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Research Article

DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RP-LC METHOD FOR THE ESTIMATION OF PROCESS RELATED IMPURITIES AND DEGRADATION PRODUCTS OF DIPYRIDAMOLE RETARD CAPSULES

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ABSTRACT

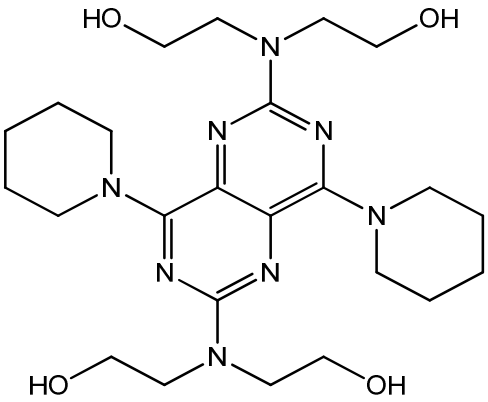
A novel stability-indicating gradient reverse phase liquid chromatographic method was developed for the quantitative determination of process related impurities and forced degradation products of Dipyridamole in Dipyridamole retard capsules. This method is able separate all seven known impurities which includes two newly identified potential degradant impurities.The method was developed by using Inertsil ODS-2 (150mm x 4.6mm) 5µm column with mobile phase containing a gradient mixture of solvent A (0.007 M potassium dihydrogen phosphate, pH adjusted to 7.0 with 5% sodium hydroxide solution) and solvent B (methanol).The flow rate was 1.0 mL/min with column temperature of 45°C and detection wavelength at 295nm. Dipyridamole was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Dipyridamole was found to degrade in all the condition where significantly degrade in acid, oxidative stress conditions and Humidity conditions. The degradation products were well resolved from Dipyridamole and its impurities, thus proved the stability-indicating power of the method. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

Keywords: Validation, Stability-indicating, Dipyridamole, Related substances, Degradation

INTRODUCTION

Dipyridamole is a platelet inhibitor chemically described as 2,2',2'',2'''-[(4,8-Dipiperidinopyrimido[5,4-d]pyrimidine-2,6-diyl) dinitrilo]-tetraethanol. (Fig. 1). Its empirical formula is $C_{24}H_{40}N_8O_4$, which corresponds to a molecular weight of 504.63. Its solid oral dosage form is available as capsule. Each modified release capsule contains Dipyridamole 200 mg.

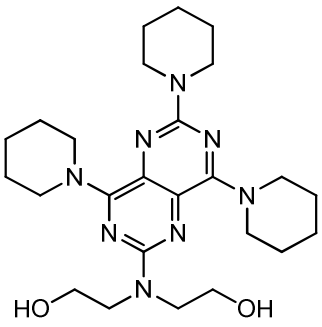
The recommended dose is one capsule twice daily, usually one in the morning and one in the evening preferably with meals. The capsules should be swallowed whole without chewing[1].



2,2,2,2-[[4,8-Di(piperidin-1-yl)pyrimido[5,4-d]pyrimidine-2,6-diyl]dinitrilo]tetraethanol

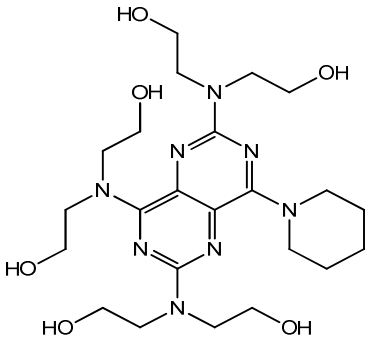
Fig. 1: It shows Structure and Chemical name of Dipyridamole

In the literature survey there were quite a few LC methods have been reported for determination of Dipyridamole in pharmaceutical preparation[2,3] and few method were reported for Dipyridamole and its degradation product[4]. However, several method were reported for determination of Dipyridamole in combination with other drug[5-7]. Estimation of Dipyridamole, and its metabolites in human plasma by LC-MS and HPLC has been performed[8-11].Beside the reported impurities, in these methods we have observed two other potential impurities (Impurity-F and Impurity-G) in our drug product during force degradation. The formation of the impurity-F has been rationalized in terms of dehydration of one mole of Dipyridamole with one mole of tartaric acid (Tartaric acid is used as one of the Excipient used in core pellets of formulation). Impurity-G is a hydrolysis degradant of Dipyridamole where pipyridine group is replaced by hydroxyl group. Tartaric acid adduct impurity (Impurity-F) and 4-Hydroxy impurity (Impurity-G) may increase during the shelf life of the drug product. So, the monitoring of these degradants is essential and very important. Hence, we have developed a stability indicating RP-LC method that can separate and determine the all seven impurities of Dipyridamole namely Impurity-A, Impurity-B, Impurity-C, Impurity-D, Impurity-E, Impurity-F and Impurity-G impurities [Fig. 2]. The developed LC method was validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness. Force degradation studies were performed on the placebo and drug products to show the stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines[14].



