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Analytical Method Development and Validation for the Simultaneous Estimation of Aspirin, Clopidogrel and Rosuvastatin in Pharmaceutical Dosage Form

Pradip Kumar Tiwari[1]*, Amit Jain[1], BK Dubey[2], GK Pandey[1], Suresh Dhakad[1]

[*1] TIT- Pharmacy Education & Research, Bhopal, Madhya Pradesh, India

[2] Technocrats Institute of Technology- Pharmacy, Bhopal, Madhya Pradesh, India

ABSTRACT

A new, simple, novel, accurate, precise, reliable, rapid and linear reverse phase high-performance liquid chromatography (RP-HPLC) method was developed and fully validated for simultaneous qualitative and quantitative estimation of Rosuvastatin (ROS), Clopidogrel (CLOP) and Aspirin (ASP) in bulk and pharmaceutical dosage form as per International Conference on Harmonization (ICH) guidelines. In the present work, good chromatographic separation was achieved by isocratic method using a Hypersil BDS C_{18} column (250 mm ×4.6, 5 µm) and a mobile phase consisting of KH_2Po_4 buffer pH-6.0: acetonitrile in the ratio 60:40, at a flow rate of 1 ml/min. The effluents obtained were monitored at 242nm with the UV-visible detector. The calibration curves obtained were linear (r^2=0.999) over the concentration range of 7.5-22.5µg/ml and 1-3µg/ml for CLOP, ASP and ROS respectively. A run time of 7.0 minutes for each sample made it possible to analyze more than 200 samples per day. The retention time of ASP, CLOP and ROS was found to be 3.103 min, 4,277 min and 5.707 min respectively. The high recovery values (99%-101%) indicate a satisfactory accuracy. The low percent relative standard deviation (% RSD) values in the precision study reveal that the method is precise therefore the method can be used for routine monitoring of CLOP, ASP and ROS in industry in the assay of bulk drug and dosage form.

Keywords: RP-HPLC, Rosuvastatin, Clopidogrel, Aspirin, Method validation, ICH guidelines.

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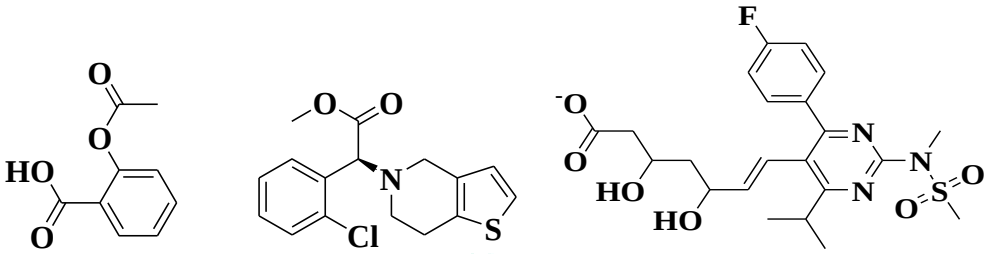
***Address for Correspondence:**

Pradip Kumar Tiwari , TIT- Pharmacy Education & Research, Bhopal, Madhya Pradesh, India

