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A Simple RP-HPLC Method Development and Validation for the Simultaneous Estimation of Acetylsalicylic Acid and Dipyridamole in API and Marketed Pharmaceutical Dosage Form

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A B S T R A C T

The present work describes a reverse phase high performance liquid chromatographic method (RP-HPLC) for the simultaneous estimation of Acetylsalicylic Acid and Dipyridamole in bulk and in tablet dosage form. Chromatographic separation was performed on Hypersil (C18) (250mm x 4.6mm, 5µm) Column, with a mobile phase comprising of a mixture of methanol and Acetonitrile in the ratio of 34:66v/v. The flow rate was 1.0 ml/min with detection at 257 nm. Retention times of Acetylsalicylic Acid and Dipyridamole were found to be 1.791min and 3.465min respectively. As per International Conference on Harmonization (ICH) guidelines the method was validated for linearity, accuracy, precision, limit of quantitation, limit of detection, and robustness. Linearity of Acetylsalicylic Acid was found to be in the range of 60-140 µg/mL and that for Dipyridamole was found to be 30-70µg/mL. The Precision (Repeatability, Intra-day and Inter-day) of the Acetylsalicylic Acid and Dipyridamole was found to be within the limits. The correlation coefficients were 0.999 and 0.999 for Acetylsalicylic Acid and Dipyridamole respectively. The mean recoveries obtained for Acetylsalicylic Acid and Dipyridamole were 100.28% and 99.79%. This demonstrates that the developed method is simple, precise, accurate, reproducible and rapid for simultaneous estimation of these drugs in bulk and in tablet dosage forms.

Keywords: Acetylsalicylic Acid and Dipyridamole, RP-HPLC, Validation, Accuracy, Robustness.

1. Introduction

Product quality is defined in terms of specifications, critical quality standards, and attributes. A critical quality attribute is a physical, chemical, biological property or characteristic that would be within an appropriate limit, range, or distribution to ensure the desired product quality. Some important critical quality attributes to ensure the quality of drug products are assay, dissolution, uniformity of dosage units, and related substances. Most of the research works were carried out in method development for assay and related substances[1]. Developing a method for content uniformity of dosage units for a combination product is a challenging process since the drug concentrations of two drugs would be varying very high. The term "uniformity of dosage unit" is defined as the degree of uniformity in the amount of the drug substance among dosage units. The test for content uniformity of preparations presented in dosage units is based on the assay of the individual content of drug substance(s) in a number of dosage units to determine whether the individual content is within limits set. It ensures that a consistent dose of the active pharmaceutical ingredient is maintained between batches so that the patient receives the correct dose [2-4]. The chemical name for Aspirin (ASP) is benzoic acid, 2- (acetyloxy)-, with molecular weight of 180.16 and a molecular formula of C9H8O4. White crystalline powder and odorless or has a faint odor. It is stable in dry air. In moist air, it gradually hydrolyzes to salicylic and acetic acids. Slightly soluble in water, freely soluble in alcohol, soluble in chloroform, and sparingly soluble in absolute ether. ASP is having a log P value of 1.18 and the pKa value of 3.5. [5]. The chemical name of Dipyridamole (DPM) is 2,2',2",2"'-[(4,8-diperidinopyrimido[5,4-d] pyrimidine-2,6- diyl) dinitrilo]- tetraethanol, with molecular weight of 504.63 and molecular formula of C24H40N8O4. Intensely yellow, crystalline powder. Very soluble in methanol, in alcohol and in chloroform, slightly soluble in water, very slightly soluble in acetone and in ethyl acetate. DPM is having the log P value of 1.5. [6] The combination of ASP and DPM is widely used to reduce thrombosis in patients with thrombotic diseases. This antithrombotic action results from additive antiplatelet effects of both drugs. ASP inhibits platelet aggregation by irreversible inhibition of platelet cyclooxygenase and thus inhibiting the generation of thromboxane A2. DPM inhibits the uptake of adenosine into platelets and endothelial cells .