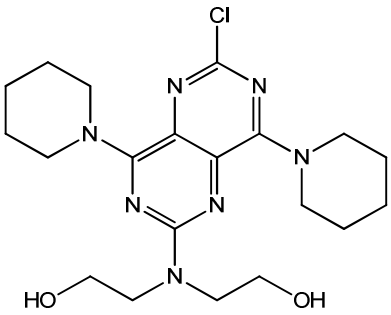
2,2-[[4,6,8-tri(piperidin-1-yl)pyrimido[5,4-d]pyrimidin-2-yl]nitrilo]diethanol

Impurity-A



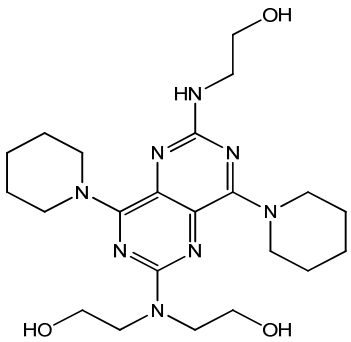
2,2,2,2,2,2[[8-(piperidin-1-yl)pyrimido[5,4-d]pyrimidine-2,4,6-triyl]trinitrilo]hexaethanol,

Impurity-B



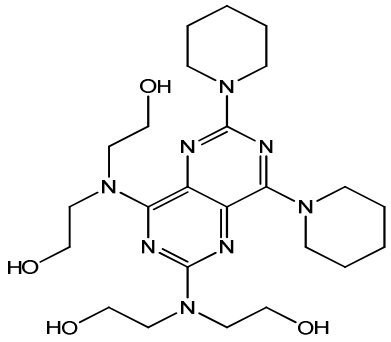
2,2-[[6-chloro-4,8-di(piperidin-1-yl)pyrimido[5,4-d]pyrimidin-2-yl]nitrilo]diethanol

Impurity-C



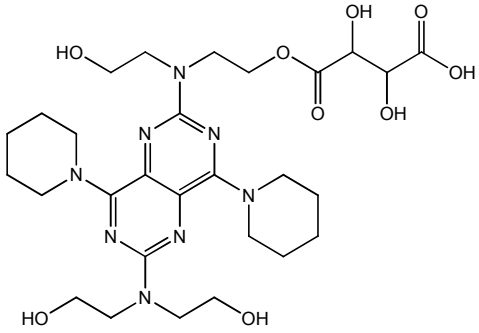
2,2-[[6-[(2-hydroxyethyl)amino]-4,8-di(piperidin-1-yl)pyrimido[5,4-d]pyrimidin-2-yl]nitrilo]diethanol

Impurity-D



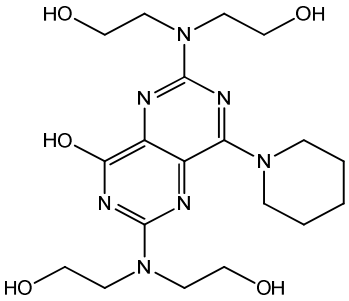
2,2,2,2-[[6,8-di(piperidin-1-yl)pyrimido[5,4-d]pyrimidine-2,4-diyl]dinitrilo]tetraethanol

Impurity-E



2,3-Dihydroxy-succinic acid mono-{2-[{6-[bis-(2-hydroxy-ethyl)-amino]-4,8-di-piperidin-1-yl-pyrimido [5,4-d]pyrimidin-2yl}-(2hydroxy-ethyl)-amino]-ethyl} ester

Impurity-F



2,6-Bis-[bis-(2-hydroxy-ethyl)-amino]-8-piperidin-1-yl-pyrimido[5,4-d]pyrimidin-4ol