INTRODUCTION

Platelet aggregation and thrombus formation play a critical role in the initiation and development of key complications of acute coronary syndromes (ACSs). HMG-CoA Reductase catalyzes the conversion of HMG-CoA to mevalonic acid, the rate-limiting step in cholesterol biosynthesis and it is used to reduce plasma cholesterol levels and prevent cardiovascular disease (CVD), antiplatelet therapy and antithrombotic therapy have been demonstrated to modify clinical outcome favorably, and recent trials of revascularization in ACSs have demonstrated a reduction in the frequency of major cardiac events[1-3]. Yet, all effective antithrombotic agents also increase the risk of bleeding. Especially bleeding that result from vascular accessories associated with surgery, including coronary artery bypass grafting (CABG)[4-6]. Aspirin (ASP) (Fig.1A) is a well-known antithrombotic, antipyretic, analgesic agent. Chemically 2-(acetyloxy) benzoic acid, it is official in USP-NF[7], BP[8] and IP[9]. It is an antiplatelet agent approved by the Food and Drug Administration, USA, for use in secondary prevention of heart attacks and stroke[10,11]. Besides it is mainly used as an analgesic, antipyretic, anti-inflammatory and antithrombotic agent. Clopidogrel (CLOP) (+)-(*S*)-methyl2- (2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*] pyridin-5(4*H*)-yl) acetate (Fig.1B), is a new thienopyridine derivative. It is an antiplatelet agent, which selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and blocks the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation[12]. It is official in USP-NF[13]. It has been shown to prevent ischemic stroke, myocardial infarction and vascular disease and has demonstrated its clinical efficacy superior to that of aspirin. Thus, clopidogrel is indicated for the patients with atherosclerosis documented by recent stroke, recent myocardial infraction or cardiovascular disease[14]. Rosuvastatin (ROS) is the calcium salt of 7-[4-(4-fluorophenyl)-2-[methyl (methylsulfonyl) amino]-6-propan-2-ylpyrimidin-5-yl]-3, 5- dihydroxyhept-6-

enoate (Fig.1C). ROS is a selective and competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate, a precursor of cholesterol. ROS is a member of the class of statins, used to treat hypercholesterolemia and related conditions and to prevent cardiovascular disease. It increases the number of hepatic LDL (Low Density Lipoprotein) receptors on the cell surface to enhance uptake and catabolism of LDL. Secondly, ROS inhibits hepatic synthesis of VLDL (Very Low Density Lipoprotein), which reduces the total number of VLDL and LDL particles[15].The combination of aspirin, rosuvastatin and clopidogrel is available in the market as a capsule and tablet dosage form and is choice of drug for treatment of heart attack prevention. Rosumac gold manufactured by Macleod pharmaceuticals was used for RP-HPLC study. The strengths of the drug in the capsule chosen are aspirin 75 mg, rosuvastatin 10 mg and clopidogrel 75 mg. Several analytical methods that have been reported for the estimation of ASP, CLOP and ROS in biological fluids and/or pharmaceutical formulations individually or combination with other drugs include UV, RP- HPLC and HPTLC[16-23]. Few methods were developed for estimation of these 3 dug in pharmaceutical dosage form by UV and HPLC. But reported method was time consuming, total RT was found to be 16 min[24-26]. Therefore, an attempt was made to develop a new, rapid and sensitive method for the simultaneous determination of ASP, CLOP and ROS with total run time 7.0 min. To access the reproducibility and wide applicability of the developed method, it was validated as per ICH norm, which is mandatory also[27, 28].



(A) (B) (C)

Figure 1 Structure of (A) Aspirin (B) Clopidogrel (C) Rosuvastatin

MATERIALS AND METHODS

Instrument

Liquid chromatographic system from Shimadzu (LC-20AT) comprising of manual injector, double reciprocating plunger pump LC-20ATVp for constant flow and constant pressure delivery and Photodiode array detector SPD-M20A connected to software LC solution for controlling the instrumentation as well as processing the data generated was used.

Chemicals and reagents

Analytically pure sample of ASP, CLOP and ROS was a generous gift from Yash Pharma Laboratories Pvt. Ltd. Mumbai, India. Potassium di hydrogen phosphates (AR grade), Disodium hydrogen phosphate (AR grade), glacial acetic acid and acetonitrile (HPLC Grade) was purchased from E. Merck Ltd. Worli, Mumbai, India. The 0.45µm nylon filters were purchased from Advanced Micro Devices Pvt. Ltd. Chandigarh, India. All excipients used were of pharmaceutical grade. Triple distilled water was generated in house.

Diluents

A mixture of acetonitrile: phosphate buffer pH- 6.0 (40:60 % v/v) was used in RP-HPLC as diluents.

Selection of mobile phase

Initially to estimate ASP, CLOP and ROS simultaneously, number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was acetonitrile: phosphate buffer pH-6.0 (40:60 % v/v), run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by Sonication. Flow rate employed for analysis was 1 ml/min.