The aim of the study is to develop new simple, sensitive, accurate and economical analytical method for the simultaneous estimation of Acetyl Salicylic Acid and Dipyridamole in bulk form and combined pharmaceutical dosage form; to validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the Acetyl Salicylic Acid and Dipyridamole in bulk form and combined pharmaceutical dosage form.

2. Materials and Methods

2.1 Chemicals and Reagents:

Working standards and impurities for ASP and DPM were obtained as gift samples from Sura labs,Hyderabad. The finished dosage form Aggrenox was procured from the pharmacy. Water and methanol of suitable HPLC, Acetonitrile for HPLC and AR grade were purchased from E. Merck Co., Mumbai.

2.2 Method Validation

Preparation of mobile phase:

Accurately measured 340 ml (34%) of Methanol, 660 ml of Acetonitrile (66%) were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

2.3 Method Validation Parameters

2.3.1 System suitability

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of the above Acetyl Salicylic Acid and 0.5ml of the Dipyridamole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

2.3.2 Specificity study of drug:

Preparation of Standard Solution:

Accurately weigh and transfer 10mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of the above Acetyl Salicylic Acid and 0.5ml of the Dipyridamole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:

Take average weight of one Tablet and crush in a mortor by using pestle and weight 10 mg equivalent weight of Acetyl Salicylic Acid and Dipyridamole sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 1ml of the sample solution from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

The mean and percentage relative standard deviation were calculated from the peak areas.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

2.3.3 Linearity

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (60ppm of Acetyl Salicylic Acid & 30ppm of Dipyridamole):

Pipette out 0.6ml of Acetyl Salicylic Acid and 0.3ml of Dipyridamole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (80ppm of Acetyl Salicylic Acid & 40ppm of Dipyridamole):

Pipette out 0.8ml of Acetyl Salicylic Acid and 0.4ml of Dipyridamole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (100ppm of Acetyl Salicylic Acid & 50ppm of Dipyridamole):

Pipette out 1ml of Acetyl Salicylic Acid and 0.5ml of Dipyridamole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (120ppm of Acetyl Salicylic Acid & 60ppm of Dipyridamole):

Pipette out 1.2ml of Acetyl Salicylic Acid and 0.6ml of Dipyridamole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (140ppm of Acetyl Salicylic Acid & 70ppm of Dipyridamole):

Pipette out 1.4ml of Acetyl Salicylic Acid and 0.7ml of Dipyridamole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

2.3.4 Precision

Preparation of Acetyl Salicylic Acid and Dipyridamole Product Solution for Precision:

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of the above Acetyl Salicylic Acid and 0.5ml of the Dipyridamole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

2.3.5 Intermediate precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

DAY 1:

The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.

DAY 2:

The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.

2.3.5 Accuracy

For preparation of 50% Standard stock solution:

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.5ml of the above Acetyl Salicylic Acid and 0.25ml of the Dipyridamole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of the above Acetyl Salicylic Acid and 0.5ml of the Dipyridamole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5ml of Acetyl Salicylic Acid and 0.75ml of Dipyridamole from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100% and 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Acetyl Salicylic Acid and Dipyridamole and calculate the individual recovery and mean recovery values.

2.3.6 Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Acetyl Salicylic Acid and 10mg of Dipyridamole working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of the above Acetyl Salicylic Acid and 0.5ml of the Dipyridamole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: Acetonitrile was taken in the ratio and 39:61, 29:71 instead (34:66), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

3. Results and Discussion

3.1 Optimized Chromatogram (Standard)

Mobile phase ratio	: Methanol: Acetonitrile (34:66% v/v)
Column	: Hypersil (C18) (250mm x 4.6mm, 5µm) Column
Column temperature	: Ambient
Wavelength	: 257nm
Flow rate	: 1.0ml/min
Injection volume	: 10µl
Run time	: 8minutes