Impurity-G

Fig. 2: It shows Structures and chemical name of Dipyridamole impurities

MATERIALS AND METHODS

Chemicals and reagents

Samples of Dipyridamole retard capsules and its impurities Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-G were supplied by Dr. Reddy's laboratories limited, Hyderabad, India. The HPLC grade acetonitrile, and analytical grade Potassium Dihydrogen phosphate and sodium hydroxide were from Merck, Mumbai, India. High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

Equipment

The chromatography analysis was performed using Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/visible detector or 2998 PDA detector (for specificity and forced degradation studies), degasser, quaternary pump and auto sampler system. The output signal was monitored and processed using Empower 2 software. Cintex digital water bath was used for hydrolysis studies. Photo-stability studies were carried out in photo-stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India). The pH of the solutions was measured by a pH meter (Mettler-Toledo, Switzerland).

Chromatographic Conditions

The method was developed using Inertsil ODS-2 (150 x 4.6 mm, 5µm) column with mobile phase containing a gradient mixture of solvent A (0.007 M Potassium dihydrogen phosphate buffer ,pH

adjusted to 7.0 with 5% sodium hydroxide solution) and Solvent B (Methanol). The gradient program (Time/%B) was set 00/50, 4/50, 25/95, 28/95, 30/50 and 35/50. The mobile phases were filtered through nylon 0.45 µm membrane filters and degassed in sonicator. The flow rate of the mobile phase was 1.0 mL/min. The column temperature was maintained at 45° C and the eluted compounds were monitored at the wavelength of 295 nm. The injection volume was 10 µl.

Preparation of system suitability Solution

Methanol and buffer (0.01M potassium dihydrogen phosphate, pH adjusted to 3.0 with ortho phosphoric acid) in the ratio of 60:40 v/v was used as diluents. A system suitability solution of Imp-A, Imp-B, Imp-C, Imp-D and Imp-E with a concentration of 3.2µg/mL and 1.6 mg/mL of Dipyridamole, was prepared by dissolving appropriate amount of drug in the diluent.

Preparation of Standard Solution

A standard stock solution (Stock A) of Dipyridamole were prepared in diluent with a concentration of 0. 8mg/mL. Working standard solution was prepared from above stock solution(stock A) by further dilution with diluent to get final concentration of 8µg/mL of dipyridamole.

Preparation of sample Solution

Mixed the content of 20 Capsule in mortal pestle. A content equivalent to 80 mg Dipyridamole was dissolved in diluents with sonication for 10 min to give a solution containing 1.6 mg/mL drug. This solution was centrifuged at 4000 rpm for 10 min.

RESULTS AND DISCUSSION

Method Development and Optimization

The main objective of the chromatographic method was to separate critical closely eluting compounds Imp-D and Dipyridamole, and to elute non-polar Imp-A with a shorter run time. The blend containing 1.6mg/ml of Dipyridamole, 8µg/ml Impurity-A, Impurity-B and Impurity-C, 3.2 µg/ml Impurity-D ,Impurity-E and Impurity-G and Impurity-F at 24 µg/ml was used for separation. An isocratic method employed using 0.01 M potassium dihydrogen phosphate (pH3.0) and acetonitrile in the ratio of 60:40 as mobile phase, Ace C18 (250 mm X 4.6 mm) 5µm column with flow rate of 1.5 mL/min on HPLC equipped with photo diode array detector. Imp-D peak was merged with Dipyridamole peak and Imp-A retained strongly. To resolve the imp-D peak and reduce the run time an attempt was made with gradient elution with mobile phase 0.01 M potassium dihydrogen orthophosphate buffer (pH 3.0) as solvent-A and acetonitrile as solvent-B using same Ace C18, 250mm x 4.6mm, 5µm column. Imp-D peak was slightly resolved but impurity-A is not eluted in shorter runtime. For shorter run time column length was reduced and to resolve imp-D from Dipyridamole, pH of mobile phase also drifted to basic side to see the impact. Solvent A 0.01M potassium dihydrogen phosphate pH changed to 7.0 and used methanol as solvent B,Inertsil ODS 2(50 mm X 4.6 mm) 5µm column with flow rate of 1.0 mL/min. On the optimization of gradient program, all peaks ware well resolved, satisfactory results were obtained also confirm the column lot to lot variation. Based on these experiments, the conditions were further optimized as described below.