Chromatographic conditions

The isocratic mobile phase consisted of acetonitrile: phosphate buffer pH-6.0 in the ratio of 40:60 (v/v), flowing through the column at a constant flow rate of 1.0 ml/min. A Hypersil BDS C_{18} column (5 µm, 150mm × 4.60mm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of method for two drugs, 242nm was selected as the detection wavelength for UV-PDA detector. The HPLC system was operated at room temperature 25°C.

Preparation of stock solution

Accurately weighed 100 mg of ASP, CLOP and ROS were transferred into 100 ml volumetric flasks separately and dissolved in 50 ml of diluent, then volume was made up to 100 ml with diluent to get a concentration of 1000µg/ml (Stock-A) for all three drugs.

Preparation of sub stock solution

5 ml of solution was taken from stock-A of ASP, CLOP and ROS and transferred into 50 ml volumetric flask separately and diluted up to 50 ml with diluent to give concentration of 100µg/ml (Stock-B). From stock-B a series of dilution was made in the range of 7.5-22.5µg/ml for ASP, CLOP and 1-3µg/ml for ROS respectively.

System suitability parameters

Separation variable was set and mobile phase was allowed to saturate the column at 1ml/min. After complete saturation of column, six replicates of reference standard, 15µg/ml and 2µg/ml of ASP, CLOP and ROS respectively were injected separately. Peak report and column performance report were recorded at 242 nm for all chromatogram.

Preparation of calibration curve standards and quality control samples

To establish the linearity of analytical method, a series of dilution ranging from 7.5-22.5µg/ml for ASP, CLOP and 1-3µg/ml for ROS was prepared in the same manner as described above. All the solution were filtered through 0.2 mm membrane filter and injected, chromatograms were recorded at 242 nm and it was repeated for five times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

Stability study

Samples prepared for repeatability study were preserved for 24 h at room temperature and analyzed on the following day to test for short-term stability.

Preparation for analysis of capsule formulation

Twenty capsules were taken and their average weight was determined. They are crushed to fine powder; amount equivalent to 10 mg of ROS was taken in 100-ml volumetric flask. The ASP and CLOP present in this amount of tablet powder was 75 mg and the ratios of all three drugs were 10: 75:75. This was than dissolve in 50 ml of diluent by sonication for about 10 minutes. The volume was made upto the mark by diluents and filtered by Whatmann filter paper (no. 41) and the filtrate was used to prepare samples of different concentration. Now all the tablet samples was scanned in multi photometric mode and the concentration of all three drugs were obtained from the equation.

Validation of method

As per ICH guideline the method was validated and following parameters were evaluated.

Linearity

Linearity of ASP, CLOP and ROS was established by response ratios of drug. The response ratios (response factor) were calculated by dividing the AUC with respective concentration. The curve was plotted between response ratios and concentration which shows the good linearity of drugs in the concentration ranging from 7.5-22.5µg/ml for ASP, CLOP and 1-3µg/ml for ROS respectively.

Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components.

Precision

Precision was determined by repeatability, Intermediate precision and reproducibility of all three drugs.

Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range for ASP, CLOP and ROS that indicates the precision under the same operating condition over short interval time.

Intermediate precision

Day to day precision

Intermediate precision was also performed within laboratory variation on different days for all three drugs simultaneously in five replicate at five concentrations.

Analyst- to- analyst precision

Analyst to analyst variation was performed by different analyst in five replicate at five concentrations.

Reproducibility

The reproducibility was performed by chemical to chemical (use of Rankem chemicals in place of Merck chemicals) variation in five replicate at five concentrations.

Accuracy (% recovery)

This study was carried out using pre analyzed tablet solution. A definite concentration of pure drug was added (80 %, 100 % and 120 % level) and then recovery was studied. A pre analyzed tablet solution containing 7.5µg/ml of ASP 1µg/ml of ROS and 7.5µg/ml of CLOP were taken in 10 ml volumetric flasks and known concentrations of pure drug solution was added to them, which were prepared from standard stock solution of ASP, CLOP and ROS. It was repeated at 5 concentrations and 3 replicate levels. Calculation was done from the label claim and the average weight of the final product.

