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DEVELOPMENT OF RP-HPLC METHOD FOR SIMULTANEOUS EVALUATION OF UNIFORMITY OF DOSAGE UNITS FROM ASPIRIN AND DIPYRIDAMOLE EXTENDED-RELEASE CAPSULES

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Keywords:

Aspirin, Dipyridamole, Content uniformity, Forced degradation, RP-HPLC, stability-indicating

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ABSTRACT: The aim of the present study is to develop a stability-indicating reverse-phase high-performance liquid chromatography method for the simultaneous estimation of Aspirin and Dipyridamole in combined extended-release dosage form 25/200mg, using a single unit of the capsule. **Materials and Methods:** Chromatographic separation was achieved with Agilent's high-performance liquid chromatography and X bridge C_8 column, with the mobile phase containing a mixture of ammonium dihydrogen phosphate buffer (pH 2.5): methanol (550:450, v/v) by isocratic elution technique. The flow rate was maintained at 1.2 ml/min, and the detection wavelength was 230nm. **Results:** Aspirin and Dipyridamole were eluted at 4.7min and 12.6min, respectively, using the developed method. The method was linear in the range of 12.5-50µg/ml for Aspirin and 100-400 µg/ml for Dipyridamole, with an r^2 value of 0.9997 and 0.9999, respectively. The sample recoveries observed were 97.63-100.96% and 97.38-98.01%, respectively, for aspirin and dipyridamole. The forced degradation studies were carried out, and the stressed samples were analyzed using the developed method. The purity angle of the peak was observed lesser than the threshold angle. **Conclusion:** The method could able to detect the potency of the product using one capsule. The recovery study results confirm the non-interference of formulation additives in the estimation. The purity angle from the forced degradation study confirms the non-interference from degradants in quantitating marketed formulation. Hence, the developed method is precise and accurate.

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 [1].

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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. 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 [2-3].

A stability-indicating method is an analytical procedure used to quantitate the decrease in the amount of the active pharmaceutical ingredient in drug product due to degradation [4].

The chemical name for Aspirin (ASP) is benzoic acid, 2- (acetyloxy)-, with molecular weight of 180.16 and a molecular formula of $C_9H_8O_4$. 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]

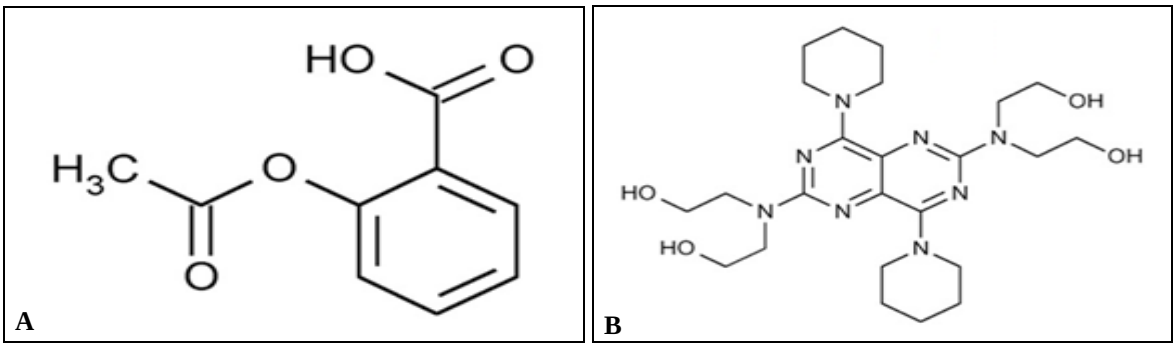


FIG. 1: CHEMICAL STRUCTURE (A) ASPIRIN AND (B) DIPYRIDAMOLE

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 $C_{24}H_{40}N_8O_4$. 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 [7].

A literature survey revealed some analytical methods were reported for the determination of ASP and DPM individually or in combination with other drugs like clopidogrel and atorvastatin using different analytical techniques like HPLC, HPTLC and LCMS from pharmaceutical formulations and other biological matrices [8-11]. Few methods were also reported for the simultaneous determination of ASP and DPM using combination of liquid chromatographic and mass spectrometric detection, second-order derivative spectrophotometry, RP-HPLC method, UP-HPLC method and spectrofluorimetric method [12-15]. No methods were established for the content uniformity test. The sample size for content uniformity was a limiting factor, whereas for evaluation of assay and related substances, the sample size shall be optimized to achieve the required final concentration of drug substance for analysis. The reported methods used for evaluation of assay, where the sample size was selected from the minimum of 10 capsules.

The developed method is to evaluate the potency of the drug in drug products using a single capsule. Literature also revealed that dipyridamole is incompatible with tartaric acid, and the impact of tartaric acid in principle peak was very high. In the current study, the method was developed to elute tartaric acid at an initial time point, so that the impact on principal peaks by tartaric acid is controlled. The aim of the present study is to develop and validate a simple, accurate, and precise stability-indicating reverse phase HPLC method for the simultaneous estimation of ASP and DPM from the single extended-release capsule and by extending the run time to confirm the non-interference of excipients and degradants.

MATERIALS AND METHODS:

Chemicals and Reagents: Working standards and impurities for ASP were obtained as gift samples from Andhra sugars, for DPM was obtained from Mylan. The finished dosage form Aggrenox was procured from the pharmacy. Excipients were obtained from Signet Chemical Corporation. Ammonium dihydrogen phosphate, orthophosphoric acid, formic acid, water, and methanol of suitable HPLC and AR grade were purchased from E. Merck Co., Mumbai.

Instrumentation: The analysis was carried out using an Agilent 1200 RP-HPLC system consisting of a pump, an injector, and a photodiode array (PDA)/UV-Visible detector, with an autosampler and column heater. Data were collected and processed using Empower software. Other instruments used for analysis were Analytical Balance, Ultrasonic Bath, Centrifuge, pH meter, Oven, and Mechanical shaker. Polyvinyl difluoride filters (0.45 micron) used for sample filtration were purchased from Rankem, India.