Inertsil ODS-2, 150 mm x 4.6mm, 5µm was used as the stationary phase. The Solvent A consist of 0.007 M Potassium dihydrogen phosphate having pH 7.0 and Solvent B contained a methanol. The flow rate was 1.0 mL/min with a gradient program (Time/%B) was set 00/50, 4/50, 25/95, 28/95, 30/50 and 35/50. The column temperature was maintained at 45°C and detection was monitored at 295 nm. The injection volume was 10µL. Using the optimized conditions, impurity-D and Dipyridamole were well separated with resolution greater than 1.5 and the typical relative retention time of Imp-A, Imp-B,Imp-C,Imp-D,Imp-E,Imp-F and Imp-G were 1.54,0.52,1.29,0.97,1.16,0.68 and 0.24 respectively. The developed method was determined to be specific for Dipyridamole and all impurities.

Method Validation

The proposed method was validated as per ICH guidelines[12]. The following validation characteristics were addressed: specificity, accuracy, precision, linearity, range and robustness.

System suitability

System suitability was checked for the conformance of suitability and reproducibility of chromatographic system for analysis. The system suitability was evaluated on the basis of retention time, the resolution between impurity D and Dipyridamole peak, ratio of Dipyridamole peak areas from two standard injections and USP tailing factor for Dipyridamole peak from standard solution. System suitability was determined before sample analysis from two replicate injections of the standard solution containing 8µg/mL Dipyridamole. The acceptance criteria were ratio should be between 0.9 and 1.1 for Dipyridamole peak areas, USP tailing factor less than 2.0 for Dipyridamole peaks from standard solution and from system suitability solution, the resolution between impurity D and Dipyridamole peak should be not less than 1.5. All critical parameters tested met the acceptance criteria (Table 1).

Table 1: System suitability test results

Parameters	Specification	Observed values
Resolution between impurity D and Dipyridamole peak in system suitability solution	>1.5	2.5
The ratio of peak areas in two standard injections	Between 0.9 to 1.1	1.0
The USP Tailing factor for Dipyridamole peak	<2.0	1.0

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. A study was conducted to demonstrate the effective separation of Dipyridamole and its impurities. Also study was intended to ensure the effective separation of degradation peaks of formulation ingredients at the retention time of Dipyridamole and its impurities. Separate portions of drug product and ingredients were exposed to following stress conditions to induce degradation.

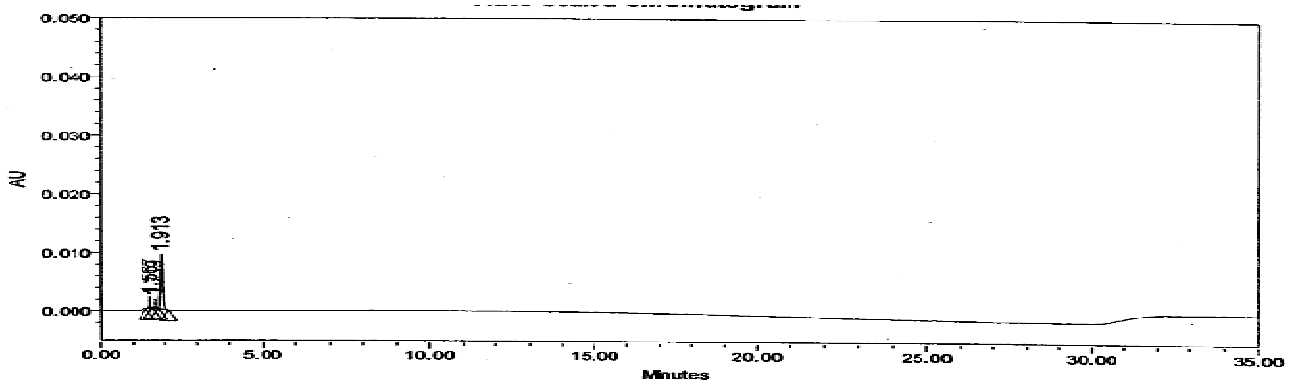
The drug product was subjected to base hydrolysis using 1 N Sodium hydroxide for duration of 27hrs at 60°C, acid hydrolysis with 1N Hydrochloric acid for duration of 2hrs at 60°C, Hydrolytic degradation performed at 60°C for 27hours. Oxidation study was performed with 1 % Hydrogen Peroxide solution at 60°c for 5 hours.