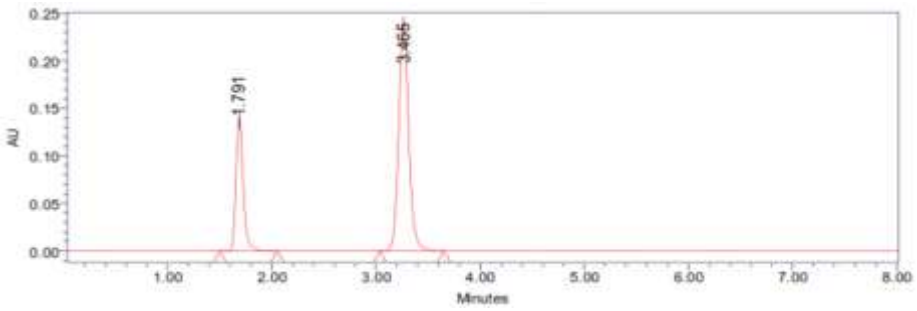

Fig.1: Optimized Chromatogram (Standard)

Table1: Optimized Chromatogram (Standard)

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Acetyl Salicylic Acid	1.791	485685	6598	1.35	6527
2	Dipyridamole	3.465	8654527	584753	1.46	7698

From the above chromatogram it was observed that the Acetyl Salicylic Acid and Dipyridamole peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

3.2 Optimized Chromatogram (Sample)

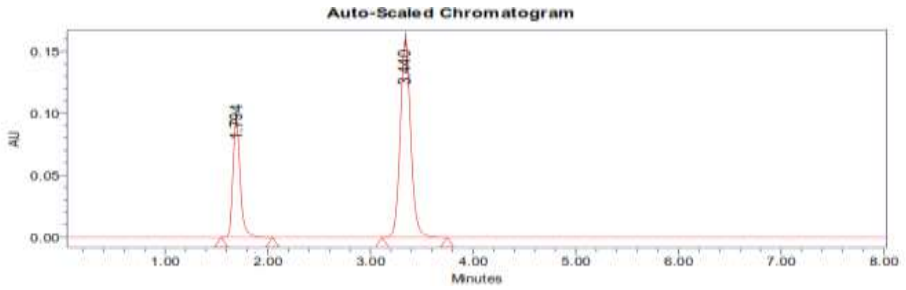

Fig.2: Optimized Chromatogram (Sample)

Table- 2: Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Acetyl Salicylic	1.794	496582	6648	1.36	6695
2	Dipyridamole	3.440	8756854	598652	1.47	7786

It was found from above data that all the system suitability parameters for developed method were within the limit.

3.3 Assay:

Table- 3: Peak results for assay standard of Acetyl Salicylic Acid

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Acetyl Salicylic Acid	1.788	486598	6539	6598	1.35
2	Acetyl Salicylic Acid	1.792	485692	6652	6652	1.36
3	Acetyl Salicylic Acid	1.793	492364	6579	6587	1.35
4	Acetyl Salicylic Acid	1.788	485254	6624	6582	1.36
5	Acetyl Salicylic Acid	1.787	493245	6784	6575	1.35
Mean			488630.6			
Std. Dev.			3853.545			
% RSD			0.788642			

Acceptance Criteria:

• %RSD of five different sample solutions should not more than 2.

• The %RSD obtained is within the limit, hence the method is suitable.

Table-4: Peak results for assay standard of Dipyridamole

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Dipyridamole	3.438	8652895	586525	7622	1.46
2	Dipyridamole	3.446	8625642	587548	7649	1.47
3	Dipyridamole	3.444	8625495	586985	7599	1.46
4	Dipyridamole	3.465	8722542	584752	7685	1.46
5	Dipyridamole	3.465	8695898	594231	7643	1.47
Mean			8664494			
Std. Dev.			43361.04			
% RSD			0.500445			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

3.4 Assay (Sample):

Table- 5 : Peak results for Assay sample of Acetyl Salicylic Acid

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Acetyl Salicylic Acid	1.794	498652	6658	1.36	6625	1
2	Acetyl Salicylic Acid	1.791	497586	6692	1.37	6692	2
3	Acetyl Salicylic Acid	1.791	497582	6674	1.36	6678	3

Table - 6: Peak results for Assay sample of Dipyridamole

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Dipyridamole	3.440	8758654	598654	1.47	7785	1
2	Dipyridamole	3.442	8745985	596548	1.48	7796	2
3	Dipyridamole	3.434	8758644	598746	1.47	7782	3

