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ANALYTICAL METHOD DEVELOPMENT & VALIDATION FOR RELATED SUBSTANCES IN DIPYRIDAMOLE BY RP-HPLC

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ABSTRACT: Simple RS method is developed and validated as reversed-phase chromatographic method for the identification and quantification of the dipyridamole related substances-A, B, C, D, E and F. Chromatographic separation has been achieved by using Shodex C18, 150 mm, 4.6 mm diameter, 5 µ column, using mobile phase 0.1 % of formic acid and acetonitrile by eluting in gradient with 1.0 ml flow, detection was achieved at 254 nm by maintaining 25 °C temperature for column. The method is validated as per the ICH guidelines. Linearity was recorded at various concentrations ranges 0.0100 - 6.0051 ppm for related substances A, B, C & 0.0040 - 2.4024 ppm of related substances D, E, F. Recovery RSD value of each related substance was <5.0 % (n=9). RS method for related substances in dipyridamole is found specific, linear, accurate, precise, rugged and robust hence the validated method is suitable to identify the related substances in dipyridamole drug.

INTRODUCTION: Dipyridamole is chemically a derivative of pyrimido-pyrimidine nuclei, which has been developed to treat blood clot aggregation through the anti-platelet property by inhibiting platelets and endothelial adenosine uptake and inhibits the stimulation of both platelet-activating and collagen factors by triggering an accretion of cyclic adenosine monophosphate (cAMP) [1]. Thorough literature reveals that only a few related substance analytical methods for dipyridamole and its related substances were reported.

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The purity evaluation method for dipyridamole [2] and demonstration of a regulatory requirement on analytical method development for drug and its related substances [3]. Dipyridamole was identified by photodecomposition and HPLC analytical methods in human plasma [4-10].

Monitoring of related substances below the threshold limit in a drug substance is important because the presence of related substances in small quantities could influence drug efficacy and safety. Owing to this, Importance has given to identify and quantify the Pharmacopoeial related substances in Dipyridamole, we developed an RS method to distinguish and regulate the related substances of Dipyridamole namely related substance-A, B, C, D, E and F [11] **Fig. 1**. Developed RS method was validated to parameters accuracy, precision, LOD, LOQ, specificity, robustness and linearity.

Degradation was completed on dipyridamole related substances to confirm the method for its stability-indication [12]. Analytical studies are achieved according to the ICH guidelines [13-17].