The photo stability study was carried out as per ICH Q1B where drug product was sufficiently spread on Petri plates (1 mm thick layer), exposed to sunlight (1.2Million Lux hours) and UV light (200 watt hours / square meter). Humidity study was performed separately by exposing the drug product to humidity at 25°C, 90% RH for 10 days.

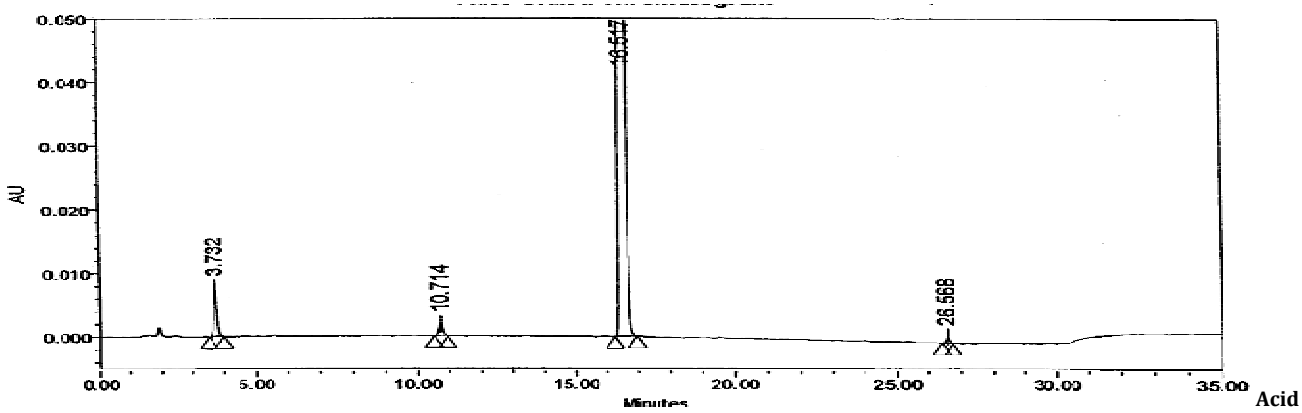
Thermal degradation study was performed by heating drug product at 65° C for 7 days. Similarly placebo samples were prepared as like as drug product by exposing formulation matrices without drug substance. Peak purity test was carried out for the Dipyridamole peak by using PDA detector in stress samples also Placebo interference was evaluated by analyzing the placebo equivalent amount present in sample, prepared as per test method. No peak due to placebo detected at the retention time of Dipyridamole and its impurities (Fig. 2A).

Table 2: Summary of forced degradation results

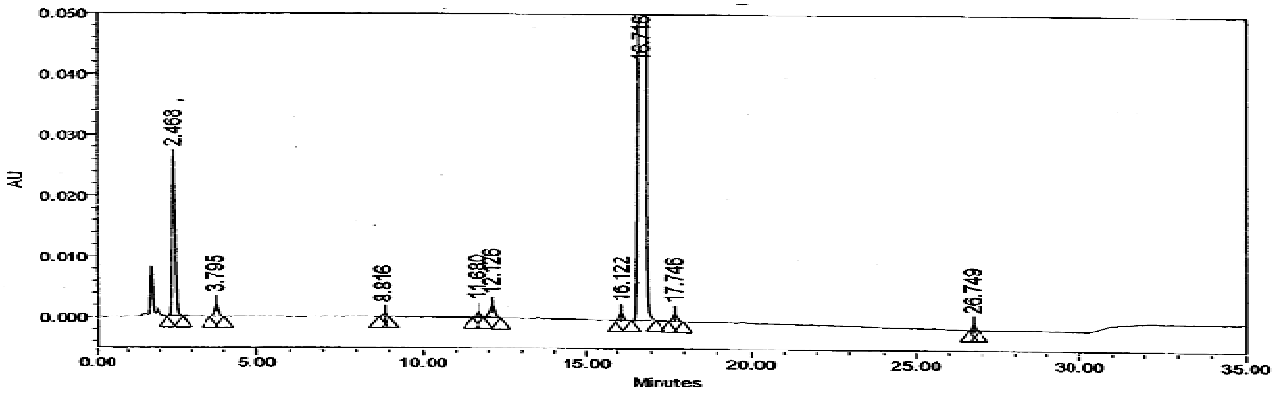
Stress Condition	% Degradation	Purity angle	Purity threshold	Purity flag
Acid hydrolysis (1 N HCl at 60°C, 2 hour)	1.18	0.034	0.257	No
Base hydrolysis (1 N NaOH at 60°C, 27 hrs)	0.20	0.028	0.250	No
Oxidation (1% H_2O_2 at 60°C, 5 hrs)	3.50	0.033	0.254	No
Thermal (At 65°C for 7 days)	0.28	0.029	0.253	No
Hydrolytic (Water at 60°C, 27 hrs)	0.98	0.032	0.254	No
Humidity (25°c,90%RH-10 days)	8.19	0.031	0.247	No
Photolytic Degradation (As per ICH Q1B)	0.33	0.033	0.255	No



