with methylamine and the second route of synthesis involves direct acid hydrolysis of final regadenoson. Both the routes gave successful results towards the impurity-D, and the synthesized impurity structure was confirmed by ^{1}H NMR, ^{13}C NMR and mass analysis. The synthetic scheme followed for the generation of impurity-D is shown in **Scheme** 6.



Scheme 6. Preparation of Impurity D

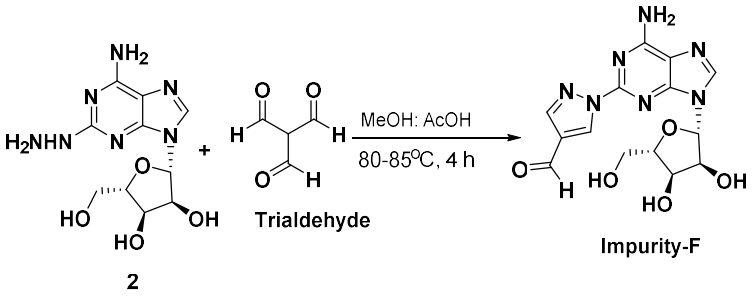
Impurity E: The N,N-dimethylamine impurity (impurity E) was observed in very trace amounts during the final stages. The origin of the impurity is from the trace amount contamination of dimethylamine in methylamine, which reacts with the carryover impurity B from stage-II. The same compound was further synthesized by the reaction of impurity-B with diethylamine in the presence of DMF. The structure of the synthesized compound was confirmed by spectral analysis. The synthetic scheme is shown in **Scheme 7**.



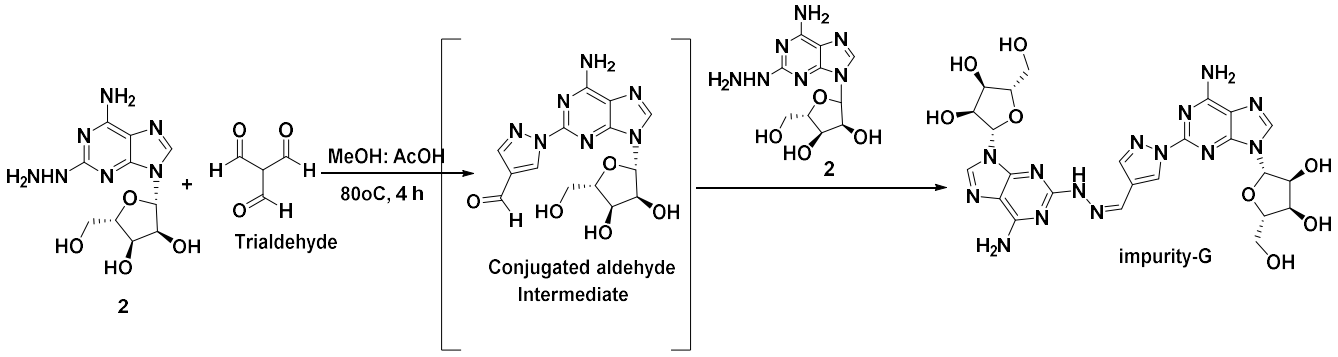
Scheme 7. Preparation of Impurity E

Impurity F: During synthesis of Regadenoson, Compound (2) reacts with diformylacetate to form Compound (3). Trialdehyde is one of the major impurity formed during preparation of diformylacetate. The impurity trialdehyde present in diformyl acetate react with compound (2) leads to formation of

process related impurity F. The formed impurity F further reacts with Compound (2) to form this process related impurity G.



Scheme 8. Preparation of Impurity F



Scheme 9: Preparation of Impurity G

3.2. Spectral Discussion

3.2.1. 2-hydrazineyl-9H-purin-6-amine (Impurity A)

^{1}H-NMR (DMSO-d_6): δ 8.40 (1H, brs), 8.14 (1H, s), 7.69 (2H, s); ^{13}C-NMR (DMSO-d_6): 162.07, 155.71, 152.74, 140.47, Mass (ESI) $[M+H]^+$ calcd. for: $[C_5H_7N_7]$ 166.17; found; 166.00; FT-IR (KBr) ν =3278.99, 3116.97, 3008.95, 2808.36, 1680.00, 1612.49, 1384.89 cm^{-1}.

