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Research Article

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A Validated RP-HPLC Method for the Determination of 2-Chloroadenosine as Process Related Impurity in Regadenosen Parenteral Dosage Form

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ABSTRACT

Regadenoson is an A_{2A} adenosine receptor agonist that is a coronary vasodilator. Regadenoson is chemically described as adenosine, 2-[4[(methylamino) carbonyl]-1H-pyrazol-1-yl]-, monohydrate. Regadenoson is a low affinity agonist (Ki ≈ 1.3 μM) for the A2A adenosine receptor, with at least 10-fold lower affinity for the A_1 adenosine receptor (Ki > 16.5 μM), and weak, if any, affinity for the A_{2B} and A_3adenosine receptors. Activation of the A_{2A} adenosine receptor by regadenoson produces coronary vasodilation and increases coronary blood flow (CBF). A simple and precise reverse phase high performance liquid chromatography (RP-HPLC) method has been developed and validated which can separate and accurately quantitate 2-chloro adenosine related compounds. The capability of the method was demonstrated through adequate separation of all potential 2-chloro adenosine related compounds from active pharmaceutical ingredient and also from each other that are present in aged and stress degraded 2-chloro adenosine stability samples. Successful separation of 2-chloro adenosine from its one process impurity and degradation impurities formed under stress conditions was achieved by using a isocratic elution at a flow rate of 1.0 mL/min on a symmetry C18-3v column (250 mm × 4.6 mm, 5 µm particle size, 100 Å pore size) at ambient temperature. Mobile phase is the mixture of 1:1 v/v of acetonitrile and methanol; (pH-adjusted to 3.2 with 10% v/v ortho-phosphoric acid) in gradient mode was found to be best suitable for separation of impurity. UV detection at 205 nm was employed to monitor the analytes. The method was successfully validated in accordance to ICH guidelines acceptance criteria for system suitability, specificity, linearity, range, precision, accuracy, limits of detection and quantification for the impurities, and robustness, following the ICH guidelines. Therefore, the proposed method was suitable for the simultaneous determination of 2-chloro adenosine and its process related impurities. Finally, the applicability of the method was evaluated in commercial dosage form analysis as well as in stability studies.

Keywords: 2-chloro adenosine; Degradants; Validation; Stability indicating method

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INTRODUCTION

Regadenoson is an A_{2A} adenosine receptor agonist that is a coronary vasodilator. Regadenoson is chemically described as adenosine, 2-[4[(methyl amino) carbonyl]-1H-pyrazol-1-yl]-, monohydrate [1,2]. Regadenoson has the empirical formula $C_{15}H_{18}N_8O_5 \cdot H_2O$ and a molecular weight of 408.37. Lexiscan® contains Regadenoson as active component, is a sterile, nonpyrogenic solution for intravenous injection. The solution is clear and colorless. Each 1 mL in the 5 mL pre-filled syringe contains 0.084 mg of regadenoson monohydrate, corresponding to 0.08 mg regadenoson on an anhydrous basis, 10.9 mg dibasic sodium phosphate dihydrate or 8.7 mg dibasic sodium phosphate anhydrous, 5.4 mg monobasic sodium phosphate monohydrate, 150 mg propylene glycol, 1 mg edetate disodium dihydrate, and Water for Injection, with pH between 6.3 and 7.7. Lexiscan® (Regadenoson) injection is a pharmacologic stress agent indicated for radionuclide myocardial perfusion imaging (MPI) in patients unable to undergo adequate exercise stress [3-5]. The novel synthetic method has been developed by concerning the yields and