The % purity of Acetyl Salicylic Acid and Dipyridamole in pharmaceutical dosage form was found to be 99.95%

3.5 Linearity

Table - 7: chromatographic data for linearity study for acetyl salicylic acid

Concentration µg/ml	Average Peak Area
60	289658
80	387568
100	478562
120	568546
140	658825

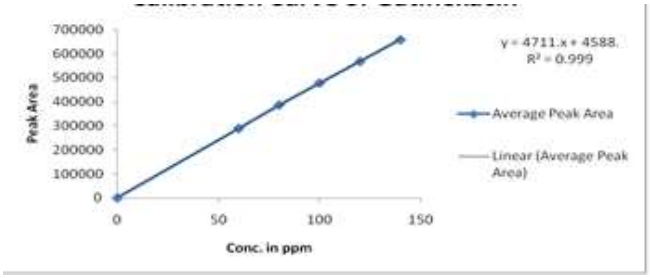

Fig.3: Chromatogram showing linearity level

Table - 8: chromatographic data for linearity study for Dipyridamole

Concentration µg/ml	Average Peak Area
30	5495852
40	7258456
50	9058654
60	10854253
70	12567853

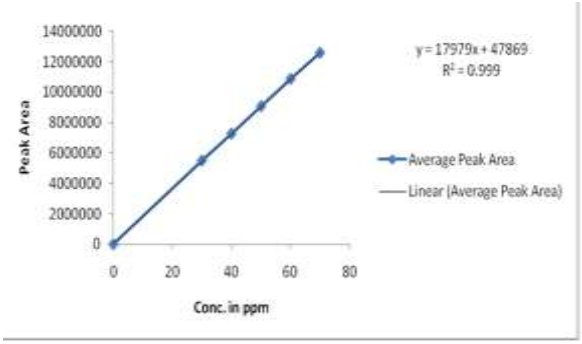

Fig.4: Chromatogram showing linearity level

3.6 Precision:

Table-9: Results of Repeatability for Acetyl Salicylic Acid

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Acetyl Salicylic Acid	1.792	485785	6598	6598	1.36
2	Acetyl Salicylic Acid	1.791	486958	6652	6574	1.35
3	Acetyl Salicylic Acid	1.790	485623	6592	6592	1.35
4	Acetyl Salicylic Acid	1.790	485986	6499	6524	1.36
5	Acetyl Salicylic Acid	1.789	487525	6583	6549	1.35
Mean			486375.4			
Std.dev			825.7289			
%RSD			0.16977			

Acceptance Criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table - 10: Results of Repeatability for Dipyridamole

S. No.	**Peak Name**	**Retention time**	**Area (µV*sec)**	**Height (µV)**	**USP Plate Count**	**USP Tailing**
1	Dipyridamole	3.435	8675845	586985	7685	1.46
2	Dipyridamole	3.428	8659852	587452	7695	1.47
3	Dipyridamole	3.419	8657543	589453	7692	1.46
4	Dipyridamole	3.414	8659254	587895	7682	1.47
5	Dipyridamole	3.408	8653542	587652	7785	1.46
Mean			8661207			
Std.dev			8545.443			
%RSD			0.098663			

Acceptance Criteria:

• %RSD for sample should be NMT 2.

• The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

3.7 Accuracy:

Table-11: Results of Accuracy for concentration-50%

S.No	Name	Rt	Area	Height	USP Tailing	USP plate count	Injection
1	Acetyl Salicylic Acid	1.786	238864	5785	1.28	5986	1
2	Dipyridamole	3.477	496252	469852	1.37	6854	1
3	Acetyl Salicylic Acid	1.788	246852	5834	1.29	5974	2
4	Dipyridamole	3.475	494654	459865	1.36	6824	2
5	Acetyl Salicylic Acid	1.791	236576	5874	1.28	5936	3
6	Dipyridamole	3.465	496875	468523	1.37	6829	3