Preparation of Mobile Phase: Preparation of buffer for mobile phase: 0.05M ammonium dihydrogen phosphate buffer was prepared by transferring 5.75 g of ammonium dihydrogen phosphate to a suitable container containing 1000 mL of water and dissolved. The pH was adjusted to 2.5 ± 0.05 using orthophosphoric acid. The solution was filtered through a 0.45 µ PVDF filter. Preparation of mobile phase: 550 mL of buffer and 450 mL of methanol were transferred into a suitable container, mixed for 5min using a stirrer, degassed through sonication.

Diluents: Three diluents were used in the analysis. Diluent-1: It was prepared by mixing 50 ml of formic acid with 950 ml of purified water, mixed for 5 min, using an overhead stirrer, degassed in a sonicator for about 10 min. Diluent-2: It was prepared by mixing 400 mL of 5% formic acid and 600 mL of methanol, using an overhead stirrer. Diluent-3: Methanol.

Preparation of Standard Solution: Preparation of ASP standard stock solution: About 25 mg of ASP was weighed accurately and transferred into a 100 ml volumetric flask, 40 ml of methanol was added and sonicated for 10 min to dissolve the material completely and 30 ml of 5% formic acid was added. The volume was made up with diluent-2 and mixed for 10 min. The resultant solution is standard stock preparation, the concentration of about 250 µg/mL of ASP.

Preparation of DPM Standard Stock Solution: About 50 mg of DPM was weighed accurately and transferred into a 50 ml volumetric flask, 20 ml of methanol was added and sonicated for 10 min to dissolve the material completely, and 15 ml of 5% formic acid was added. The volume was made up with diluent-2 and mixed for 10 min. The resultant solution is standard stock preparation, the concentration of about 1000 µg/mL of DPM.

Standard Preparation: 5 mL of ASP standard stock preparation and 10 mL of DPM standard stock preparation was transferred into a 50 mL volumetric flask. Volume was made with diluent-2 and mixed well for 10 min. The standard preparation concentration of about 25 µg/mL of ASP and 200 µg/mL of DPM.

Preparation of Test Solution: One capsule was opened and dropped the contents of the capsules into a 50 mL volumetric flask, and 20 mL of methanol was added, sonicated for about 10 min with intermittent shaking. 15 mL of 5% formic acid was added, sonicated for about 15 min with intermittent shaking, or till the capsule disperses completely. The solution was mixed using a mechanical shaker for 15 min at 200 rpm. The volume was made up with diluent-2 and mixed well. A portion of the solution was centrifuged at 3500 rpm for 10 min. 5 mL above supernatant solution was diluted to 100 mL with diluent-2 and mixed well. A portion of the above solution was filtered through a 0.45 µm PVDF membrane filter by discarding the first 4 ml of the filtrate.

Chromatographic System: HPLC analysis was performed on the Agilent HPLC system with a UV detector. Chromatographic separation of ASP and DPM was carried on X Bridge C_8 column with 250 × 4.6 mm and 5 µm particle size. The isocratic condition with the mobile phase containing a mixture of buffer (pH 2.5): methanol (55:45) was programmed, and 1.2 ml/min flow rate was used for analysis, with a run time of 20 min. The detection wavelength was set at 230 nm, with the

sample volume of 15µL. HPLC column was maintained at a temperature of 30 °C, and the sample was maintained at a temperature of 25 °C. The retention time was observed with 4.7 min for ASP and 12.6 min for DPM

System Suitability: Standard solutions were prepared and injected. Peak area responses for five replicate injections of the standard solutions were recorded, and system suitability was calculated. The USP tailing factor should be not more than 2.0 for ASP and DPM peak from standard preparation. The USP plate count should be not more than 1500 for ASP and DPM peak from standard preparations. The RSD of ASP and DPM peak area is NMT 2.0 from five replicate injections of standard preparations. A typical chromatogram of standard and sample were presented

Test samples were injected, and the chromatograms were recorded for the response of the analyte peak. The % content of both the drugs was calculated using the formula presented below:

Quantity of ASP present in a capsule as % of the labelled amount

$$\% \text{ label claim for Aspirin} = A_T \times WS \times 5 \times 50 \times P \times 100 / A_S \times 100 \times 50 \times LC \times 5 \times 100$$

A_T = Peak area of ASP for Test preparation
A_S = Peak area of ASP for Standard preparation
WS = Weight of ASP working standard/reference standard taken, in mg
P = Potency of ASP standard, in percent, as-is basis
L = Labelled amount of ASP in mg, per capsule

Quantity of DPM present in portion of capsule as

$$\% \text{ of labelled amount} = A_T \times WS \times 5 \times 100 \times 50 \times P \times 100 / A_S \times 250 \times 50 \times 1 \times 100 \times L$$

A_T = Peak area of DPM for Test preparation
A_S = Peak area of DPM for Standard preparation
WS = Weight of DPM working standard/reference standard taken, in mg.
P = Potency of DPM standard, in percent as is basis
L = labelled amount of DPM in mg, per capsule

Calculation for the Acceptance Value:

$$\text{Acceptance value (AV)} = [M-X] + ks$$

k = Acceptability constant, for 10 units, the acceptability constant is 2.4

s = Sample standard deviation
X = mean of the individual contents (expressed as % of label claim)
M = is based on the X value.

If $98.5\% \leq X \leq 101.5\%$, then M = X. if X > 101.5%, then X = 101. 5%. If X< 98.5, then M = 98.5%.

If the AV value is less than 15.0, 10 units value for content uniformity is adequate.

Analytical Method Validation: [16-17] HPLC method was validated to ensure consistent, reliable, and accurate results were obtained to determine the levels of two drugs in all the samples. The validation parameters linearity, accuracy, precision, limit of detection, the limit of quantitation, and specificity were evaluated.