mild reaction conditions to synthesize regadenoson from stable starting material such as 2-chloro adenosine as stable starting material. Besides the simple route of synthesis for regadenoson, we have observed that 2-chloroadenosine is present as potential impurity in our synthetic process and also appears in finished product. The aim of this paper was to develop validate a simple and reliable RP-HPLC method for the determination of 2-chloroadenosine as process related impurity in regadenoson parenteral dosage form. Hence, a RP-HPLC method for determination of 2-choloroadenosie was developed and validated as per International Conference on Harmonization (ICH) guidelines [6-9]. This manuscript describes the development and validation, in accordance with ICH guidelines, of a rapid, economical, precise, and accurate reversed-phase HPLC method in gradient mode for analysis of 2-chloro adenosine as process related impurity [10]. To the best of our knowledge, no analytical methods are currently available to separate and quantitate the known process related compounds of Regadenoson. Furthermore, there is no RP-HPLC method reported in the literature that can adequately quantitate impurities present in finished pharmaceutical dosage forms. Synthesis of regadenosone from 2-chloro adenosine is depicted in Figure 1.

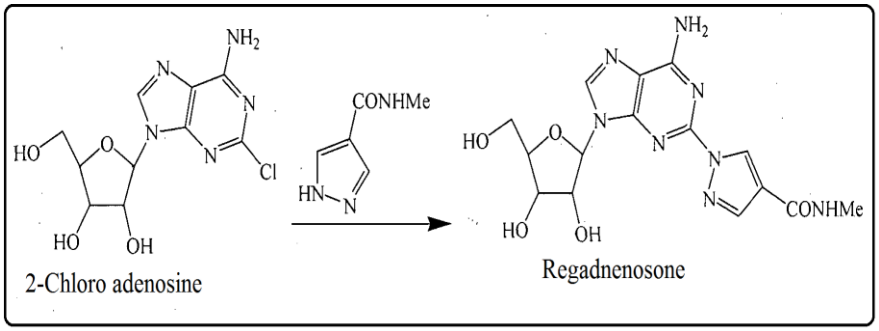

Figure 1: Synthesis of regadenosone from 2-chloro adenosine

EXPERIMENTAL SECTION

Materials and Reagents

Regadenosone as active pharmaceutical ingredient, 2-Chloro adenosine (Regadenoson impurity A), 2-Methoxy adenosine, 2, 6-Dichloropurine, 2-Amino adenosine and 2-Chloro adenosine were obtained from Gland Pharma as reference standards for process related substances.

Instrumentation and Software

Shimadzu 2010CHT liquid chromatograph separation module equipped with PDA detector (for specificity and degradation studies) with LC solutions software was used for the analysis. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldbronn, Germany) computer system. Chromatographic separation of 2-chloro adenosine as process related impurity was achieved Symmetry C-8 (250 × 4.6 mm, 5 µ particle size). The pH measurements were carried out with Elico, model LI 120, pH meter equipped with a combined glass-calomel electrode calibrated using standard buffer solutions of pH 4.0, 7.0 and 9.2.

Chemicals and Reagents

All the reagents were of analytical-reagent grade unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnstead, USA), HPLC-grade acetonitrile, potassium dihydrogen ortho phosphate, and ortho-phosphoric acid (S.D.Fine chem, Mumbai, India) were used. Pure sample of 2-chloro adenosine (reference standard), and other process related substances viz. 2,6-dichloro adenosine, 2-methoxy adenosine and 2-amino adenosine were a kind of gift from Gland Pharma Pvt. Ltd, Hyderabad. Acetonitrile (HPLC grade) and potassium dihydrogen orthophosphate were obtained from Loba Chem, Mumbai, and Sd fine-Chem ltd, Mumbai, respectively. All other chemicals used in the analysis were AR grade.

Chromatographic Conditions

Separation was achieved on a Symmetryc-C18-column (250 × 4.0 mm, 5 µm) using a gradient mode by the mobile phase consists of a mixture of Buffer and acetonitrile- methanol as equimolar mixture at 1:1 v/v with pH-3.2 adjusted with 10% o-phosphoric acid in water. The column was then re-equilibrated for 10 min with mobile phase. The flow rate of the mobile phase was 1.0 mL/min and the total elution time, including the column re-equilibration, was approximately 55 min. The UV detection wavelength was carried at 205 nm. The injection volume was 20 µL

and experiments were conducted at ambient temperature. Gradient program for acceptable resolution was given below (Table 1)