3.2.2. Methyl 1-(6-amino-9H-purin-2-yl)-1H pyrazole-4-carboxylate (Impurity B)

^{1}H-NMR (DMSO-d_6): δ 8.93 (1H, s), 8.73 (1H, s), 8.16 (1H, s, C14-H), 8.09 (1H, brs), 5.61(2H, brs), 3.81 (3H, s); ^{13}C-NMR (DMSO-d_6): 172.24, 162.32, 154.39, 151.08, 150.85, 142.36, 139.88, 132.11, 115.66, 51.59; Mass (ESI) $[M+H]^+$ calcd. for: $[C_{10}H_{10}ClN_7O_2]$ 260.2; found; 260.1; FT-IR (KBr) ν =3361.93, 3197.98, 1722.43, 1670.3 5, 1568.13, 1267.23 cm^{-1}.

3.2.3. N'-(6-amino-9-((2S, 3S, 4R, 5S)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)acetohydrazide (Impurity C)

^{1}H-NMR (DMSO-d_6): δ 8.05 (H, s), 7.98 (1H, brs), 6.96 (2H, brs), 5.74 (1H, d), 4.54-4.58 (1H, m), 4.14-4.16 (1H, m), 3.89-3.90 (1H, m), 3.49-3.66 (2H, m), 1.90 (3H, s); ^{13}C-NMR (DMSO-d_6): δ 169.50 , 160.29 , 156.49 , 151.20 , 137.82 , 115.13 , 87.78 , 85.86 , 73.66 , 71.10 , 62.28 , 20.64; Mass (ESI) $[M+H]^+$ calcd. for: $[C_{12}H_{17}N_7O_5]$ 340.3; found; 340.2; FT-IR (KBr) ν =3271.27, 2935.66, 1645.28, 1598.99, 1267.23 cm^{-1}.

3.2.4. 1-(6-Amino-9H-purin-2-yl)-*N*-methyl-1H-pyrazole-4-carboxamide (Impurity D)

^{1}H-NMR (DMSO-d_6): δ 8.96 (1H, s), 8.64 (1H, s), 8.39 (1H, q), 8.10 (1H, s), 7.96 (2H, s) 2.75 (3H, d); ^{13}C-NMR (DMSO-d_6): δ 161.64, 154.72, 151.22, 151.02, 141.11, 139.92, 129.51, 120.21, 113.36, 25.54; Mass (ESI) $[M+H]^+$ calcd. for: $[C_{10}H_{11}ClN_8O]$ 259.2 ; found; 259.1; FT-IR (KBr) ν = 3325.28, 3151.69, 1680.00, 1647.21, 1583.56, 1288.45 cm^{-1}.

3.2.5. 1-(6-amino-9H-purin-2-yl)-N,N-dimethyl-1H-pyrazole-4-carboxamide (Impurity E)

^{1}H-NMR (DMSO-d_6): δ 8.74 (1H, s), 8.33 (1H, s), 8.01 (1H, s), 7.73 (1H, brs), 4.24 (3H, brs), 3.20 (3H, s) 2.99 (3H, s); ^{13}C-NMR (DMSO-d_6): δ 162.85, 155.32, 150.56, 141.98, 140.05, 131.73, 130.08, 118.66, 40.67, 40.46; Mass (ESI) $[M-H]^+$ calcd. for: $[C_{11}H_{12}N_8O]$ 271.2 ; found; 271.0; FT-IR (KBr) ν = 3377.36, 3120.82 , 2893.22 , 1662.64 , 1608.63 , 1556.55 , 1166.93 cm^{-1}.

3.2.6. 1-(6-amino-9-((2S, 3S, 4R, 5S)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)-1H-pyrazole-4-carbaldehyde (Impurity F)

^{1}H-NMR (DMSO-d_6): δ 9.96 (1H, s), 9.18 (1H, s), 8.43 (1H, s), 8.21 (1H, s), 7.87 (2H, brs), 5.94 (1H, d), 5.50 (1H, d), 5.24 (1H, d), 5.02 (1H, t), 4.60-4.65 (1H, m), 4.16-4.19 (1H, m), 3.94-3.97 (1H, m), 3.55-3.71 (2H, m); ^{13}C-NMR (DMSO-d_6): δ 185.5 , 156.43,150.15 , 150.05 , 140.76 ,140.30 ,134.13, 124.82,118.18, 87.12, 85.66, 73.58,70.42,61.41; Mass (ESI) $[M+H]^+$ calcd. for: $[C_{14}H_{15}N_7O_5]$ 362.3; found; 362.12; FT-IR (KBr) ν = 3392.79, 3184.48, 2926.01, 1666.50, 1610.56 cm^{-1}.

