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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE BY RP-HPLC

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ABSTRACT: A novel, precise, accurate, rapid and cost effective isocratic reverse-phase high performance liquid chromatographic (RP-HPLC) method was developed, optimized and validated for the estimation of Arterolane maleate and Piperaquine phosphate in pharmaceutical dosage forms (tablet). The drugs were estimated using hypersil C18 (250 mm x 4.6 mm i.d- 5 µm particle size) column. A mobile phase composed of phosphate buffer, acetonitrile, methanol in proportion of 40:30:30 v/v, at a flow rate of 1.0 ml/min was used for the separation. Detection was carried out at 244 nm. The linearity range obtained was 20-70 µg/ml for ART and 100-350 µg/ml for PIP with retention times ($_R$t) of 3.353 min and 2.389 min for ART and PIP respectively. The correlation coefficient values were found to be 0.999. Precession studies showed % RSD values less than 2 % for both the drugs in all the selected concentrations. The percentage recoveries of ART and PIP were in the range of 99.47-100.72% and 99.30-100.18% respectively. The assay results of ART and PIP were 99.57% and 99.75 % respectively. The limit of detection (LOD) and limit of quantification (LOQ) were 0.172 µg/ml 0.524 µg/ml for ART and 0.542 µg/ml and 1.641 µg/ml for PIP respectively. The method was validated as per the International Conference on Harmonization (ICH) guidelines. The proposed validated method was successfully used for the quantitative analysis of commercially available dosage form.

INTRODUCTION: Arterolane maleate (**fig. 1**) is chemically cis- admantane-2-spiro-3'-8'-[[[(2'-amino-2'methyl Propyl) amino] carbonyl] methyl] 1'2'4'-trioxaspiro [4, 5]decane hydrogen maleate. Arterolane maleateis a Synthetic peroxide which acts as anti-malarial agent by rapidly acting as blood schizonticide against all blood stages of P. *falciparum* without having effect on liver stages. Arterolane acts by inhibition of PfATP6, a sarcoplasmic endoplasmic reticulum calcium ATPase encoded by *P. falciparum*. Arterolane accumulates in the parasite's food vacuole whereas this is not a feature of the subcellular distribution of artemisinins.[1]

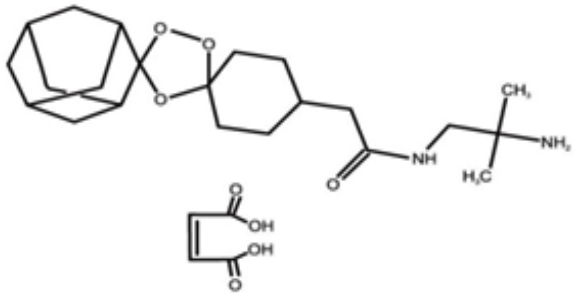

FIG. 1: STRUCTURE OF ARTEROLANE MALEATE

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Piperaquine phosphate (**fig. 2**) is chemically 1, 3-Bis (4-(7-chloroquinolin-4-yl)piperazin-1-yl) propane tetraphosphate tetrahydrate. Piperaquine phosphate is bisquinoline anti-malarial drug which shows good activity against chloroquine-resistant Plasmodium strains. Evidence suggests the inhibition of the heame-digestion pathway in the parasite food vacuole is most convincing. Piperaquine's bulky bisquinoline structure may be important for activity against chloroquine resistant strains and may act by inhibition of the transporters that efflux chloroquine from the parasite food vacuole [2].

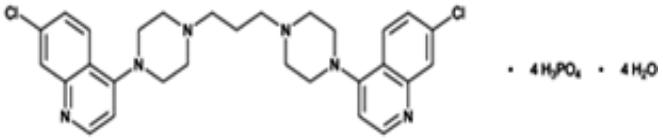

FIG. 2: STRUCTURE OF PIPERAQUINE PHOSPHATE

Arterolane maleate and Piperaquine phosphate in combination are available in tablet dosage forms in the ratio of 150:750. Literature survey reveals Automated Solid Phase Extraction and Liquid Chromatographic Method [3], Capillary Zone Electrophoresis [4], HPLC [5] and LC/MS/MS [6-12] methods for the estimation of Piperaquine phosphate alone or in combination with other drugs in pharmaceutical formulation and biological samples where as headspace gas chromatographic [13] methods for the estimation of Arterolane maleate alone in pharmaceutical formulation and biological samples. There are very few reported methods for the simultaneous determination of Arterolane maleate and Piperaquine phosphate by RP-HPLC in pharmaceutical dosage forms [14, 15].