Table 1: Gradient program for acceptable resolution

Time (min)	Solvent- A (%)	Solvent B (%)
0.01	95	5
20	83	17
35	55	45
40	50	50
42	95	5
55	95	5
55.01	Controller	Stop

Preparation of System Suitability Solution (Standard Solution)

Standards of 2-Chloro adenosine as process related impurity (20 mg each) was accurately weighed, transferred into 20 ml volumetric flasks, dissolved in diluents and made up to the mark with the mobile phase to get 1000 ppm of 2-Chloro adenosine primary standard solution. Transfer the 2.0 ml of the above stock solution and was adequately diluted up to 20 ml in volumetric flask with mobile phase to get the concentration of 80 microgram/mL as working standard solution.

Preparation of Sample Solutions

Samples of Regadenoson powder for injection containing 2-chloro adenosine as process related impurity were prepared as by dissolving equivalent amount of 20 mg is dissolved in 25 ml volumetric flask containing diluents solution. Transfer the 2.0 ml of the above stock solution and was adequately diluted up to 20 ml in volumetric flask with mobile phase to get the concentration of 80 microgram/mL as working sample solution.

Method Validation

Validation of the developed method for the determination of 2-Chloro adenosine present as process related impurity in Regadenosone sterile powder for injection was performed according to the ICH guidelines with standards, and related substance. Thus, system suitability along with method selectivity, specificity, linearity, range, precision, accuracy, limits of detection and quantification for the impurities, short term and long term stability of the analytes in the prepared solutions and robustness were demonstrated.

System suitability:

The system suitability was conducted using 1.5% (w/w) of the impurity spiked to 2-chloro adenosine (80.0 µg/ml) and evaluated by making three replicate injections. The system was suitable for use if the tailing factors for 2-chloro adenosine and its impurity were <1.55 (observed value is 1.06) and the resolution was >1.90 (Observed value is 7.33).

Specificity:

Specificity of a method can be defined as the absence of any interference at retention times of peaks of interest, and is normally evaluated by observing the chromatograms of blank samples and samples spiked with the pure sample of process related impurity in the presence of other related substance and impurities in the mobile phase and the sterile powder for injection of Regadenoson. The known impurities were injected at specification limit and the retention times were tabulated along with retention time of 2-chloro adenosine. It was observed that there is no interference at retention time of 2-chloro adenosine peak. A separate solution of blank, standard and batch samples of 2-chloro adenosine were evaluated along with impurity solutions (Figure 2).