3.2.7. 2-(6-amino-2-(2-((1-(6-amino-9-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)-1H-pyrazol-4-yl)methylene)hydrazineyl)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (Impurity G)

^{1}H-NMR (DMSO-d_6): δ 10.58 (1H, brs) , 8.82 (1H, s), 8.39 (1H, s) 8.21 (1H, s), 8.0- 8.06(2H, s), 7.72 (2H, brs), 7.06 (2H, brs), 5.94 (1H, d), 5.77(1H,d), 5.55 (1H, d), 5.42-5.43 (2H, m), 5.03-5.06 (1H, t), 4.61-4.75 (2H, m), 4.19-4.23 (2H, m), 3.97-4.01 (2H, m), 3.55-3.71 (2H, m); ^{13}C-NMR (DMSO-d_6): δ 156.38 , 156.20 , 150.72 , 150.33 , 140.31 , 139.83 , 138.17 , 132.56 , 127.48, 120.68, 117.66 ,115.45 , 88.18, 87.01, 86.32,85.63, 73.61, 72.81, 71.18, 70.56, 62.12,61.47; Mass (ESI) $[M+H]^+$ calcd. for: $[C_{24}H_{28}N_{14}O_8]$ 641.5; found; 641.23; FT-IR (KBr) ν = 3332.99, 3265.49, 2929.87, 1595.13, 1485.19, 1396.46 cm^{-1}.

4. CONCLUSION

Five novel degradation products were found under degradation conditions along with process related impurities which are not reported earlier. All the degradation products were well characterized by using advanced spectroscopic analysis like IR, ^{1}H NMR, ^{13}C NMR and Mass .The identification of these impurities will be productive for the quality control during the production and stability behavior of the regadenoson drug substance. Based on the study, regadenoson is sensitive towards acidic, basic and photolytic conditions.

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Supporting information

The supplementary information consists of the following data ^{1}H, ^{13}C, and DEPT-135 data of impurities A, B, C, D, E, F and G (S2-S12).

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Table 1: Known Impurities of Regadenoson

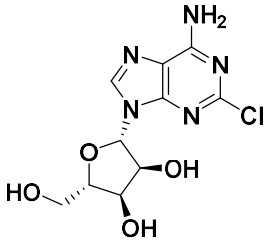
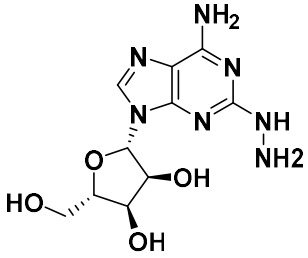
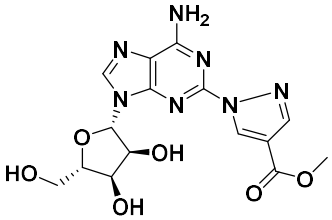
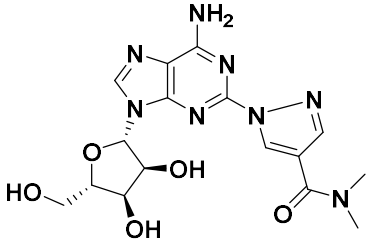
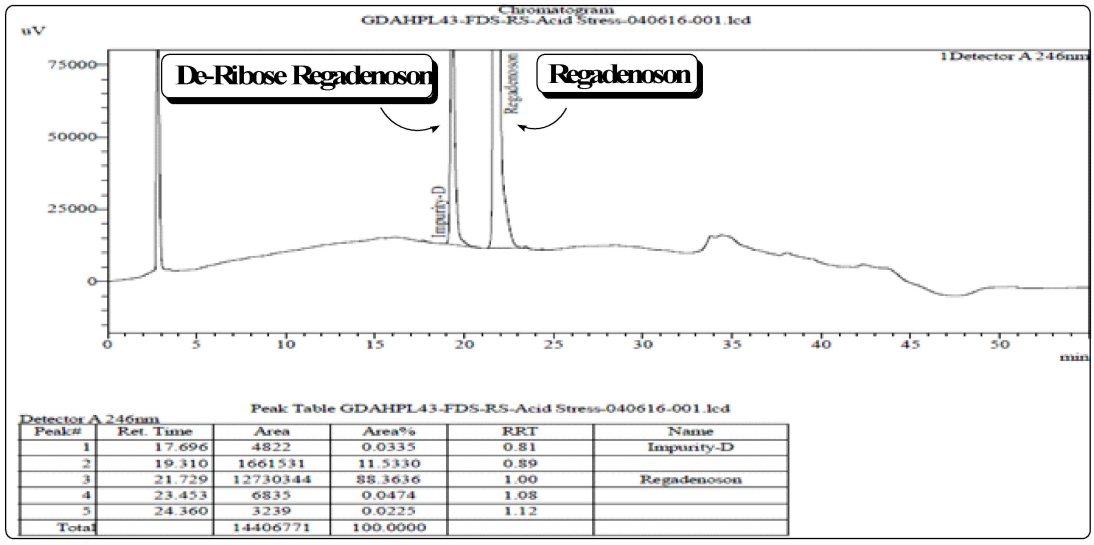
S. No	Name of the impurity	Structure
1	2-Chloroadenosine	
2	2-(hydrazino)adenosine	
3	1-[6-Amino-9-((2R,3R,4S,5R)-3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-9H-purin-2-yl]-1H-pyrazole-4-carboxylic acid methyl ester	
4	2- {4-[(Dimethylamino)carbonyl]- 1H- pyrazol-1-yl} adenosine	