Linearity: Solutions of ASP and DPM at concentration levels from about 50% to 200% of standard solution were injected into HPLC system. The linearity graph was plotted from 50% to 200%. Six injections were performed at 50% level and at 200% level and the chromatograms were recorded.

Precision: The system precision was carried out to ensure that the analytical system is working properly by injecting standard solution preparation containing ASP 25 µg/mL and DPM 200 µg/mL six times into the HPLC system as per the test procedure. The retention time and peak areas for both the drugs in all the sample solutions were measured, and % RSD was calculated**.** In method precision, a homogenous sample containing of ASP and DPM of a single capsule was analyzed six times, and % RSD was calculated.

Accuracy: Accuracy was performed by calculating percentage recovery by the standard addition method. A known amount of ASP and DPM were spiked with Aspirin/Extended-release dipyridamole capsules 25mg/200mg, in order to produce recovery at 50%, 100% and 150% levels of the ASP working concentration of 25 µg/mL and DPM working concentration 200 µg/mL. Spiked samples were prepared in triplicate, injected in duplicate, and the percentage recovery was calculated.

Limit of Detection (LOD): Limit of detection is the lowest concentration of the analyte that can be

detected by injecting decreasing amount, not necessarily quantity by the method, under the stated experimental conditions. The minimum concentration at which the analyte can be detected is determined from the linearity curve.

The detection limit (DL) may be expressed as:

$$DL = 3.3\ \sigma/S$$

Where, σ = the standard deviation of the response. S = the slope of the calibration curve

Limit of Quantification (LOQ): Limit of quantification is the lowest concentration of the analyte in a sample that can be estimated quantitatively by injecting a decreasing amount of drug with acceptable precision and accuracy under the stated experimental conditions of the method. The limit of quantitation can be obtained from the linearity curve. The detection limit (DL) may be expressed as:

$$DL = 10\ \sigma/S$$

Where, σ = the standard deviation of the response. S = the slope of the calibration curve

Specificity: Blank, standard, placebo, sample solutions, and impurity were prepared and injected into the chromatographic system for identification and interference of the ASP and DPM Peak.

Solution Stability: The standard and sample solutions were prepared and injected. Replicate injections of the standard and sample solutions were made at the following time intervals at 5 °C: Initial, 24 h, and 48 h. The concentration of standard at 24 h and 48 h were compared to that of the initial.

Forced Degradation Studies (Stress Testing): In order to develop a stability-indicating method for estimation of ASP and DPM, stress studies were carried out and validated the stability-indicating property of the proposed method. The chromatograms of the stressed samples were evaluated for peak purity of ASP and DPM peak using Empower networking software

Acid Degradation Studies: Weighed and transferred five capsules of Aspirin/Extended-Release Dipyridamole Capsules into a 250 mL volumetric flask. Added about 20 ml of methanol, sonicated for about 10 min with intermittent shaking. Added 15 mL of 5% formic acid, sonicated for about 15 min with intermittent shaking till the capsules disperse completely. Shaken on a mechanical shaker for 15 min at 200 rpm. Diluted the volume with 5mL of 1N methanolic HCl. Benchtop kept for 1.5 h. Neutralized with 5 mL of IN methanolic NaOH,

Base Degradation Studies: Weighed and transferred five capsules of Aspirin/Extended-Release Dipyridamole Capsules into a 250 mL volumetric flask. Added about 20 ml of methanol, sonicated for about 10 min with intermittent shaking. 15 mL of 5% formic acid was added and sonicated for about 15 min with intermittent shaking till the capsules disperse completely. Shaken on a mechanical shaker for 15 min at 200 rpm. Diluted the volume with 5mL of IN Methanolic NaOH. Benchtop kept 1.0 h neutralized with 5 mL of IN Methanolic HCl.

Oxidation Stress Studies: Weighed and transferred five capsules of Aspirin/Extended-Release Dipyridamole Capsules into a 250 mL volumetric flask and added about 20 ml of methanol, sonicated for about 10 min with intermittent shaking. Added 15 mL of 5% formic acid, sonicated for about 15 min with intermittent shaking till the capsules disperse completely. They were shaken on a mechanical shaker for 15 min at 200 rpm. Diluted the volume with 5 mL of 30% hydrogen peroxide and heated on a water bath at 60 °C for 15 min.

Photolytic Degradation Studies: Weighed and transferred five capsules of Aspirin/Extended-Release Dipyridamole Capsules (stressed under UV light for 24 h) into a 250 mL volumetric flask. Added about 100 ml of methanol, sonicated for about 10 min with intermittent shaking.

Thermal Degradation Studies: Weighed and transferred five capsules of Aspirin/Extended Release Dipyridamole Capsules (Heated at 105°C in an oven for 1.5 h) into a 250 mL volumetric flask. Added about 100 ml of methanol, sonicated for about 10 min with intermittent shaking.

Filter Study: A sample was prepared as per the method for the filter study. This sample was divided into three portions. One portion of the

prepared sample was centrifuged at 3500 RPM for 10 min. The centrifuged sample was used as a control for the filter study. The second portion of sample was filtered through 0.45µ PVDF filter, and the filtrate was collected after discarding the first 4 mL, 5 mL, 6 mL, and 7 mL of the filtrate. The third portion of sample was filtered through 0.45 µ nylon filter and the filtrate was collected after discarding the first 4 mL, 5 mL, 6 mL and 7 mL of the filtrate. The centrifuged and filtered samples were injected.

Robustness: Standard solution was prepared and injected into the chromatographic system as per the conditions specified in the method. The same standard solution was re-injected by changing one parameter at a time, keeping other parameters constant. Method Parameters:

1. Flow Rate (Normal -1.2 mL/min), a. Flow minus ~ 1.1 mL/min,b. Flow plus ~ 1.3 mL/min.

2. Column Operating Temperature (Normal temperature is 30 °C), a. Temperature minus ~ 25 °C, b. Temperature plus ~ 35 °C.