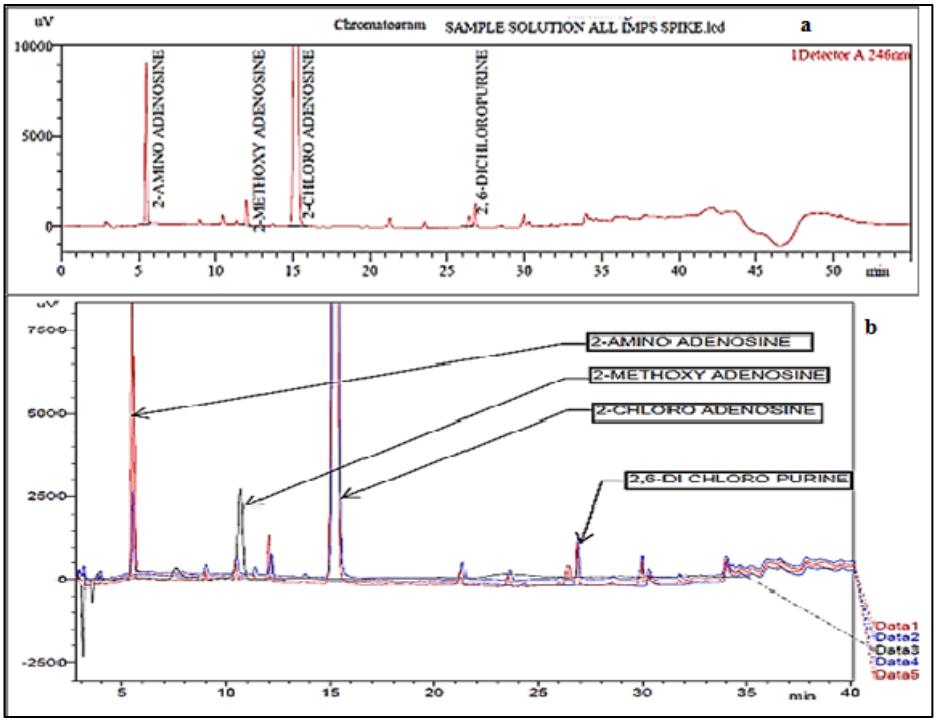

Figure 2: Estimation of specificity

Linearity and range:

The linearity of 2-chloro adenosine was studied by preparing standard solutions at five different levels ranging from 40 µg/ml to 120 µg/ml. The data were subjected to statistical analysis using a linear-regression model; the regression equations and coefficients (r^2) are given in calibration curves of 2-chloro adenosine and its impurity in Figure 3.

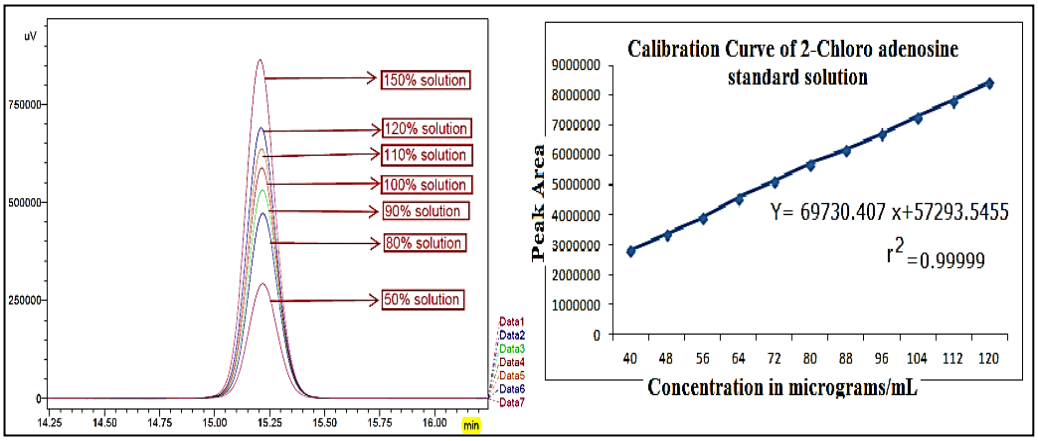

Figure 3: Calibration graphs of 2-chloro adenosine as process related impurity

Precision:

Precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested. The system precision was conducted using 80.0 micrograms/ml of the 2-chloro adenosine (i.e., 100% level) and evaluated by making six replicated injections. The method precision was conducted by using 10% (w/w) (i.e., 8 µg/ml) of the impurity spiked to 2-chloro adenosine (80 mg/ml) (i.e.,-100% level) and evaluated by making duplicate injections (Table 2).

Table 2: Precision studies of 2-chloroadenosise as impurity in regadenosine

No. of Injections	Peak Area	Retention time (min)
Inj-1	5256405	15.21
Inj-2	5255765	15.2
Inj-3	5255536	15.2
Inj-4	5254927	15.21
Inj-5	5253552	15.2
AVERAGE	5254945	15.20125
SD	993.7226307	0.0025
% RSD	0.02	0.02

Accuracy:

To determine the accuracy of the test method samples were prepared by spiking 2-chloro adenosine raw material with the equivalent amount of placebo at 50%, 75%, 100%, 125% and 150% of the target concentration. Six samples were prepared at lower and higher concentration levels and remaining levels in triplicate. The average % recovery of 2-chloro adenosine was found to be within the limits. The results were summarized in Table 3 (Figure 4).