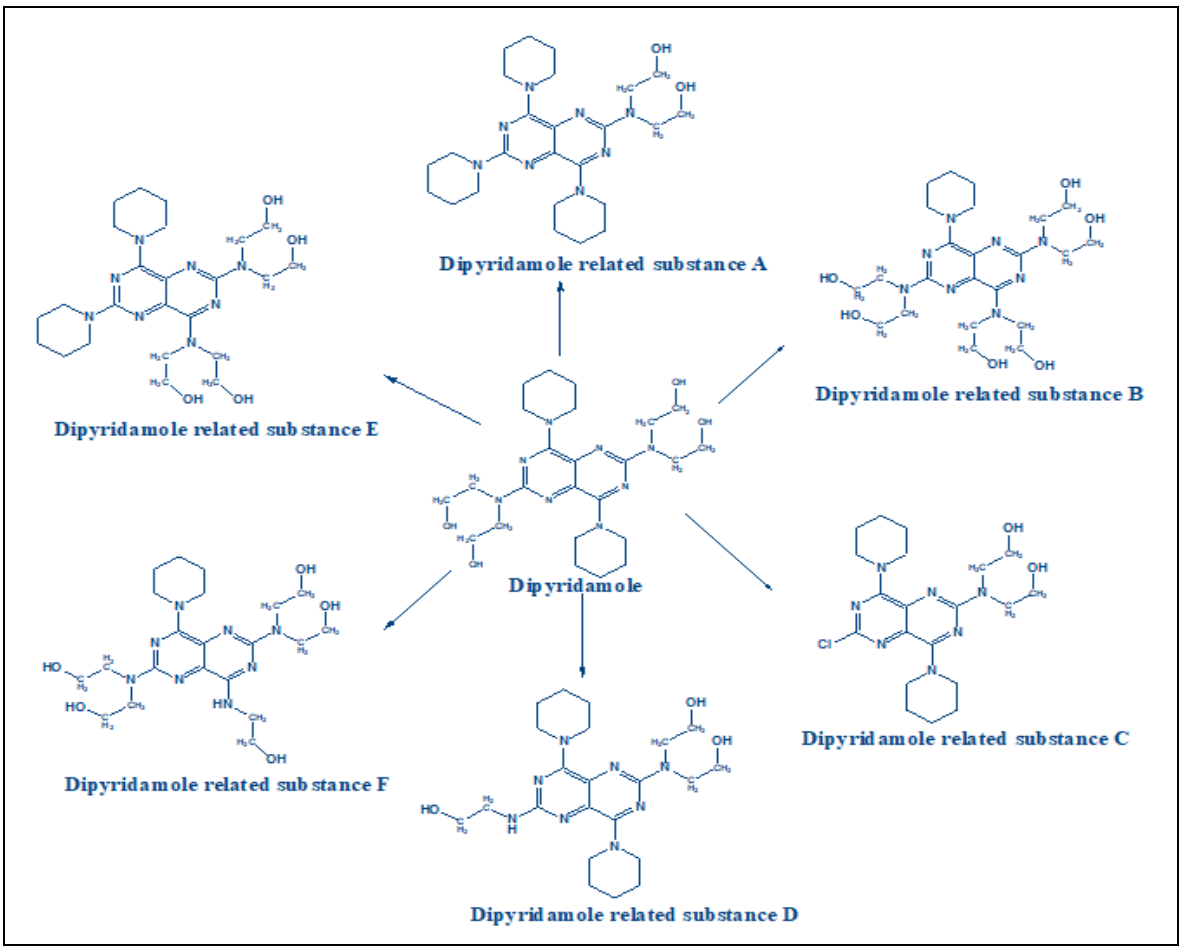

FIG. 1: CHEMICAL STRUCTURE OF DIPYRIDAMOLE AND ITS RELATED SUBSTANCES

MATERIALS AND METHODS: Dipyridamole and related substances - A, B, C, D, E and F were bought from PADM Laboratories Pvt. Ltd. Bangalore. Analytical grade acetonitrile was procured from standard reagent, Bangalore Milli-Q water purifying system by PADM lab (Bangalore). Analytical grade Formic acid was purchased from the Venus lab (Bangalore). Agilent 1100 HPLC linked with PDA detector, degasser, quaternary pump, auto-injector and column oven, data acquisition by Empower-2 software. The Shodex C18, 150 mm, 4.6 mm diameter; 5 µm column. Test and standard solutions were sonicated by sonicator (Branson's). Photostability was done in Mack Pharma tech. Thermal studies were done by Geotech dry air oven (Geotech).

Preparation of Standard Solution: Standard of dipyridamole 100 µg/ml stock was prepared using methanol as diluent. From the above stock solution working standard solution of 20 µg/ml was prepared using the diluent methanol. A concentration of 50 µg/ml related substances A, B and C, 20 µg/ml concentration of D, E and F prepared from a stock solution of 100 µg/ml for the identification of related substances. The spiked sample solution was prepared by mixing dipyridamole and each individual related substance concentration of 400 and 0 .4 µg/ml, respectively.

Optimization of Analytical Method: Gradient programme with flow of 1.0 ml/min, 25 °C for column temperature maintained and Gradient mixture of 0.1% Formic acid (phase-A) and acetonitrile (phase-B). Flow set as 0-2, 2-3.5, 3.5-5, 5- 7, 7- 10, 10- 15, 15- 18, 18- 20, 20- 25, 25-28, 28-30, 30-33, 33-35, 35-37 min and gradient as 95% A and 5% B, 90% A and 10% B, 85% A and 15% B, 75% A and 25% B, 65% A and 35% B, 55% A and 45% B, 50% A and 50% B, 40% A and 60% B, 30% A and 70% B, 20% A and 80% B, 30% A and 70% B, 50% A and 50% B, 80% A and

20% B, 95% A and 5% B respectively. Injection volume 40 µl of the sample was injected at 25 °C and the analytes were recorded at 254 nm wavelength.