Table 2: Forced degradation results for related substances and purity

Name of the Impurity	RRT	Peroxide stress	Acid stress
Unknown impurity	0.310	0.1159	ND
Unknown impurity	0.370	0.0599	ND
Unknown impurity	0.490	0.0734	ND
Unknown impurity	0.700	0.0954	ND
Deribose-Regadenoson	0.890	0.2092	11.862
Unknown impurity	0.900	0.1865	ND
Unknown impurity	0.950	0.1004	ND
Unknown impurity	1.080	0.0397	0.049



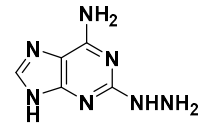
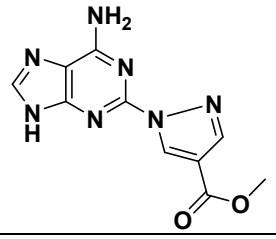
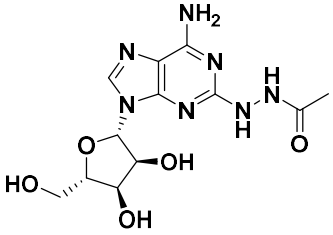
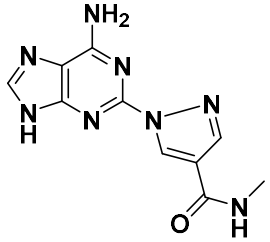
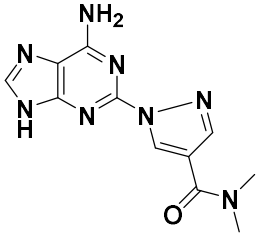
Peak Table GDAHPL43-FDS-RS-Acid Stress-040616-001.lcd

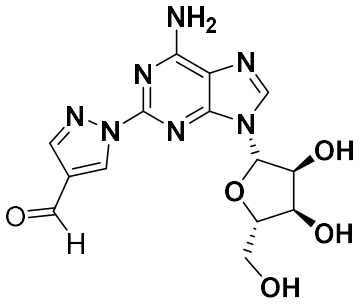
Detector A 246nm

Peak#	Ret. Time	Area	Area%	RRT	Name
1	17.696	4822	0.0335	0.81	Impurity-D
2	19.310	1661531	11.5330	0.89	
3	21.729	12730344	88.3636	1.00	Regadenoson
4	23.453	6835	0.0474	1.08	
5	24.360	3239	0.0225	1.12	
Total		14406771	100.0000		

Figure 2: HPLC chromatogram of acid stressed regadenoson

Table 3: **Identified and possible unknown impurities of Regadenoson.**

S. No.	Name of the Impurity	Structure
1	2-hydrazineyl-9H-purin-6-amine **(impurity A)**	
2	Methyl 1-(6-amino-9H-purin-2-yl)-1H pyrazole-4-carboxylate **(Impurity B)**	
3	N'-(6-amino-9-((2S,3S,4R,5S)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)acetohydrazide **(Impurity C)**	
4	1-(6-Amino-9H-purin-2-yl)-*N*-methyl-1H-pyrazole-4-carboxamide **(Impurity D)**	
5	1-(6-amino-9H-purin-2-yl)-N,N-dimethyl-1H-pyrazole-4-carboxamide **(Impurity E)**	

6.	1-(6-amino-9-((2S,3S,4R,5S)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)-1H-pyrazole-4-carbaldehyde (**Impurity F**)	
7.	2-(6-amino-2-(2-((1-(6-amino-9-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-2-yl)-1H-pyrazol-4-yl)methylene)hydrazineyl)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**Impurity G)**