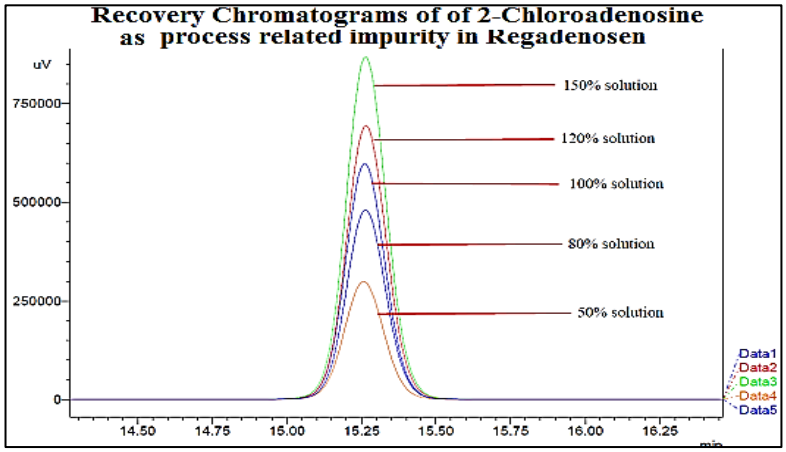

Figure 4: Accuracy studies of 2-chloroadenosine as process related impurity in regadenosen

Table 3: Accuracy studies of 2-chloroadenosine as process related impurity in regadenosen

Concentration in (MG/ML)	Dilution Level (%)	Spike Area	Stnd. Area	Amount Added	Amount Found	Recovery
0.04	50%	2880241	5255237	0.039525	0.041	102.1662343
		2897168	5255237	0.039525	0.041	102.772536
		2881458	5255237	0.039525	0.041	102.2098256
		2892466	5255237	0.039525	0.041	102.6041169
		2882482	5255237	0.039525	0.041	102.2465038
		2897995	5255237	0.039525	0.041	102.802158
0.06	80%	4621005	5255237	0.06324	0.065	102.4487402
		4626713	5255237	0.06324	0.066	102.5765231
		4624701	5255237	0.06324	0.065	102.5314812
0.08	100%	5747431	5255237	0.07905	0.081	102.9325
		5759667	5255237	0.07905	0.082	102.1516382
		5748976	5255237	0.07905	0.081	102.9601698
		5757604	5255237	0.07905	0.082	102.1146913
		5748371	5255237	0.07905	0.081	102.9493347
		5754368	5255237	0.07905	0.081	102..0567368
0.1	120%	6740123	5255237	0.09486	0.095	100.5924372
		6741562	5255237	0.09486	0.095	100.6139135
		6742171	5255237	0.09486	0.095	100.6230024
0.12	150%	8473809	5255237	0.118575	0.12	101.1733584
		8472305	5255237	0.118575	0.12	101.1554013
		8474348	5255237	0.118575	0.12	101.1797938
		8459743	5255237	0.118575	0.12	101.0054169
		8462886	5255237	0.118575	0.12	101.0429428
		8455534	5255237	0.118575	0.12	100.9551634

LOD and LOQ:

The LOD and LOQ were determined for 2-chloro adenosine and its process related impurity based on the standard deviation of (SD) of the response and slope (S) of the regression line as per ICH guidelines according to the formulae give below [11,12].