Method Validation: As per the ICH guidelines, validated the developed method for the parameters-specificity, precision, linearity, range, accuracy, robustness, forced degradation and solution stability [11-13].

Specificity: Weigh accurately about 2.07 mg of Dipyridamole related substances A, B and C into the standard flask (10 ml) add methanol to final volume and mix, dilute 5 ml stock solution into the volumetric flask (10 ml) and diluted with methanol. Weigh accurately about 2.01 mg of dipyridamole related substances D, E and F into the volumetric flask (10 ml) add methanol to final volume and mix, dilute with 2 ml of stock in 10 ml volumetric flask.

Precision: Replicates of six injections of the standard were injected into chromatographic system. The single batch homogenous sample was analyzed in 6 times to specify the method reliability results for a single batch. Six test solutions of the single batch were analysed as per the proposed methodology.

Linearity: Performed the linearity with dipyridamole standard and its known related substances and range of LOQ to 300% related substance limit. Weigh accurately 2.01 mg of dipyridamole standard in a volumetric flask (20 ml), transfer 3 ml of methanol, sonicate for 3 minutes, add methanol to the final volume and mix. Weigh accurately 2.07 mg of each dipyridamole related substances into the individual volumetric flask (10 ml), transfer 3 ml of methanol, sonicate for 3 minutes, add methanol to final volume and mix. Pipette out 0.8 ml stock solution from standard, 2 ml from related substance-A, B and C standard stock and 0.8 ml from related substance-D, E and F standard stock into 10 ml volumetric flask and mixed thoroughly.

Final dilutions of 12 linearities standard solution of concentration ranging from 0.0020 to 1.2012 µg/ml, related substance concentration 0.0100 to 6.0051 µg/ml for A, B, C and 0.0040 to 2.4024 µg/ml for D, E, F and G.

LOD and LOQ Level: LOD: Detection of a lower level of an analyte in a sample that can be detected as per the developed chromatographic conditions. LOQ: Quantification at the lowermost of analyte in a sample shall be quantified with acceptable accuracy and precision in the developed chromatographic conditions. Calculated slope, intercept and correlation coefficient and the residual standard deviation from the linearity curve and recorded the results for a limit of detection and quantification formula mentioned.

$$LOD = 3.3 \text{ (Residual Standard deviation)} / \text{slope}$$

$$LOQ = 10 \text{ (Residual Standard deviation)} / \text{slope}$$

Accuracy: Performed by a known amount of dipyridamole standard into the diluents at LOQ level, 50%, 100%, 200% and 300% with respect to the concentration of sample solution. Accurately weigh about 2.01 mg of dipyridamole into volumetric flask (20 ml) transfer methanol and diluted with methanol. Further, dilute 2 ml of stock with 10 ml methanol and mix. Weigh accurately about 2.07 mg of each dipyridamole related substances into the individual volumetric flask (10 ml), transfer methanol, mix and final volume make up with methanol. Further, dilute 5 ml of standard stock into 10ml flask respectively and final volume made with methanol. Weigh accurately about 2.01 mg of each dipyridamole related substances into individual volumetric flask (10 ml) transfer methanol, mix and final volume make up with methanol. Further, dilute 2 ml of methanol to 10ml flask and mix.

Robustness: Robustness was verified by checking the system suitability parameters by deliberately varying the parameters (± 0.2) such as Flow rate, detector wavelength, and column temperature. The sample solution was analysed under each condition. Standard and sample were arranged according to the method of analysis.

Solution Stability: Injected the standard solution as per the method at different intervals up to 39 hours at 25 ± 2 °C (room temperature).