Robustness

As per ICH norms, small, but deliberate variations in flow rate, pH and concentration of the mobile phase were made to check the method's capacity to remain unaffected.

1. Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.

2. pH of Mobile phase was changed (± 0.2) 6.2 and 5.8.

3. Ratio of Mobile phase was changed (±2) Buffer: Acetonitrile (58:42) and Buffer: Acetonitrile (62:38)

LOD and LOQ

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

RESULTS AND DISCUSSION

Method development

The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of ASP, CLOP and ROS from capsule dosage form. Initially to estimate ASP, CLOP and ROS simultaneously number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter (Table 1) like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was acetonitrile: KH_2Po_4 buffer pH-6.0 (40:60 % v/v), run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min. Separation variable (Table 2) was set and mobile phase was allowed to saturate the column at 1.0 ml/min. After complete saturation of column, six replicates of reference standard, 15µg/ml of ASP, CLOP and 2µg/ml of ROS were injected separately. Peak report and column performance report were recorded. The chromatogram was recorded at 242 nm Figure 2. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph Figure 3-5. The result of their optical characteristics and linearity data of all three drugs has been reported in the Table 3.

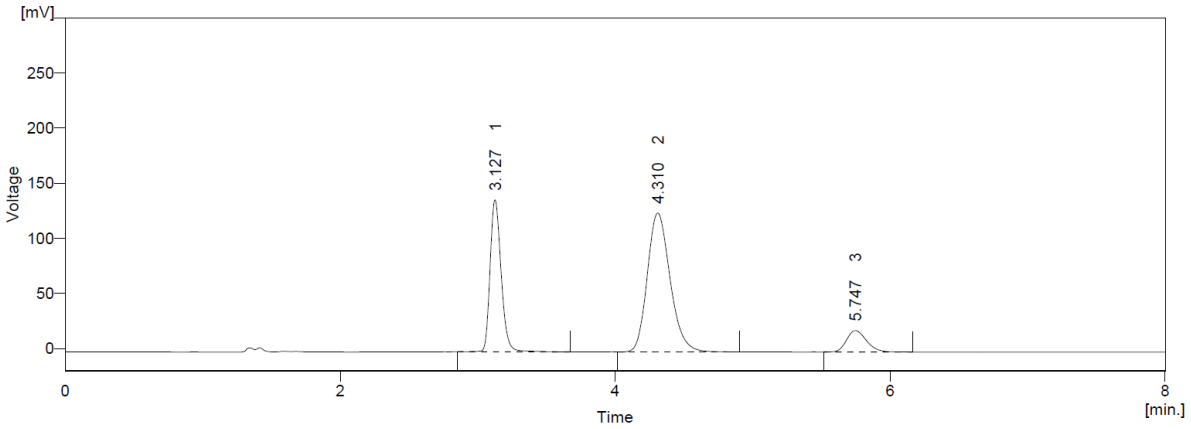


Figure 2: HPLC chromatogram of CLOP 15µg/ml, ROS 2µg/ml and ASP 15µg/ml standard

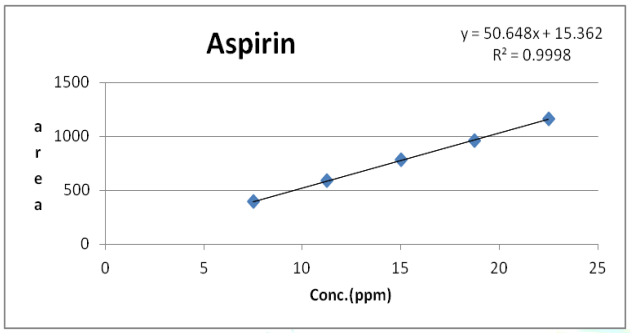


Figure 3: Calibration curve of ASP

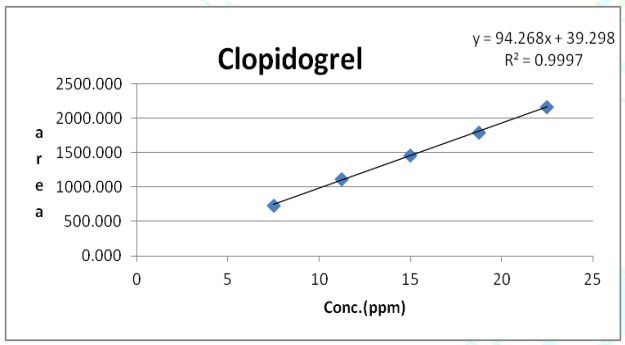


Figure 4: Calibration curve of CLOP

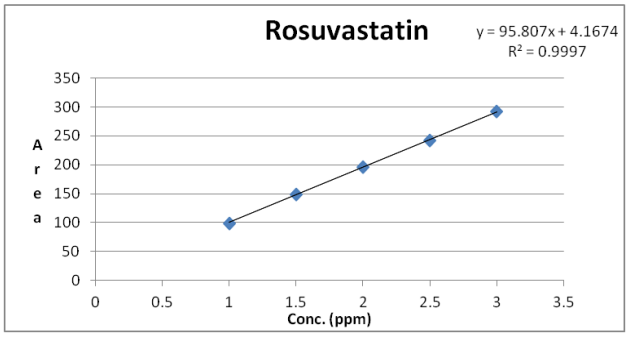


Figure 5: Calibration curve of ROS

Method Validation

Linearity