Table-12: Results of Accuracy for concentration-100%

S.No.	Name	Rt	Area	Height	USP Tailing	USP plate count	Injection
1	Acetyl Salicylic Acid	1.788	471258	6658	1.36	6659	1
2	Dipyridamole	3.465	942324	584752	1.47	7698	1
3	Acetyl Salicylic Acid	1.787	482426	6599	1.37	6599	2
4	Dipyridamole	3.465	941585	596212	1.46	7742	2
5	Acetyl Salicylic Acid	1.786	482354	6647	1.38	6642	3
6	Dipyridamole	3.465	942452	598475	1.48	7628	3

Table-13: Results of Accuracy for concentration-150%

S.No	Name	Rt	Area	Height	USP Tailing	USP plate count	Injection
1	Acetyl Salicylic Acid	1.786	712352	7256	1.45	7152	1
2	Dipyridamole	3.468	1395824	642658	1.53	8265	1
3	Acetyl Salicylic Acid	1.784	715874	7365	1.46	7265	2
4	Dipyridamole	3.467	1398568	658365	1.54	8246	2
5	Acetyl Salicylic Acid	1.783	704253	7426	1.45	7169	3
6	Dipyridamole	3.466	1402565	648578	1.53	8364	3

Table - 14: The accuracy results for Acetyl Salicylic Acid

% Concentration (at Specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	240764	50	50.132	100.264%	100.28%
100%	478679.3	100	100.634	100.634%	
150%	710826.3	150	149.912	99.941%	

Acceptance Criteria:

The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table - 15: The accuracy results for Dipyridamole

%Concentration (at Specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	495927	25	24.921	99.684%	99.79%
100%	942120.3	50	49.738	99.476%	
150%	1398986	75	75.149	100.198%	

Acceptance Criteria:

The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

3.8 Limit Of Detection

Acetyl Salicylic Acid =0.99µg/ml

Dipyridamole =1.35µg/ml

3.9 LOQ

Acetyl Salicylic Acid =2.97µg/ml

Dipyridamole = 4.05µg/ml

3.10 Robustness

Table. 16: Results for Robustness -Acetyl Salicylic Acid

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	485685	1.791	6527	1.35
Less Flow rate of 0.8mL/min	546523	1.867	7256	1.43
More Flow rate of 1.0mL/min More Flow rate of 0.9mL/min	498652	1.744	6248	1.34
Less organic phase (about 5 % decrease in organic phase)	478524	1.831	6423	1.32
More organic phase (about 5 % Increase in organic phase)	465382	1.874	6355	1.31

Table. 17: Results for Robustness-Dipyridamole

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	8654527	3.465	7698	1.46
Less Flow rate of 0.8mL/min	9152684	3.721	8254	1.42
More Flow rate of 1.0mL/min	8526472	3.097	7326	1.43
Less organic phase (about 5 % decrease in organic phase)	8245635	6.242	7298	1.45
More organic phase (about 5 % Increase in organic phase)	8365824	2.402	7199	1.40

Acceptance Criteria:

The Tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

4. Conclusion

A new method was established for simultaneous estimation of Acetylsalicylic Acid and Dipyridamole by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Acetylsalicylic Acid and Dipyridamole by using Hypersil (C18) (250mm x 4.6mm, 5µm) Column, flow rate was 1ml/min, mobile phase ratio was Methanol: Acetonitrile in the ratio of 34:66 v/v and the detection wave length was found to be 257nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 1.791mins and 3.465mins. The % purity of Acetylsalicylic Acid and Dipyridamole was found to be 99.95%. The system suitability parameters for Acetylsalicylic Acid and Dipyridamole such as theoretical plates and tailing factor were found to be within limits. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study in Acetylsalicylic Acid and Dipyridamole was found in concentration range of 60µg-140µg and 30µg-70µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % recovery was found to be 100.28% and 99.79%, %RSD for repeatability was 0.169 and 0.098. The precision study was precise, robust, and repeatable. LOD value was 0.99 and 1.35, and LOQ value was 2.97 and 4.05 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Acetylsalicylic Acid and Dipyridamole in bulk and Combined Pharmaceutical dosage form..

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