Forced Degradation Study: Degradation study conducted by exposing dipyridamole solution to acidic (2 ml of 5N Hydrochloric acid, further cooled and neutralized with 2 ml of 5N sodium

hydroxide), basic (2 ml of 5N Sodium Hydroxide solution, further cooled and neutralized with 2 ml of 5N HCl), oxidizing agent (2 ml of 30% Hydrogen Peroxide), Thermal (105 for 48 h), humidity (25 °C/90% RH for 7 days), Photolytic and Neutral (2 ml of H_2O at Room temperature for 30 min) conditions.

RESULTS AND DISCUSSION: The purpose was to develop a new RS method to identify and separate close eluting dipyridamole related substances like D, E, F and C with dipyridamole. To elute related substance A in a smaller run time. To achieve the objective, different methods were adopted by considering variables like column, mobile phase composition and elution mode, the optimization process was tabulated in **Table 1**. All the analytes are detected at 254 nm, well resolved and system suitability chromatogram shows method specificity **Fig. 2** hence the developed method is applied for the study.

Specificity chromatograms with purity plot and purity threshold for dipyridamole related substance A-F **Fig. 3** respectively, indicating no interference of blank and diluents with the retention time of dipyridamole peak summarised in **Table 2**.

TABLE 1: METHOD DEVELOPMENT DETAILS WITH DIFFERENT VARIATIONS IN CHROMATOGRAPHIC CONDITIONS

Trial no.	Column	Mobile phase composition	Elution mode	Result
1	Shodex ODS C-18, 150×4.6-mm, 5microns at Temp. 45 °C	Buffer: Formic acid (0.1%)-Methanol, 50:50 v/v	Isocratic	Related substances are coeluted
2	Shodex ODS C-18, 150×4.6-mm, 5microns at Temp. 45 °C	Buffer: Dihydrogen Potassium Phosphate-Methanol	Gradient	Related substance A and C are merged, and Related substance F and Dipyridamole coeluted
3	Inertsil ODS C-18, 4.6-mm, 5 microns at Temp. 45 °C	Buffer: Formic acid (0.1%)-Acetonitrile	Gradient	Related substance B and F are merged, and split peaks observed in Related substance E
4	Hypersil-BDS C-18, 4.6-mm, at Temp. 45 °C	Buffer: Formic acid (0.1%)-Methanol	Gradient	Out of six Related substances two Related substances are resolved, remaining are not
5	Inertsil ODS-3V C-18, 4.6-mm, at Temp. 45 °C	Buffer Formic acid (0.1%)-Methanol 40:60 v/v	Isocratic	Related substances are not separated

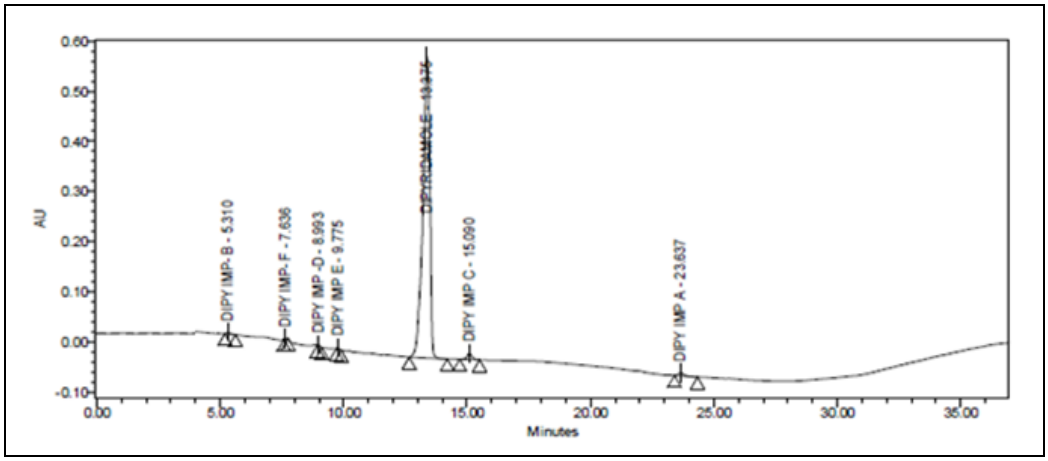

FIG. 2: SYSTEM SUITABILITY CHROMATOGRAM OF DIPYRIDAMOLE AND RELATED SUBSTANCE A, B, C, D, E AND F

System precision, the retention time and responses are reliable as evidenced by RSD (NMT 1.0% and NMT 5.0% respectively). Hence, the system precision meets the condition for the validated method and from the reliable results concluded that method is precise.