3. Buffer pH variation (Normal Buffer pH 2.5), a. pH minus ~ pH 2.3 b. pH plus ~ pH 2.7.

4. Mobile Phase Composition Variation (Normal Composition is Buffer: Methanol, 55:45) a. MPVl ~ Buffer: Methanol (57:43); b. MPV2 ~ Buffer: Methanol (53:47) c. MPV3 ~ Buffer: Methanol (56:44); d. MPV4 ~ Buffer: Methanol (54:46)

RESULTS AND DISCUSSION: The initial screening for diluents was performed based on the chemical nature of the molecule. Since ASP is sensitive to basic conditions, diluents selected are of acidic nature with lower pH values. Chromatographic parameters were preliminary optimized to develop a stability-indicating method for ASP and DPM with a short analysis time (20 min). To separate the degradants from main analyte, isocratic system was developed to elute the impurities, thus capturing all the possible degradants of both the components. All degradants shall be separated using with increased column length. Hence, a longer column (250 × 4.6 mm, 5 µm particle size) was selected to have a shortest possible runtime without compromising on the resolution. In order to identify a suitable organic modifier, various organic solvents like acetonitrile and methanol were tested. Methanol produced better selectivity with low column back pressures. Ammonium dihydrogen phosphate buffer gave sharp peaks for both the components compared to other buffers. Diluents selected for the preparation standard and sample solutions were based on the extraction and stability of both the drugs.

The system suitability was performed by injecting 15µL combined standard preparation into the chromatographic system for five times, the chromatograms were recorded, and responses were measured for the ASP and DPM peaks. The system suitability parameters summary is presented in **Table 1** and the typical chromatogram for standard is presented in **Fig. 2a**. The % RSD for peak area of five replicate injections was observed with 0.11% for ASP and 0.07% for DPM. The marketed product Aggrenox 25/200mg was analysed using the developed method and results were presented in **Table 2**. The sample chromatogram was presented in **Fig. 2b**. The acceptance value is meeting the acceptance criteria of below 15.

TABLE 1: SYSTEM SUITABILITY FOR ASPIRIN AND DIPYRIDAMOLE

Parameters	Drug	Mean ± SD	% RSD
Retention time (R_t)	ASP	4.42 ± 0.052	1.19
	DPM	12.67 ± 0.059	0.46
Peak area	ASP	802848 ± 867	0.11
	DPM	7253432 ± 4893	0.07
Tailing factor(T)	ASP	1.15 ± 0.008	0.73
	DPM	1.50 ± 0.016	1.09
Theoretical plates (N)	ASP	7974 ± 48	0.60
	DPM	6738 ± 82	1.22

TABLE 2: CONTENT UNIFORMITY FOR ASPIRIN AND DIPYRIDAMOLE EXTENDED RELEASE CAPSULES

S. no.	Content uniformity (BH30472)	
	ASP	DPM
1	100.2	99.8
2	99.8	100.1
3	100.3	99.5
4	100.2	98.9
5	100.5	101.2
6	99.7	101.1
7	99.5	100.5
8	100.1	99.8
9	100.5	98.9
10	100.3	100.3
Average (X)	100.11	100.01
S	0.34	0.80
% RSD	0.34	0.80
K	2.4	2.4
M	100.11	100.01
AV	0.81	1.92

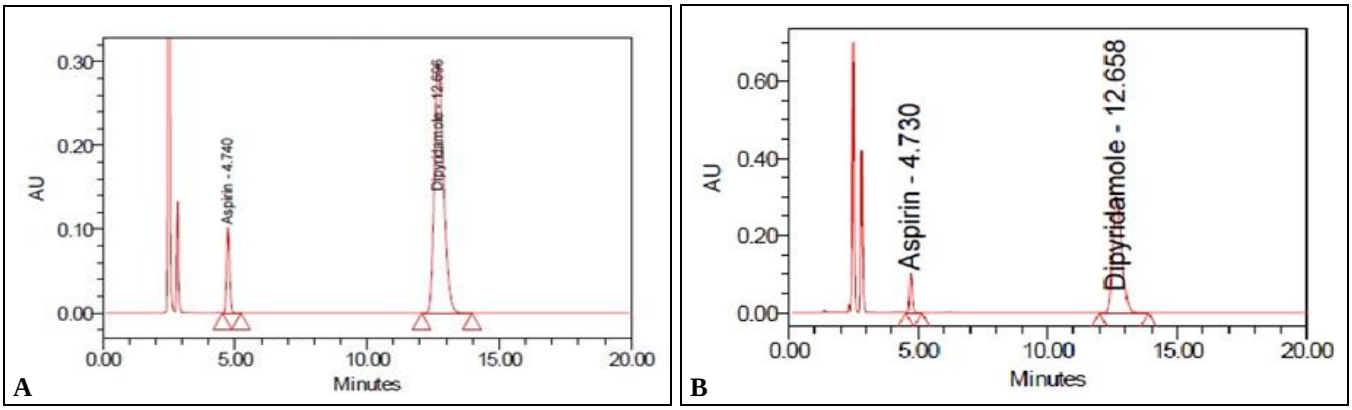


FIG. 2: A TYPICAL CHROMATOGRAM OF (A) STANDARD FOR ASPIRIN AND DIPYRIDAMOLE, AND (B) SAMPLE FOR ASPIRIN AND DIPYRIDAMOLE EXTENDED-RELEASE CAPSULE 25/200mg

The linearity of the method was established by injecting solutions of ASP and DPM. The data is shown in **Tables 3** and **4**. The linearity plot of ASP and DPM was presented in **Fig. 3** and **4**. Correlation coefficient square (r^2) for ASP and DPM met the acceptance criteria of more than 0.997. RSD of peak area at 50% and 200% is less than 2.0%. The linear regression data shows that the method is linear over the entire concentration range (50% to 200% of the standard concentration 25 µg/mL of ASP and 200 µg/mL of DPM), and it is adequate for its intended concentration range. The LOD values for ASP and DPM were determined to be 0.56 µg/ml and 3.95 µg/ml, and the LOQ values were 1.69µg/ml and 11.96 µg/ml, respectively.