$$LOD = \frac{3.3 \times SD}{S}$$

$$LOQ = \frac{10 \times SD}{S}$$

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

To optimize the chromatographic conditions, different combinations of methanol–water (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70), acetonitrile–methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70), and acetonitrile–potassium dihydrogen phosphate buffer (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70) were tested. Acetonitrile: methanol (1:1 v/v)with phosphate buffer (pH 3.5) was preferred because it resulted in a greater response to 2-chloro adenosine and also to its related impurity after several preliminary investigatory runs compared with the other mobile phases. The composition, pH, and flow rate of the mobile phase were changed in gradient mode to optimize the separation conditions. Increasing the organic modifier content resulted in a decrease in the retention time of the analyte. The effect of the flow rate was studied in the range 0.8 to 1.2 mL/min and higher acetonitrile content high flow rate resulted, as expected, in rapid analysis before dead volume. A high buffer concentration was therefore used at a flow rate of 1.0 mL/ min, keeping in mind the possibility that potential minor degradation products could appear after stress studies and might co-elute with the drug because of the reduced RT if the flow rate was increased. High flow rates also reduce the life time of both column and pump. Changing the buffer pH from 3.5 to 5.5 (by addition of potassium dehydrogenate phosphate (0.01 M) or *ortho*-phosphoric acid) affected the RT and shape of the 2-chloro adenosine impurity peak. The effect of pH on analyte elution was related to the degree of ionization. Reducing the pH resulted in a shorter RT of process-related impurity because of ionization of its basic site. A pH of 3.2 was regarded as optimum because at this pH the analyte peak was sharp and well-defined. The preferred brand of HPLC column should be selected primarily based on the long term stability and lot-to-lot reproducibility. Preliminary development trials have performed with various octadecyl columns (C18 columns) of different types and dimensions from different manufacturers were tested for the peak shape and the number of theoretical plates of 2-chloro adenosine raw material at 500 µg/mL concentration. Finally by switching to Symmetry C18-3V column (250 mm × 4.6 mm, 5 µ) column there was a substantial increase in the theoretical plates (~50000) with a significant improvement in the peak shapes with 1.14 tailing factor. It also produced adequate resolution between 2-chloro adenosine and its two process related impurity. As a result, it was selected as an optimum one and used throughout this investigation.

Linearity

The calibration curve showed good linearity in the range of 40-120 µg/ml, for 2-chloro adenosine API and its related substance with correlation coefficient R^2 =of 0.9998 and 1.0000 respectively indicating strong correlation (Figure 3).

Precision

The results of system precision (% RSD=0.02 for peak area 0.47 for retention time of 2-chloro adenosine as process related impurity respectively), method precision (% RSD=1.4 and 1.0 for 2-chloro adenosine and its process related impurity respectively) are found within the prescribed limit of ICH guidelines (% RSD <1%, and % RSD <2% respectively in case of system precision and method precision.

Accuracy

The accuracy of the method was determined by measurement of recovery. The results of accuracy studies from standard solution and process related impurity were shown in Table 3; recovery values demonstrated that the method was accurate within the desired range. The results revealed an excellent correlation between the amount added and the amount found.

CONCLUSION

The HPLC method was developed using Symmetry C8 (250 mm × 4.6 mm ID × 5 µm) column with mobile phase and Transfer 2.0 mL of 85% ortho phosphoric acid into 1000 mL of water. Adjust the pH of the solution to 4.0 ± 0.05 with Triethylamine. Flow rate was 1.0 ml/min, with injection volume 50 µl detection done by using UV detector at 250 nm. The run time was 35 minutes which enables rapid quantitation of many samples in routine and quality control analysis of tablet formulations. The proposed method was found to be simple, sensitive, rapid and economical for the determination of Regadenosine parenterals. The developed method was checked for the performance characteristics and has also been validated. The method was validated by using various validation parameters like linearity, accuracy, precision, specificity, solution stability and robustness. The method was found to be linear (R^2>0.999) precise (%RSD: 0.02) and accurate (mean % recovery fields 99.1% - 103.00%). The proposed HPLC method was simple, precise because of commonly used buffer and shorter run time.

The mean percentage recovery above 95% indicates the reproducibility and accuracy of new developed method compared. The result of study include the proposed method is highly accurate, simple, precise and specific. The simple recoveries in all formulations were in good agreement with their respective label claims. After validating proposed method as per ICH guidelines and correlating obtained values with the standard values, satisfactory results were obtained. From the specificity studies it was concluded that the developed method was specific for 2-chloro adenosine.

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