The apparent lack of simple method for the estimation of Arterolane maleate and Piperaquine phosphate by RP-HPLC in pharmaceutical dosage forms prompted us to develop this method. The proposed method was optimized and validated as per the International Conference on Harmonization (ICH) guidelines [18-22].

MATERIALS AND METHODS:

Instrumentation: To develop a high pressure liquid chromatographic method for quantitative estimation of Arterolane maleate & Piperaquine phosphate using Waters HPLC system a Hypersil C18 column (250 mm x 4.6 mm, 5µ) was used. The instrument is equipped with an auto sampler and DAD or UV detector. A 20 µL rheodyne injector port was used for injecting the samples. Data was analyzed by using Empower 2 software.

Chemicals and solvents

The working standard of Arterolane maleate and Piperaquine phosphate were provided as gift samples from Active Pharma Labs, Hyderabad, India. The market formulation SYNIRIM tablets (Arterolane maleate (150mg) and Piperaquine Phosphate (750mg)) were procured from local market. HPLC grade water, methanol [HPLC Grade] and Acetonitrile [HPLC Grade] were purchased from E.Merck (India) Ltd, Mumbai, India. Potassium dihydrogen phosphate and orthophosphoric acid of AR grade were obtained from S.D. Fine Chemicals Ltd, Mumbai, India.

Chromatographic conditions

Column : Hypersil C18 (250 mm x 4.6 mm i.d., 5 µm particle sizes)

Mobile phase : Phosphate buffer, acetonitrile, Methanol in proportion of 40:30:30 v/v

Detector : 244nm

Injection volume : 20µl

Flow rate : 1ml/min

Temperature : Ambient

Run time : 7min

Diluent : Methanol

Preparation of Phosphate buffer: 2.72 grams KH_2PO_4 was weighed accurately and transferred into a 1000 ml volumetric flask. About 500 ml water (HPLC grade) was added to dissolve KH_2PO_4. After complete dissolution final volume was adjusted to 1000 ml with same water. pH of the buffer was checked and adjusted to 3 with Ortho phosphoric acid.

Preparation of mobile phase: Mobile phase was prepared by mixing 400 ml phosphate buffer (pH 3), 300 ml Acetonitrile [HPLC grade] and 300ml of

Methanol. Above mixture (40:30:30) was degassed in an ultrasonic water bath for 5 minutes and filtered through 0.45 µ filter under vacuum.

Diluent Preparation: Methanol was used as diluent.

Preparation of the Arterolane maleate and Piperaquine phosphate Standard & Sample Solution:

Standard Solution Preparation: The standard solutions were prepared by weighing accurately 10 mg of Arterolane maleate and 10 mg of Piperaquine phosphate [working standards] and transferred into two 10 mL clean dry volumetric flasks. About 7 mL of methanol was added to each flask and sonicated to dissolve the powders completely. Final volumes were adjusted to the mark with methanol. From the Stock solutions 0.4 ml Arterolane maleate and 2.0 ml Piperaquine phosphate solutions were transferred into 10ml volumetric flasks and diluted up to the mark with same diluent.

Sample Solution Preparation: Accurately weighed 20 tablets, average weight is taken and powdered. Amount equivalent to Arterolane maleate 100 mg and Piperaquine phosphate 500 mg was accurately weighed and taken in a 100 ml volumetric flask and 50 ml of methanol was added. The mixture was then subjected to sonication for 20 min with intermediate shaking for complete extraction of drugs, filtered through a Whatmann filter paper and cooled to room temperature and solution was made up to mark with methanol. From the above filtrate, pipetted out 0.4 ml into a 10 ml volumetric flask and diluted with methanol

Injection of Standards and Samples into the Chromatographic system: 20 µL of each standard and sample solution were injected into the chromatographic system and measured the areas of Arterolane maleate and Piperaquine phosphate peaks. % Assay of both the drug was calculated using the appropriate formulae.