TABLE 3: LINEARITY STUDY FOR ASPIRIN AND DIPYRIDAMOLE

S. no.	Aspirin		Dipyridamole	
	Conc. (µg/ml)	Peak Area	Conc. (µg/ml)	Peak Area
1	12.5	389955	100	3665260
2	20	620314	160	5839184
3	25	772524	200	7265677
4	30	935915	240	8815845
5	38	1171020	300	11033348
6	50	1553957	400	14614871

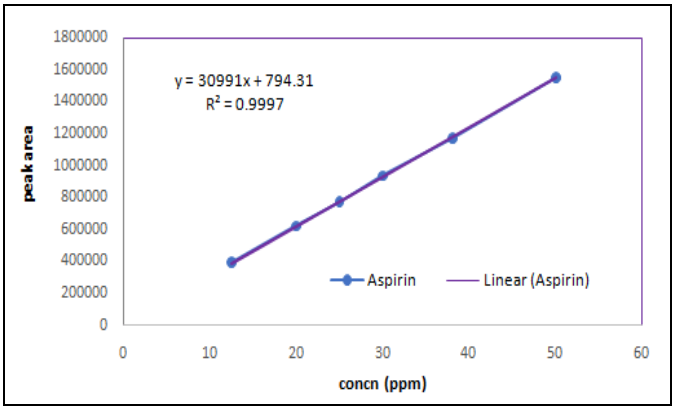


FIG. 3: LINEARITY OF ASPIRIN

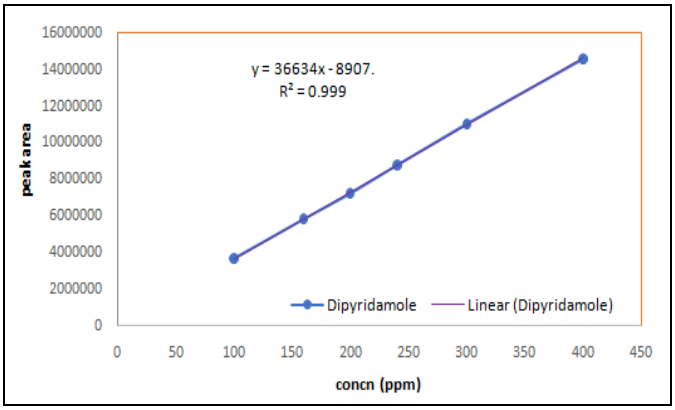


FIG. 4: LINEARITY OF DIPYRIDAMOLE

TABLE 4: VALIDATION PARAMETERS ESTABLISHED BY LINEARITY AND PRECISION

Parameters	ASP	DPM
Linearity (µg/ml)	12.5-50	100 -400
correlation co-efficient (r2)	0.9997	0.9999
Regression equation	y = 30991x + 794.31	y = 36634x - 8907.2
Method precision (% RSD)	1.02	0.57
System precision (at 50% level) (% RSD)	0.18	0.27
System precision (at 200% level) (% RSD)	0.18	0.13
LOD (µg/ml)	0.56	3.95
LOQ (µg/ml)	1.69	11.96

The precision of the test method was evaluated by repeatability studies by evaluating ten test samples of ASP and DPM extended-release capsules 25mg/200mg. The % relative standard deviation of ASP and DPM is presented in **Table 4**, which was observed within the acceptance criteria limit of not more than 15% according to the ICH guideline.

The recovery experiments were performed by adding a known quantity of pure standard drug into the solution of the capsule. The sample was spiked

with the standard at levels 50%, 100%, and 150% of test concentration were evaluated for content uniformity in triplicate, which was observed with the % RSD less than 2%, and the results are presented in **Table 5**.

TABLE 5: ACCURACY (RESULTS OF RECOVERY STUDY)

Level of Recovery (%)	Conc. actual (µg/ml)		Conc. Added (µg/ml)		Mean % recovery ± SD (n=3)		% RSD	
	ASP	DPM	ASP	DPM	ASP	DPM	ASP	DPM
50	25	200	12.5	100	100.8 ± 0.20	97.68 ± 0.30	0.20	0.30
100	25	200	25	200	100.61± 0.26	97.66 ± 0.12	0.26	0.12
150	25	200	37.5	300	97.74 ± 0.10	97.94 ± 0.07	0.10	0.07

Placebo Interference: Chromatograms of placebo showed no peaks at the retention times of ASP and DPM peaks. This indicates that the excipients used in the formulation do not interfere in the estimation of ASP and DPM in capsules. The placebo chromatogram is shown in **Fig. 5a**.

Impurity Interference: The chromatogram recorded by spiking the standard preparation with all impurities in the concentration of 0.3% of test preparation was found that all the impurities are separated from the main analyte ASP and DPM. Chromatogram of impurity interference is shown in **Fig. 5b**. The specificity data is presented in **Table 6**.