Statistical calculation of linearity data shows the responses of dipyridamole, and the related substances are linear at LOQ level to 300% level of specification limit and results are listed in **Table 3**. The R-square of dipyridamole and the related substances are 0.99 **Fig. 4**.

Validated method is precise for related substances in Dipyridamole and concluded at LOQ level and it is proved that dipyridamole and related substance A, B, C, D, E and F are precise at LOQ Level and distinctly visible peak was observed at LOD level concentration and summary of all validated parameters are listed in **Table 4**.

TABLE 2: SPECIFICITY RESULTS OBTAINED FOR PEAK PURITY OF STANDARD SOLUTION AND SAMPLE SOLUTION

Sample Details	RT	RRT	Purity Angle	Purity Threshold	Purity Flag (tick mark)
Dipyridamole	13.388	NA	0.092	0.280	No
Dipyridamole Related substance A	23.643	1.77	0.048	0.262	No
Dipyridamole Related substance B	5.236	0.39	0.088	0.288	No
Dipyridamole Related substance C	14.913	1.11	0.147	0.274	No
Dipyridamole Related substance D	8.982	0.67	0.129	0.309	No
Dipyridamole Related substance E	9.817	0.73	0.245	0.533	No
Dipyridamole Related substance F	7.784	0.58	0.079	0.287	No

RT: retention time. RRT: relative retention time. NA: not applicable

TABLE 3: LINEAR REGRESSION DATA

Linearity Parameter	Standard	Rel. sub. A	Rel. sub. B	Rel. sub. C	Rel. sub. D	Rel. sub. E	Rel. sub. F
Con. Range	0.0020-1.2012 ppm	0.0100-6.0051 ppm	0.0100-6.0051 ppm	0.0100-6.0051 ppm	0.0040-2.4024 ppm	0.0040-2.4024 ppm	0.0040-2.4024 ppm
Corr. coefficient	0.999	1.000	0.999	0.999	1.000	1.000	0.999
R-Square	0.998	0.999	0.999	0.999	0.999	0.999	0.999
Slope	36229.823	35109.125	10706.446	60350.004	19464.731	13278.276	12446.709
% Intercept	-2.9	-1.0	1.2	-1.3	-3.0	-2.3	-2.3