Assay Calculation for Arterolane maleate and Piperaquine phosphate:

Percentage purity=

$$\frac{Spl_{area} \times Std_{dil} \times Avg.\ wt \times P \times 100}{Std_{area} \times Spl_{dil} \times L.C}$$

Where, P= (%) potency of working standard use, L.C. = Label claim, Avg. Wt = Average weight of tablets.

The assay results, expressed as % of the label claim, are in **table 1**. This indicates that the amount of each drug in the product meets the requirements.

TABLE 1: ASSAY OF ARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE

Drug % Assay Amount in mg	Label recovered in mg	Sample Peak area	Standard Peak area	Standard Purity	Assay %
Arterolane maleate	150mg	520157.33	520295	99.60%	99.57%
Piperaquine phosphate	750mg	2015320	2016301	99.80%	99.75%

Method Validation:

1) **System Suitability:** System Performance parameters of developed HPLC method were determined by injecting standard solutions. Parameters such as number of theoretical plates (N), tailing factor, resolution(R), retention time (RT) were determined. The results are shown in **table 2**, it indicates good performance.

TABLE 2: SYSTEM SUITABILITY PARAMETERS OF ARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE

Drug	Theoretical plates	Tailing factor	Retention time	Resolution
Arterolane maleate	5852	1.72	2.389	5.314
Piperaquine phosphate	3051	1.23	3.353	

2) **Linearity:** The linearity of the analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample.

Preparation of stock solution: The stock solutions were prepared by weighing accurately and transferred 25 mg Arterolane maleate and 25 mg Piperaquine phosphate [working standard] into two 25 mL clean dry volumetric flasks. About 15 mL of diluent was added to each flask and sonicated to dissolve the powders completely. Final volumes were adjusted to the mark with the same solvent. Above prepared Stock Solutions were used for the further dilution to prepare the following Levels:

a. **Preparation of Level – I (20 ppm of** Arterolane maleate **& 100 ppm** Piperaquine phosphate**):** 0.2 ml Arterolane maleate and 1ml Piperaquine phosphate stock solution was transferred into a 10 ml volumetric flask and diluted up to the mark with diluent.

b. Preparation of Level – II (30 ppm of Arterolane maleate **& 150 ppm** Piperaquine phosphate**):** 0.3 ml Arterolane maleate and 1.5 ml Piperaquine phosphate stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluent

c. **Preparation of Level – III (40 ppm of** Arterolane maleate **& 200 ppm** Piperaquine phosphate**):** 0.4 ml Arterolane maleate and 2 ml Piperaquine phosphate stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluent

d. **Preparation of Level – IV (50 ppm of** Arterolane maleate **& 250 ppm** Piperaquine phosphate**):** 0.5 ml Arterolane maleate and 2.5 ml Piperaquine phosphate stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluent

e. **Preparation of Level – V (60 ppm of** Arterolane maleate **& 300 ppm** Piperaquine phosphate**):** 0.6 ml Arterolane maleate and 3 ml Piperaquine phosphate stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluents

f. **Preparation of Level – VI (70 ppm of** Arterolane maleate **& 350 ppm** Piperaquine phosphate**):** 0.7 ml Arterolane maleate and 3.5 ml Piperaquine phosphate stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluent.

Injecting the Solutions to the Chromatographic System: Each level of solution was injected to the chromatographic system and the peak area was measured. The calibration curves **(fig.3&4)** were constructed by plotting absorbance versus concentration and the regression equations were calculated**.** The results are shown in **tables 3& 4**.

Acceptance Criteria: The Correlation coefficient should be not less than 0.99.