TABLE 6: SPECIFICITY (INTERFERENCE OF BLANK, PLACEBO AND IMPURITIES)

Sample Name	Retention Time (min)	Interference
Salicylic acid	6.486	Nil
Dipyridamole Impurity-B	3.438	Nil
Dipyridamole Impurity-F	3.947	Nil
Dipyridamole Impurity-D	15.158	Nil
Dipyridamole Impurity-E	26.25	Nil
Dipyridamole Impurity-C	ND	Nil
Dipyridamole Impurity-A	ND	Nil
Spiked sample Aspirin	4.639	Nil
Spiked sample Dipyridamole	13.06	Nil
Blank	ND	Nil

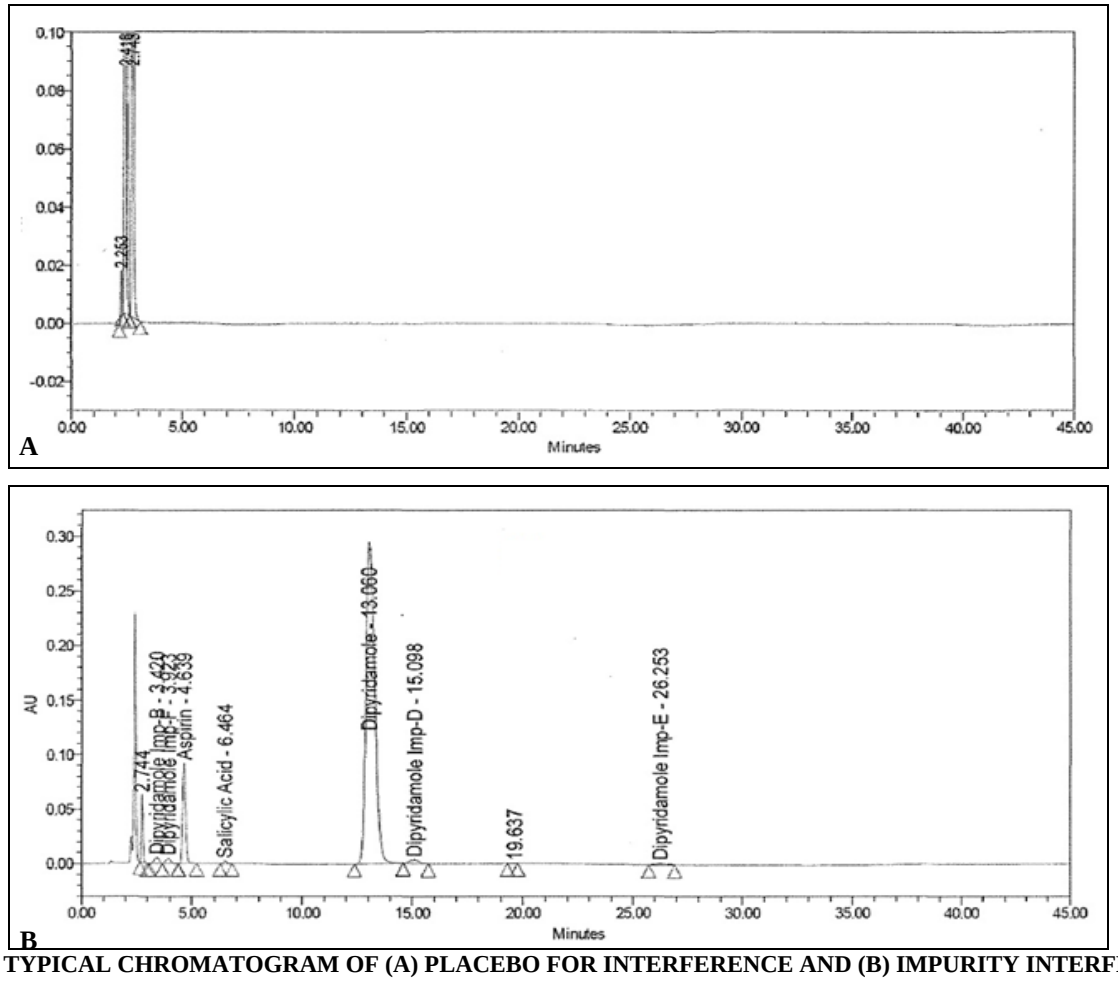


FIG. 5: A TYPICAL CHROMATOGRAM OF (A) PLACEBO FOR INTERFERENCE AND (B) IMPURITY INTERFERENCE

The solution stability study reveals the concentration of the 48 h injections of standard solution differed by less than 2.0%, and 48 h of sample solution differed by less than 2.0% when compared to the initial sample solution. Hence, the standard and sample solutions can be used up to 48 h after its preparation if it is stored at 5 °C. The solution stability results are presented in **Table 7**.

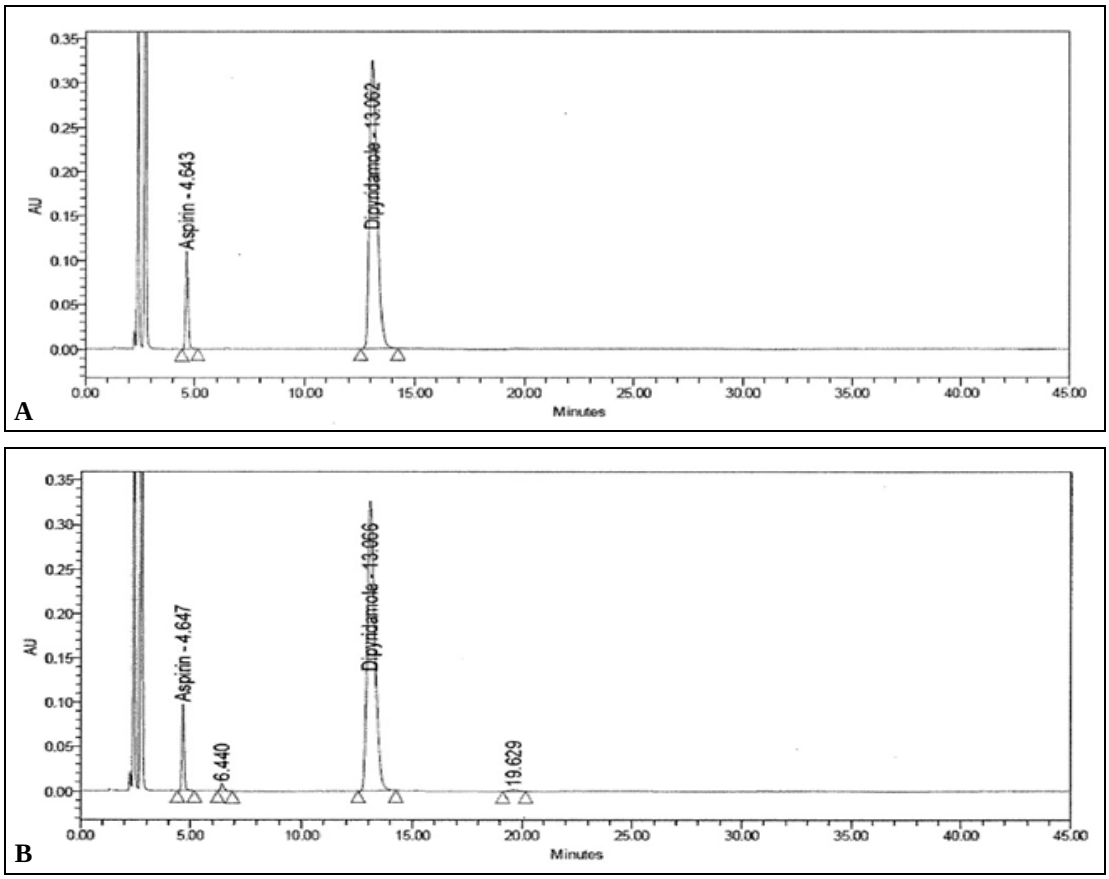
TABLE 7: SOLUTION STABILITY OF ASPIRIN AND DIPYRIDAMOLE