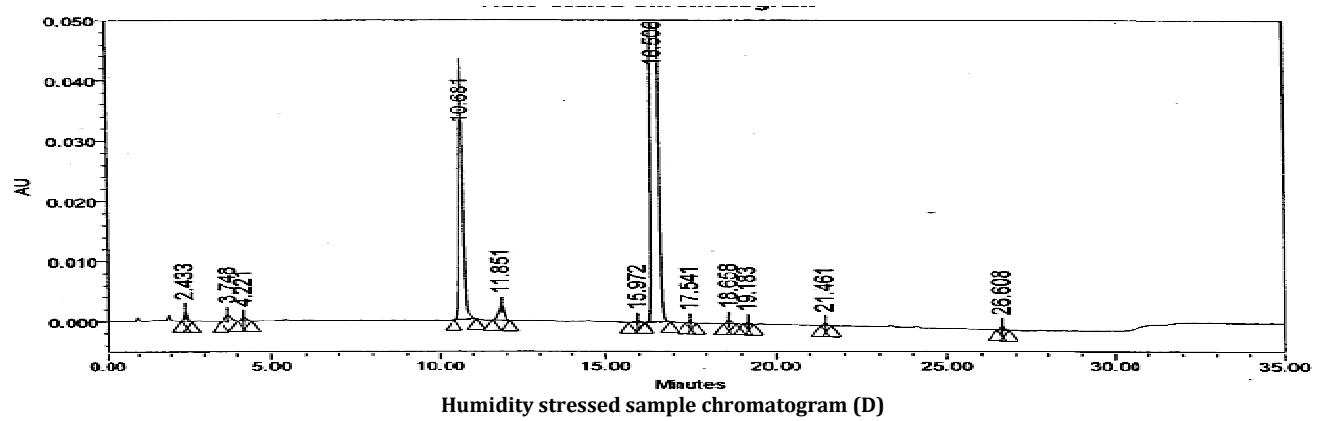
Placebo Chromatogram (A)



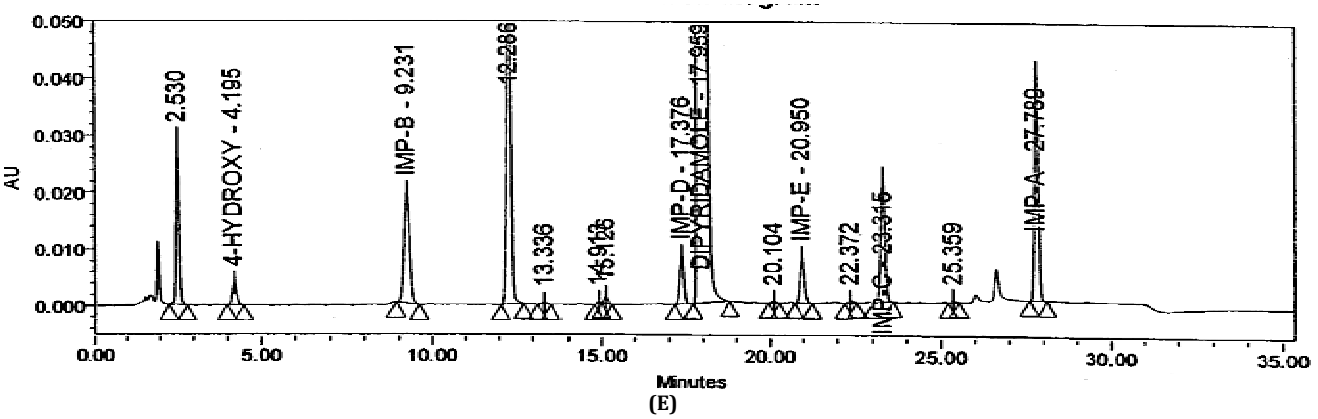
Degradation Chromatogram (B)