The proposed method was found to be linear in the range of 7.5-22.5µg/ml and 1-3µg/ml for ASP, CLOP and ROS respectively with correlation coefficient 0.9998, 0.9997 and 0.9997 for ASP, CLOP and ROS respectively. Linearity of ASP, CLOP and ROS were established by response ratios of drug. Response ratio of three drugs was calculated by dividing the absorbance or peak area with respective concentration (Table 4).

Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as matrix components. The result of specificity is shown in Figure 6 and Figure 7 as compare to blank, there was no interference seen in chromatogram.

Precision

Precision of the methods was studied at three levels as at repeatability, intermediate precision (Day to Day and analyst to analyst) and reproducibility (Table 5).

Accuracy

The validity and reliability of proposed methods were assessed by recovery studies. The recovery of added standards (80 %, 100 % and 120 %) was found at five replicate and five concentrations level. The values of % mean just close to 100, SD and % RSD were less than 2 which indicate the accuracy of method. Result of recovery study is shown in Table 6.

Robustness

The robustness of developed method was checked by changing in the deliberate variation in solvent. Result of robustness is shown in Table 7-9.

LOD and LOQ

Detection limit and Quantitation limit of described method were observed as 0.300 mg/ml and 0.909 µg/ml for ASP, 0.372 µg/ml and 1.127 µg/ml for CLOP, 0.050 µg/ml and 0.153 µg/ml for ROS, based on the SD of response and slope, which meet the requirement of new method.

Assay of marketed formulation

The results of the analysis of capsule formulation (Rosumac-Gold) were reported. The assay value of ASP, CLOP and ROS were close to 100, SD and % RSD are less than 2 which indicate that the no interference of excipient in the estimation of ASP, CLOP and ROS was observed. The statistical evaluation of tablet analysis by methods has been reported in Table 10.