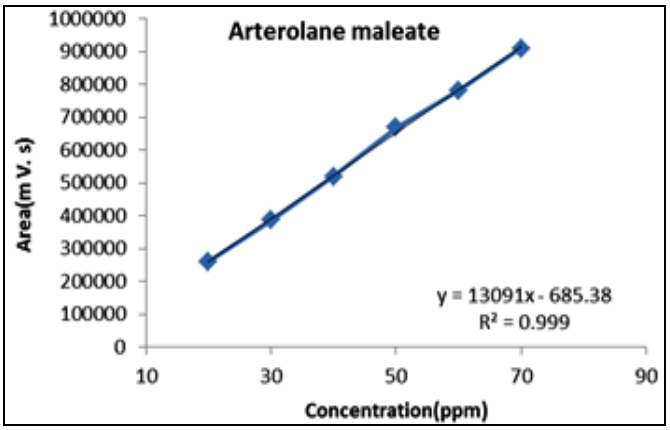

FIG. 3: LINEARITY CURVE FOR ARTEROLANE MALEATE

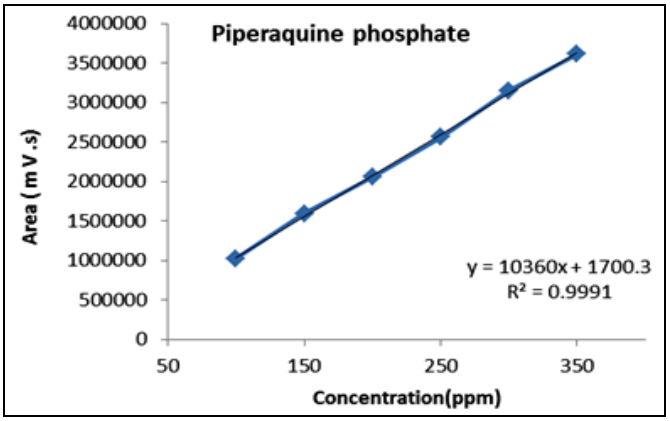

FIG. 4: LINEARITY CURVE FOR PIPERAQUINE PHOSPHATE

TABLE 3: LINEARITY OFARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE BY RP-HPLC

S. No.	Conc. Taken in µg/ml (pip)	Conc. Taken in µg/ml (ART)	Peak area of ART	Peak area of PIP
1	100	20	261038	1023409
2	150	30	387187	1591891
3	200	40	520157	2056201
4	250	50	669381	2561485
5	300	60	781834	3148919
6	350	70	910745	3614079

3) Precision: The precision of an analytical procedure expresses the closeness of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

a. **System Precision:** The system precision was carried out to ensure that the analytical system is working properly. The results obtained are tabulated as **table 5**.

b. **Method Precision:** In method precision, a homogenous sample of a single batch should be analyzed five times and was checked whether the method is giving consistent results for a single batch. The results are tabulated as **table 6.**

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

Acceptance Criteria: The % RSD for the area of five standard injections results should not be more than 2 %.

TABLE 5: SYSTEM PRECISION OF ARTEROLANEMALEATEANDPIPERAQUINE PHOSPHATE

S. No.	Arterolane maleate		Piperaquine phosphate	
	Rt	Area	Rt	Area
1	3.392	520943	2.389	2059870
2	3.378	519678	2.391	2057890
3	3.356	525154	2.369	2051321
4	3.389	520780	2.381	2048765
5	3.389	520165	2.351	2055876
AVG	3.38	521344	2.3762	2050744
STDEV	0.0148	2188.641	0.0165	4607.602
%RSD	0.439	0.4198	0.695	0.2246

TABLE 6: METHOD PRECISION OF ARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE

S. No.	Arterolane maleate		Piperaquine phosphate	
	Rt	Area	Rt	Area
1	3.389	519980	2.391	2058987
2	3.378	518970	2.398	2056741
3	3.389	520143	2.379	2059184
4	3.396	524356	2.365	2051029
5	3.369	522522	2.347	2053643
AVG	3.3828	521194.2	2.376	2055916
STDEV	0.01033	2195.814	0.02049	3529.9
%RSD	0.3054	0.4213	0.87	0.1717

4) Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and value found.