Rel. sub: related substance. Con: concentration. Corr. correlation

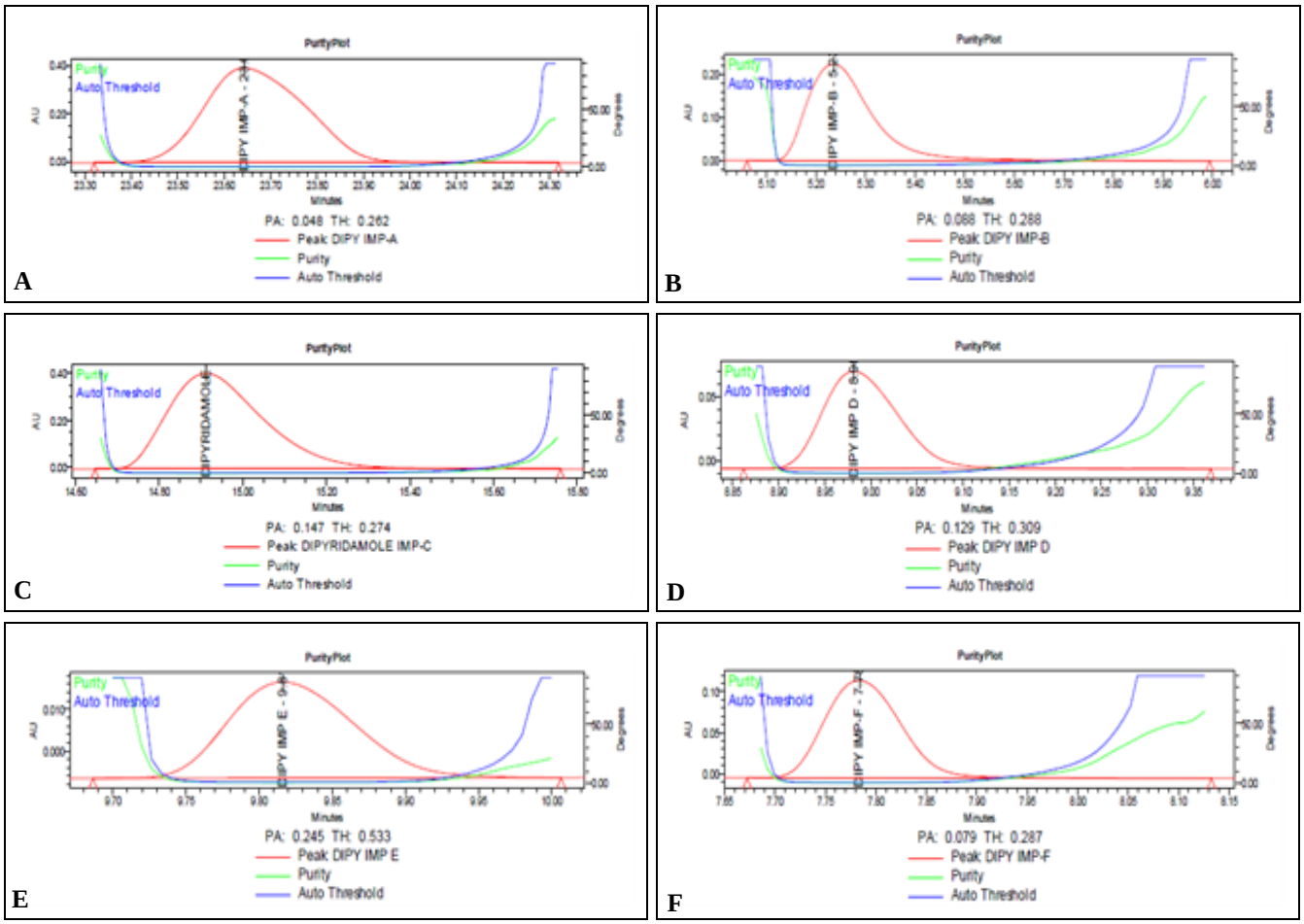

FIG. 3: PURITY PLOTSAND PURITY THRESHOLD OF DIPYRIDAMOLE RELATED SUBSTANCES. a. Related substance A; b. Related substance B; c. Related substance C; d. Related substance D; e. Related substance E; f. Related substance F