Time (hrs)	Standard conc. (µg/ml)		Difference from initial (%)		Sample Assay (%)		Difference from initial (%)	
	ASP	DPM	ASP	DPM	ASP	DPM	ASP	DPM
Initial	25.409	199.399	N/A	N/A	101.34	100.54	N/A	N/A
24 h	25.296	198.814	0.44	0.29	101.47	100.35	0.13	0.19
48 h	25.221	198.69	0.74	0.36	101.66	100.14	0.32	0.40

Forced Degradation Studies (Stress Testing): From the forced degradation sample chromatograms, all degradants peaks were resolved from ASP and DPM peak in the chromatograms of all samples. For all forced degradation samples, the purity angle found to be less than the threshold angle, which indicates that there is no interference from degradants in quantitating the ASP and DPM in capsules. The percentage drug content after forced degradation, purity threshold, and purity angle was performed for all the stressed samples, and unstressed samples were presented in **Table 8,** and chromatogram were presented in **Fig. 6**.

TABLE 8: ASSAY AND PEAK PURITY OF FORCED DEGRADATION STUDIES

Stress study	Aspirin			Dipyridamole		
	% Assay	Purity Angle	Purity threshold	% Assay	Purity Angle	Purity threshold
Control Sample	100.3	0.105	0.7	100.25	0.019	0.3
Acid stress	88.75	0.138	0.363	99.98	0.019	0.244
Base stress	86.35	0.152	0.857	83.15	0.018	0.322
peroxide stress	75.73	0.157	0.599	99.95	0.023	0.281
UV stress	100.2	0.109	0.798	100.56	0.025	0.323
Heat stress	83.76	0.153	0.475	100.95	0.021	0.275