Oxidation degradation chromatogram (C)



Humidity stressed sample chromatogram (D)



(E)

Fig. 2: Typical chromatograms of (A) Placebo, (B-D) Forced degradation samples and (E) Dipyridamole test spiked with its impurities

Precision

The precision of method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual preparations of Dipyridamole retard capsule spiked with its seven impurities; Impurity-A, Impurity-B and Impurity-C at 0.50% level, Impurity-D ,Impurity-E and Impurity-G at 0.20% level, Impurity-F at 1.5% (% of impurities with respect to 1.6mg/mL Dipyridamole). %RSD of area for each impurity was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument and performing the analysis on different days.

The % RSD for the area of Impurity-A, Impurity-B, Impurity-C , Impurity-D ,Impurity-E ,Impurity-G and Impurity-F in repeatability study was within 3.1% and in intermediate precision study was within 0.6%, which confirms the good precision of the method. The %RSD values for each impurity are presented in Table 3.

Limits of Detection and Quantification

The LOD and LOQ for Dipyridamole impurities were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations of dipyridamole impurities and calculated the %RSD of the area. The determined limit of detection, limit of quantification and precision at LOQ values for Impurity-A, Impurity-B, Impurity-C, Impurity-D ,Impurity-E ,Impurity-G and Impurity-F are reported in Table 3.

Linearity

Linearity test solutions were prepared by diluting the stock solutions to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 200% of specification level (LOQ, 0.20%. 0.40%, 0.60%, 0.85% and 1.0% for impurity-A, impurity-B and impurity-C, LOQ, 0.08%. 0.16%, 0.24%, 0.35% and 0.40% for impurity-D, impurity-E and impurity-G and LOQ, 0.6%. 1.2%, 1.8%, 2.5% and 3.0% for impurity-F). Calibration curves were plotted between the responses of peak versus analyte concentrations. The correlation coefficient obtained was greater than 0.998 and %bias at 100% level are within ± 2%for all seven Impurities. The above result shows that an excellent correlation existed between peak area and concentration of Impurity-A, Impurity-B, Impurity-C, Impurity-D, Impurity-E, Impurity-G and Impurity-F. The coefficient correlation, slope and y-intercept of the calibration curve and bias at 100% response are summarized in Table 3.

Table 3: Linearity and precision data

Parameter	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G
LOD (µg/mL)	0.032	0.064	0.08	0.08	0.08	0.112	0.192
LOQ (µg/mL)	0.144	0.336	0.256	0.288	0.336	0.256	0.528
Correlation coefficient	0.99910	0.99863	0.99874	0.99851	0.99847	0.99843	0.99860
Intercept (a)	2730.77	28791.41	2212.91	292.74	872.48	6092.81	-644.59
Slope (b)	34198.45	28791.41	19312.38	20032.08	18226.61	19189.30	10771.96
Bias at 100% response	0.81	0.76	1.14	0.36	1.18	1.08	-1.54
Precision (%RSD)	0.6	3.1	0.5	0.5	0.5	1.1	0.5
Intermediate precision (%RSD)	0.6	0.5	0.5	0.4	0.4	0.6	0.3
Precision at LOQ (%RSD)	0.0	3.3	12.1	4.1	8.6	4.6	1.9

Accuracy

Accuracy of the method for Imp-A, Imp-B, Imp-C, Imp-D ,Imp-E, Imp-F and Imp-G were evaluated in triplicate using six concentration levels LOQ, 4.0, 6.0,8.0, 10.0 and 12.0µg/mL for Imp-A, imp-B, Imp-C and LOQ, 1.6, 2.4, 3.2, 4.0 and 4.9 µg/mL for Imp-D, Imp-E and Imp-G, for Imp-F level at 2.4,12.0,17.8,23.8 and 35.7 µg/mL. The percentage recoveries for all seven impurities were calculated and varied from 88.2 to 111.1. The LC chromatogram of spiked sample at 0.5% level for Imp-A, Imp-B and Imp-C, 0.2% level Imp-D, Imp-E, Imp-G and 1.5% level Imp-F in Dipyridamole retard capsule sample is shown in Fig. 2F. The recovery values for all seven Dipyridamole impurities are presented in Table 4.