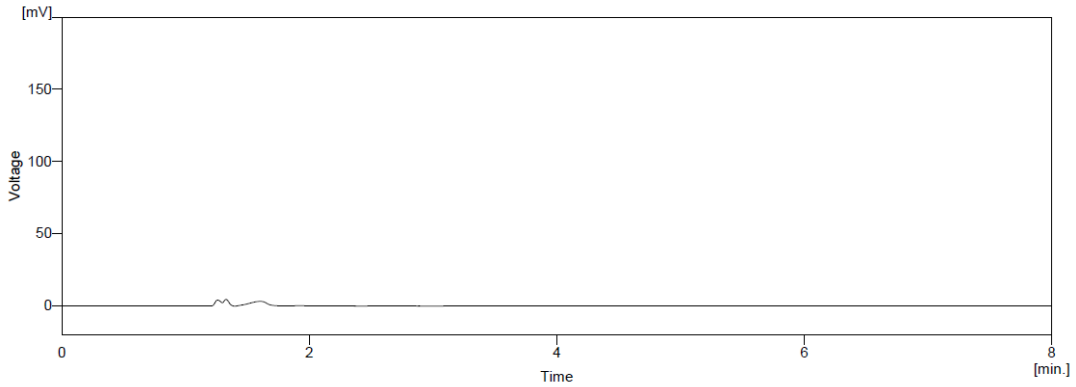


Figure 6: Chromatogram of blank

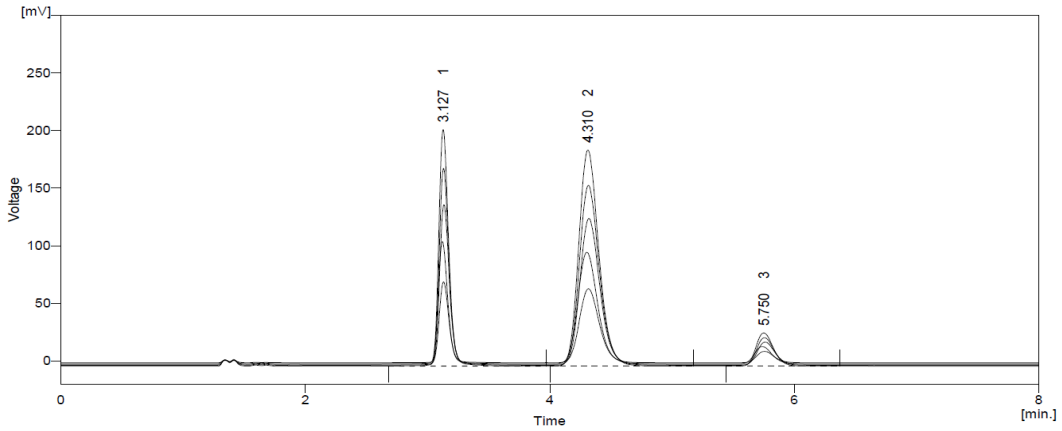


Figure 7: Chromatogram of ASP, CLOP and ROS at 242 nm

Table 1: Results of system suitability parameters

Parameters	ASP	CLOP	ROS
Retention time	3.103 ± 0.5	4,277± 0.5	5.707 ± 0.5
Number of theoretical plates	7103	3127	7047
Asymmetry	1.333	1.349	1.389
Resolution	-	5.178	4.950
HETP	0.036±0.003	0.080±0.005	0.036±0.004
Tailing factor	1.10 ± 0.017	1.60 ± 0.02	1.85 ± 0.03

Table 2: Separation variable of RP-HPLC method

Variable	Condition
Column	
Dimension	250 mm x 4.60 mm
Particle size	5 µ
Bonded phase	Octadecylsilane (C_{18})
Mobile phase	
Acetonitrile	40%
Phosphate buffer (pH- 6.0)	60%
Diluent	ACN: phosphate buffer pH-6.0 (40:60 v/v)
Flow rate	1.0 ml/min
Temperature	25^0C
Sample size	20 µl
Detection wavelength	242 nm
Retention time	
ASP	3.103 ± 0.5 min
CLOP	4.277± 0.5 min
ROS	5.707 ± 0.5min

Table 3: Optical characteristics and linearity data of ASP, CLOP and ROS

S. No.	Parameters	RP-HPLC Method		
		ASP	CLOP	ROS
1	Working λ	242	242	242
2	Concentration (µg/ml)	7.5-22.5	7.5-22.5	1-3
3	Correlation Coefficient (r^2)*	0.9998	0.9997	0.9997
4	Slope (m)*	50.648	94.268	95.807
5	Intercept (c)*	15.362	39.298	4.167