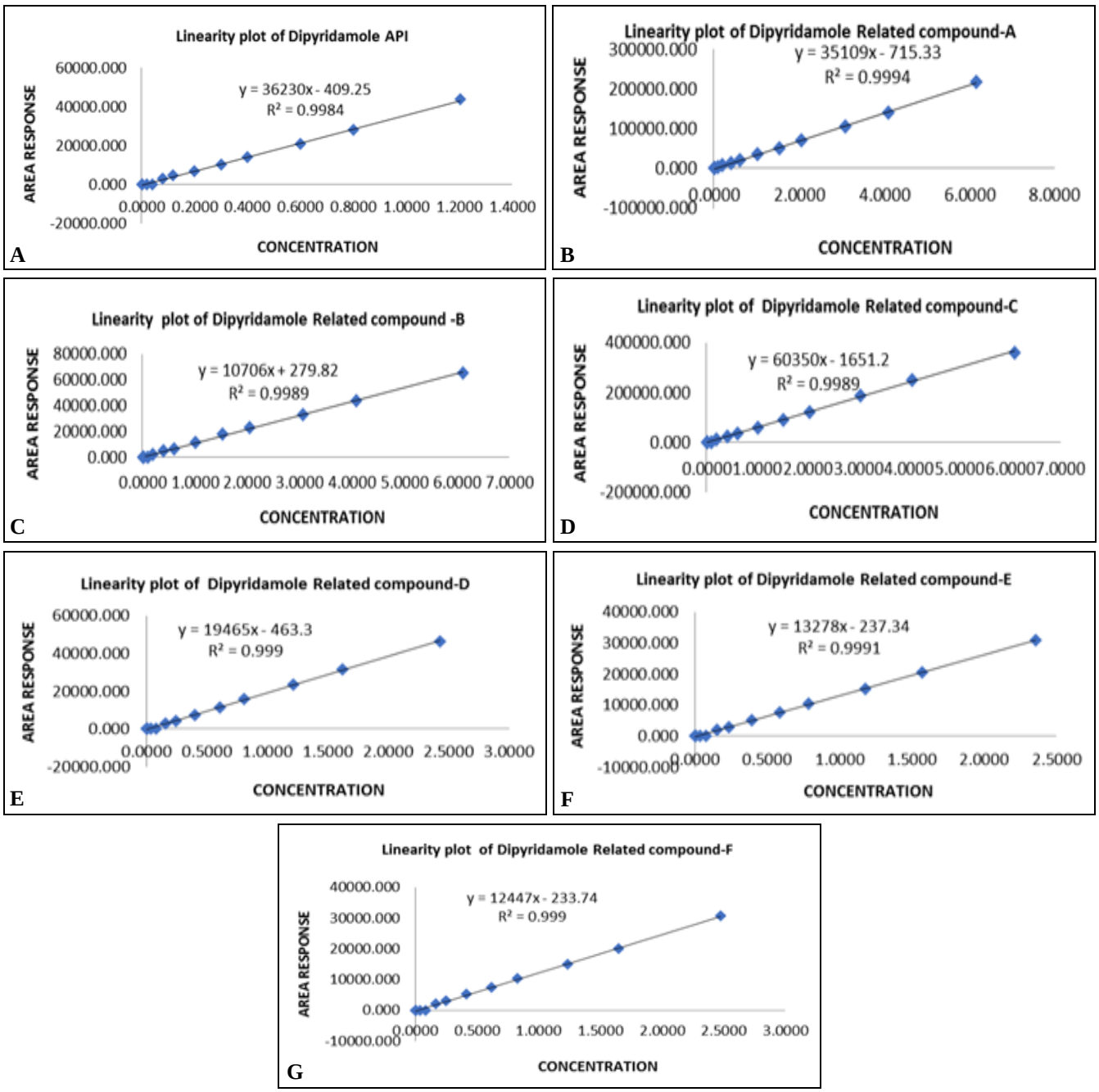

FIG. 4: LINEARITY PLOT OF DIPYRIDAMOLE AND RELATED SUBSTANCES. a. For dipyridamole b. Related substance A; c. Related substance B; d.Related substance C; e. Related substance D;f. Related substance E; g. Related substance F

TABLE 4: SUMMARIES OF VALIDATION PARAMETERS

Parameters (units)	Dipyridamole	Dipyridamole Related substances					
		A	B	C	D	E	F
Method Precision (%RSD)	3.7	3.7	3.8	4.4	2.0	3.3	2.5
Precision at Higher Level (%RSD)	1.2	1.9	1.0	1.0	1.2	0.9	1.5
Precision at LOQ Level (%RSD)	3.4	1.7	4.3	3.3	2.5	2.9	3.5
LOD (µg/ml)	0.06	0.08	0.13	0.11	0.09	0.09	0.10
LOQ (µg/ml)	0.17	0.24	0.39	0.33	0.27	0.28	0.29
Range (Accuracy/recovery) correlation- coefficient	1.000	1.000	0.999	1.000	1.000	1.000	1.000
Range (Linearity) Correlation coefficient	1.000	1.000	1.000	0.999	1.000	1.000	1.000
RRF	1.00	0.97	0.30	1.67	0.54	0.38	0.34

LOQ: limit of quantitation. LOD: limit of detection. RRF: relative response factor

Recovery results are found in the limit for dipyridamole, related substance-A, B, C, D, E, and F respectively, hence the developed method is accurate, tabulated the % mean recovery and % RSD results are listed in **Table 5**.