Table 4: Recovery data

Amount spiked [a]	% Recovery [b]						
	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G
LOQ	89.6 ± 0.0	96.9 ± 6.0	89.1 ± 0.0	101.9 ± 3.5	104.1 ± 10.2	103.0 ± 3.8	95.4 ± 5.4
50%	105.1 ± 0.8	94.7 ± 1.1	102.1 ± 0.5	96.8 ± 1.0	106.8 ± 0.9	88.2 ± 0.5	92.9 ± 6.0
75%	99.3 ± 0.4	97.9 ± 0.2	101.1 ± 0.2	101.5 ± 0.3	99.1 ± 0.4	101.0 ± 0.8	97.2 ± 9.2
100%	99.2 ± 0.1	97.3 ± 0.1	102.1 ± 0.2	100.0 ± 0.3	101.1 ± 0.3	99.5 ± 0.0	111.1 ± 0.5
125%	100.9 ± 0.8	96.3 ± 0.6	101.9 ± 0.6	100.1 ± 0.6	100.0 ± 0.8	87.8 ± 0.1	106.3 ± 0.0
150%	99.6 ± 0.8	97.9 ± 0.5	101.9 ± 0.7	101.2 ± 0.7	100.7 ± 0.7	92.9 ± 0.6	94.7 ± 3.8

[a] Amount of seven impurities spiked with respect to specification level(imp-A, imp-B, Imp-C at 0.5% ,imp-D, imp-E and Imp-G at0.2%and imp-F at 1.5%level)

[b] Mean ± %RSD for three determinations

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Imp-D and Dipyridamole; and system suitability parameters for Dipyridamole standard were recorded. The variables evaluated in the study were pH of the mobile phase buffer, column temperature, and flow rate. The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the resolution, flow was changed from 0.8 to 1.2 mL/min. The effect of pH of mobile was studied at 6.8 to 7.2 instead of 7.0 in solvent A. The effect of the column temperature on resolution was studied at 40°C and 50° C instead of 45° C. In all the deliberate varied chromatographic conditions, all analytes were adequately resolved and elution order remained unchanged.

The resolution between critical pair, i.e. for imp-D and Dipyridamole was greater than 2.3 and tailing factor for dipyridamole peak from standard solution was 1.0 and the ratio of peak areas was 1.0. The system suitability parameters evaluated are shown in Table 5.

Table 5: Robustness results of HPLC method

Variation in chromatographic condition	Observed system suitability parameters		
	Resolution[a] > 1.5	USP Tailing for Dipyridamole peak < 2.0	The ratio of peak areas[b] >0.9-1.1<
Column Temperature 40°C	2.5	1.0	1.0
Column Temperature 50°C	2.5	1.0	1.0
Flow rate 0.8 mL/min	2.3	1.0	1.0
Flow rate 1.2 mL/min	2.8	1.0	1.0
Solvent A pH 6.8	2.6	1.0	1.0
Solvent A pH 7.2	2.6	1.0	1.0

[a] Resolution between impurity-D and Dipyridamole peak from system suitability solution.

[b] The ratio of peak areas in two standard injections

Stability in Solution and in the Mobile Phase

The solution stability of Dipyridamole and its impurities was determined by keeping test solution and standard solutions in tightly capped volumetric flasks at room temperature for 48 hrs and measured the amount of seven impurities at every 24 hrs against freshly prepared standard solution. The stability of mobile phase was also determined by freshly prepared solutions of Dipyridamole and its impurities at 24 hrs interval for 48 hrs. The mobile was not changed during the study. The variability in the estimation of all seven Dipyridamole impurities was within ± 10% during solution stability and mobile phase stability. The results from solution stability and mobile phase stability experiments confirmed that mobile phase was stable up to 48 hrs and sample solution and standard solutions were stable up to 24 hrs and 48 hrs, respectively on bench top.

CONCLUSIONS

A novel simple and efficient reverse-phase HPLC method was developed and validated for quantitative analysis of Dipyridamole impurities which includes potential impurities of Dipyridamole retard capsule pharmaceutical dosage forms. The method found to be precise, accurate, linear, robust and rugged during validation. Satisfactory results were obtained from the validation of the method. The method is stability indicating and can be used for routine analysis of production samples and to check the stability of the Dipyridamole retard capsules[13].

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Authors' Statements

Competing interests

The authors declare no conflict of interest.

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