*Average of five determinations

Table 4: Response ratios of ASP, CLOP and ROS

	Concentration (µg/ml)			RP-HPLC Method					
				ASP		CLOP		ROS	
	ASP	CLOP	ROS	AUC	RR	AUC	RR	AUC	RR
1	7.5	7.5	1	391.770	52.24	737.476	98.33	99.041	99.05
2	11.25	11.25	1.5	589.880	52.44	1110.143	98.68	149.238	99.49
3	15	15	2	777.287	51.82	1460.509	97.37	196.454	98.23
4	18.75	18.75	2.5	960.235	51.22	1796.795	95.83	241.987	96.80
5	22.5	22.5	3	1156.244	51.39	2161.677	96.08	292.183	97.40

Table 5: Results of precision

Parameter	MEAN ± SD*			% RSD*		
	ASP	CLOP	ROS	ASP	CLOP	ROS
Repeatability	766.039 ±6.151	1440.355±9.749	193.717 ±1.407	0.803	0.677	0.726
Intermediate precision						
Day to day	772.891±1.812	1450.116±6.167	195.342±0.457	0.234	0.425	0.234
Analyst to Analyst	770.302±6.662	1444.768±12.82	194.136±2.583	0.865	0.887	1.331
Reproducibility	768.302±3.362	1445.106±4.167	195.142±0.257	0.334	0.625	0.534

*Value of five replicate and five concentrations

Table 6: Results of recovery study

% Level			% Mean ± SD*			% RSD*		
ASP	CLOP	ROS	ASP	CLOP	ROS	ASP	CLOP	ROS
80%	80%	80%	101.44 ± 1.31	100.52±1.02	101.44 ±1.30	1.29	1.01	1.28
100%	100%	100%	100.61 ± 0.35	99.68 ± 0.48	99.53 ± 1.26	0.34	1.26	1.26
120%	120%	120%	100.47 ± 0.43	99.49 ± 1.01	100.15 ± 0.43	0.42	0.42	0.42

*Value of five replicate and five concentrations

Table 7: Robustness data for ASP

S. NO.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at pH (- 0.2)	Area at pH (+ 0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	791.799	750.453	755.756	782.697	788.641	757.377
2	789.433	748.149	744.792	772.638	784.491	746.286
3	793.253	742.879	746.564	788.252	789.617	748.982
% R.S.D	0.244	0.520	0.786	1.013	0.346	0.770

Table 8: Robustness data for CLOP

S. NO.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	1489.304	1409.786	1420.121	1470.654	1482.282	1422.862
2	1481.987	1405.584	1406.366	1469.489	1485.583	1414.605
3	1490.791	1388.418	1403.054	1481.036	1485.181	1407.215
% R.S.D	0.317	0.808	0.642	0.431	0.121	0.553

Table 9: Robustness data for ROS

SR NO.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at pH (- 0.2)	Area at pH (+ 0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	200.307	189.632	190.997	197.808	199.378	191.379
2	198.056	189.070	190.247	198.015	200.368	190.809
3	200.508	186.485	188.723	199.201	199.758	189.267
% R.S.D	0.682	0.891	0.610	0.379	0.250	0.574

Table 10: Analysis on marketed formulation

Capsule	Rosumac Gold		
Label claim	CLO (75mg)	ROS (10mg)	ASP (75mg)
Assay (% of label claim*) Mean ± S. D.	99.060±0.384	99.485±0.311	100.036±0.679

CONCLUSION

In summary, we have developed and validated a rapid, specific, reproducible RP-HPLC method to quantify ASP, CLOP and ROS simultaneously. So far no published methods are available for the simultaneous quantification of these three drugs in capsule dosage form. To the best of our knowledge, this is the first time that all three analytes were estimated simultaneously in any of the capsule dosage form. The cost-effectiveness, simplicity of the assay is that sample turnover rate of less than 7 minutes per sample; make it an attractive procedure in high-throughput analysis of ASP, CLOP and ROS. From the results of all the validation parameters, we can conclude that the developed method can be useful for routine analysis and therapeutic drug monitoring with desired precision and accuracy.

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