TABLE 5: ACCURACY RESULTS

Sample	% Level				
	LOQ	50%	100%	200%	300%
Dipyridamole					
% Mean Recovery	99.1	98.3	101.7	100.4	102.0
% RSD	1.6	2.9	3.8	1.9	0.5
Imp-A					
% Mean Recovery	101.4	101.7	106.0	100.7	99.4
% RSD	2.4	3.0	4.7	4.9	4.3
Imp-B					
% Mean Recovery	102.6	100.7	102.9	97.9	102.0
% RSD	2.6	4.2	4.5	4.2	4.1
Imp-C					
% Mean Recovery	100.0	99.7	103.1	103.5	103.1
% RSD	0.0	0.6	6.5	4.2	2.8
Imp-D					
% Mean Recovery	96.3	100.0	104.7	104.7	103.8
% RSD	0.0	0.0	1.8	1.0	4.2
Imp-E					
% Mean Recovery	98.8	100.0	102.6	103.6	101.7
% RSD	4.2	2.6	3.8	1.9	2.1
Imp-F					
% Mean Recovery	93.1	97.4	100.4	99.6	101.6
% RSD	0.0	2.6	2.0	2.4	1.7

Accuracy evaluated for five concentration levels of LOQ, 50, 100, 200 and 300 % sample test level

TABLE 6: ROBUSTNESS PARAMETERS AND RESULTS

Parameter	Tailing	Plate Count	% RSD
PlusFlow (1.2 ml/min)	0.9	20742	1.52
Minus Flow (0.8 ml/min)	0.9	19449	0.71
Plus Temperature (30 °C)	0.9	19948	1.92
Minus Temperature (20 °C)	0.8	18327	1.69
Plus Wavelength (256 nm)	0.9	10391	1.69
Minus Wavelength (252 nm)	0.9	8216	2.0

Deliberate conditions like Plus Flow (1.2 ml/min), Minus Flow (0.8 ml/min), Plus Temperature (30 °C), Minus Temperature (20 °C), Plus Wavelength (256 nm) and Minus Wavelength (252 nm).

Robustness of the method is studied for all the deliberate conditions like Plus Flow (1.2 ml/min), Minus Flow (0.8 ml/min), Plus temperature (30 °C), Minus temperature (20 °C), Plus wavelength (256 nm) and Minus wavelength (252 nm) all related substances are well separated with the variations of robust parameters and results were listed in **Table 6**.

Forced degradation study was performed for acid, humidity, photolytic, base, oxidation, thermal and neutral, results obtained are given in **Table 7**. The purity angel and purity threshold shows that there are no co-eluting peaks for dipyridamole in all degradation conditions. Mass balances show that the developed method is specific.

TABLE 7: FORCED DEGRADATION RESULTS FOR DIPYRIDAMOLE

Degradation mode	Condition	Purity Angle	Purity Threshold	Mass Balance
Control	NA	0.092	0.280	100
Acid stress test	2 ml, 5 N HCl for 30 min	0.104	0.275	90.75
Humidity stress test	25 °C/90% RH for NLT 7 days	1.616	0.397	92.85
Photolytic stress test	1.2 mill. Lux hrs / 200-wat. hrs/ sq.m	0.104	0.295	94.39
Base stress test	2 ml, 5N NaOH for 30 min	0.408	0.929	95.91
Oxidation stress test	2ml, 30% v/v H_20_2 for 30 min	0.092	0.271	91.40
Thermal stress test	105°C for 48 h	0.090	0.289	96.92
Neutral	2 ml, water for 30 min. at RT	0.094	0.285	90.42

NA: not applicable

CONCLUSION: It is concluded that the developed and validated RS method for related substances in dipyridamole is simple, specific, stable, linear, accurate, precise, rugged and robust. The method was found to meet the entire predetermined acceptance criteria as per ICH guidelines. Based on the validation study results, it has been concluded that the developed HPLC method for related substances in dipyridamole is suitable for routine analysis to identify and quantify the related substances in the pharmaceutical industry.

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CONFLICTS OF INTEREST: Nil

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