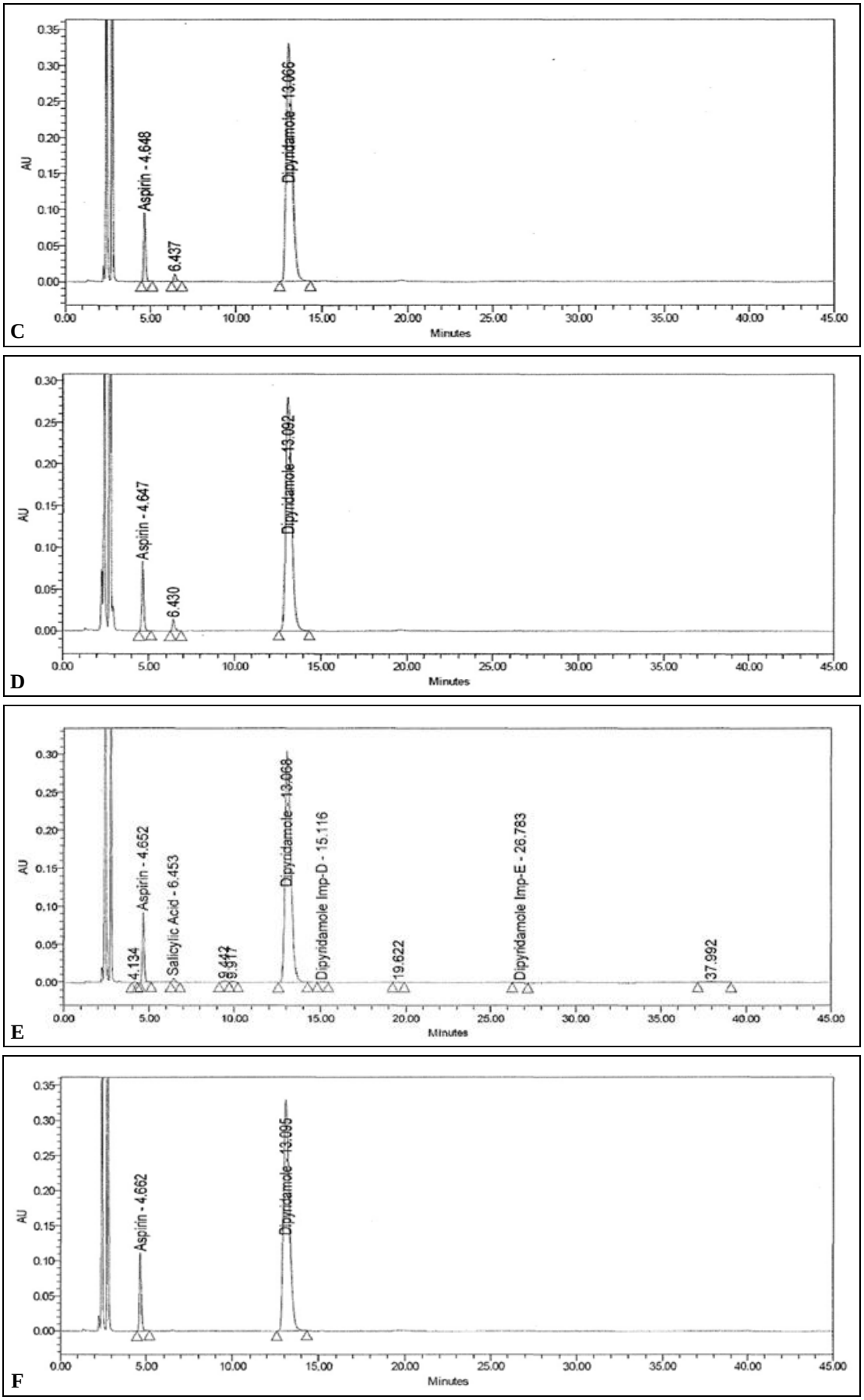


FIG. 6: CHROMATOGRAM OF ASPIRIN AND DIPYRIDAMOLE SAMPLE (A) UNSTRESSED (B) ACID STRESSED, (C) BASE STRESSED, (D) OXIDATION STRESSED, (E) HEAT STRESSED AND (F) UV LIGHT STRESSED SAMPLES

Filter Study: The area found in the filtered fractions of the sample solution was comparable to the area found in the centrifuged portion of the sample solution. There is no significant difference in the area between different volumes filtered. Therefore, the filters are suitable for use, and the discarding of 4 mL of sample solution as filtrate, as stated in the method, is a suitable volume to discard before collecting for analysis by HPLC. The results are presented in **Table 9**.

TABLE 9: FILTER STUDY WITH PVDF AND NYLON FILTERS

Sample Name	% Difference of Assay from unfiltered sample	
	ASP	DPM
Centrifuged Sample (10 min @, 3500rpm)	N/A	N/A
0.45µ PVDF filtrate sample, 4 mL discarded	0.04	0.03
0.45µ PVDF filtrate sample, 5 mL discarded	0.21	0.12
0.45µ PVDF filtrate sample, 6 mL discarded	0.19	0.25
0.45µ PVDF filtrate sample, 7 mL discarded	0.15	0.14
0.45µ Nylon filtrate sample, 4 mL discarded	0.49	0.34
0.45µ Nylon filtrate sample, 5 mL discarded	0.29	0.18
0.45µ Nylon filtrate sample, 6 mL discarded	0.28	0.15
0.45µ Nylon filtrate sample, 7 mL discarded	0.52	0.62

Robustness: No significant change was observed in retention time after individually changing the conditions of the flow rate of mobile phase by ±0.1mL/min, column operating temperature by ±5 °C, pH of the buffer by ±0.2 units and mobile phase composition variation by ±1% absolute. However, a significant difference in retention time observed while varying the mobile phase composition by ±2% absolute. Calculations for all other system suitability parameters met the acceptance criteria, and the data generated are comparable with the normal conditions. Based on the above result, it is concluded that the method is unaffected by small, deliberate variations in flow rate, column temperature, pH of buffer, and mobile phase composition variation.The results are presented in **Table 10**.

TABLE 10: ROBUSTNESS STUDY- COMPARISON OF SYSTEM SUITABILITY AND RETENTION TIME

Parameters	Condition	Retention time		Peak area (mean ± SD) (n=5)		USP tailing factor		USP plate count	
		ASP	DPM	ASP	DPM	ASP	DPM	ASP	DPM
Normal Condition	(Buffer pH 2.5: Methanol (55:45), 1.2mL/min, 30°C)	4.79	12.670	851115 ±0.10	7259212 ±0.23	1.43	1.79	8113	5800
Flow Rate Minus	1.1 mL/min	5.22	15.148	930245 ±0.20	7907613 ±0.69	1.44	1.79	8300	5672
Flow Rate Plus	1.3 mL/min	4.42	12.965	790440 ±0.40	6788328 ±0.57	1.42	1.78	7558	5404
pH	2.3	4.80	13.245	859316 ±0.39	7449066 ±0.25	1.51	1.77	7230	5516
pH	2.7	4.68	13.536	836791 ±0.16	7508209 ±0.22	1.52	1.72	7173	5789
Column Temperature	25°C	5.05	16.009	859807 ±1.34	7324933 ±0.24	1.39	1.72	7045	5089
Column Temperature	35°C	4.55	12.377	856848 ±0.28	7378215 ±0.13	1.43	1.77	8467	6353
MPV1	Buffer: Methanol, (57:43)	5.17	18.421	846392 ±0.08	7507376 ±0.12	1.5	1.83	7528	5746
MPV2	Buffer: Methanol, (53:47)	4.41	10.682	848255 ±0.28	7382697 ±0.12	1.42	1.72	7775	5648
MPV3	Buffer: Methanol, (56:44)	4.97	15.313	849761 ±0.25	7352812 ±0.16	1.46	1.77	7520	5549
MPV4	Buffer: Methanol, (54:46)	4.62	11.878	847599 ±0.07	7333893 ±0.06	1.46	1.7	7383	5476

CONCLUSION: Novelty in this research work involves establishing better isolation and elution of active ingredients and degradants in a short run time of 20 min, using 2 diluents, and mobile phase at lower pH of around 2, which resulted in longer solution stability and better resolution. The developed method was capable of eluting degradation products. The drug peaks observed from chromatograms were not interfered by degradants and formulation additives. The method was validated in compliance with the ICH guidelines. Hence, this developed method can be conveniently adopted for routine quality control analysis of content uniformity of ASP and DPM extended release capsules.

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