Preparation of Sample solutions:

a. **Preparation of 50 % solution (with respect to target Assay concentration):** 0.2 ml Arterolane maleate and 1 ml Piperaquine phosphate stock solution was transferred into a 10 ml volumetric flask and diluted up to the mark with diluent.

b. **Preparation of 100 % solution (with respect to target Assay concentration):** 0.4 ml Arterolane maleate and 2 ml Piperaquine phosphate stock solution was transferred into a 10 ml volumetric flask and diluted up to the mark with diluent.

c. **Preparation of 150 % solution (with respect to target Assay concentration):** 0.6 ml Arterolane maleate and 3 ml Piperaquine phosphate stock solution was transferred into a 10 ml volumetric flask and diluted up to the mark with diluent.

Procedure: The standard solution was injected with Accuracy 50%, 100% and 150% solutions. The amount found was calculated and amount added for Arterolane maleate and Piperaquine phosphate was estimated. The individual recovery and mean recovery values were also calculated in **table 7**.

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

TABLE 7: ACCURACY AND % RECOVERY OF EACH ANALYTE

Accuracy Level %	Mean recovery of Arterolane maleate (%)	Mean recovery of Piperaquine phosphate (%)
50	100.72	99.30
100	99.47	99.44
150	100.39	100.18

5) **Robustness:** The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameter and provides an indication of its reliability during normal usage. As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The typical variations are given below: Variation in flow rate by ± 0.2ml/min. Variation inorganic composition in the Mobile phase varied from ± 10%.

The results are shown in **table 8**.

TABLE 8: ROBUSTNESS PARAMETERS OF ARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE

S. No.	Parameter	Arterolane maleate			Piperaquine phosphate		
		Rt	Area	Tailing Factor	Rt	Area	Tailing Factor
1	Initial Sample	2.389	520567	1.345	3.353	2059780	1.445
2	Flow (+0.2ml/min)	2.254	517896	1.465	3.181	2018743	1.570
3	Flow (-0.2ml/min)	2.723	537865	1.790	3.755	2107432	1.290
4	Organic phage 10% more	2.431	539873	1.323	3.399	2176541	1.641
5	Organic phage 10% less	2.466	520987	1.179	3.521	2047623	1.279

6) **Ruggedness:** The ruggedness of an analytical method is determined by analysis of aliquots from homogenous lots by different analysts using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The assay was performed in different conditions, different analysts, and different days. The results are given in **table 9**.

TABLE 9: RUGGEDNESS OF ARTEROLANE MALEATE AND PIPERAQUINE PHOSPHATE

S. No.	Drug	Analyst-1 (area)	Analyst-2 (area)	SD	%RSD (Limit NMT 2.0%)
1	Arterolane maleate	520789	519876	645.5885	0.124
2	Piperaquine phosphate	2059765	2051876	5578.365	0.2713

7) **Limit of Detection (LOD):** The limit of detection (LOD) is the smallest concentration that can be detected but not necessarily quantified as an exact value. LOD is calculated from the formula; $LOD = 3.3\sigma / S$

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

8) **Limit of Quantitation (LOQ):** The limit of quantitation is the lowest amount of analyte in the sample that can be quantitatively determined with precision and accuracy. LOQ is calculated from formula;

$$LOQ = 10\sigma / S$$

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

LOD, LOQ are shown in the **table10.**

TABLE 10: LOD & LOQ OF ARTEROLANEMALEATEANDPIPERAQUINE PHOSPHATE

Drug	LOD	LOQ
Arterolane maleate	0.172	0.542
Piperaquine phosphate	0.524	1.641

TABLE 11: SUMMARY OF SYSTEM SUITABILITY AND VALIDATION PARAMETERS OF ART AND PIP

Parameter	Results	
	ART	**PIP**
Linearity range (µg/mL)	20-70 µg/ml	100-350 µg/ml
Correlation coefficient	0.9990	0.9991
Theoretical plates (N)	5852	3051
Tailing factor	1.72	1.23
LOD (µg/mL)	0.172	0.542
LOQ (µg/mL)	0.524	1.641

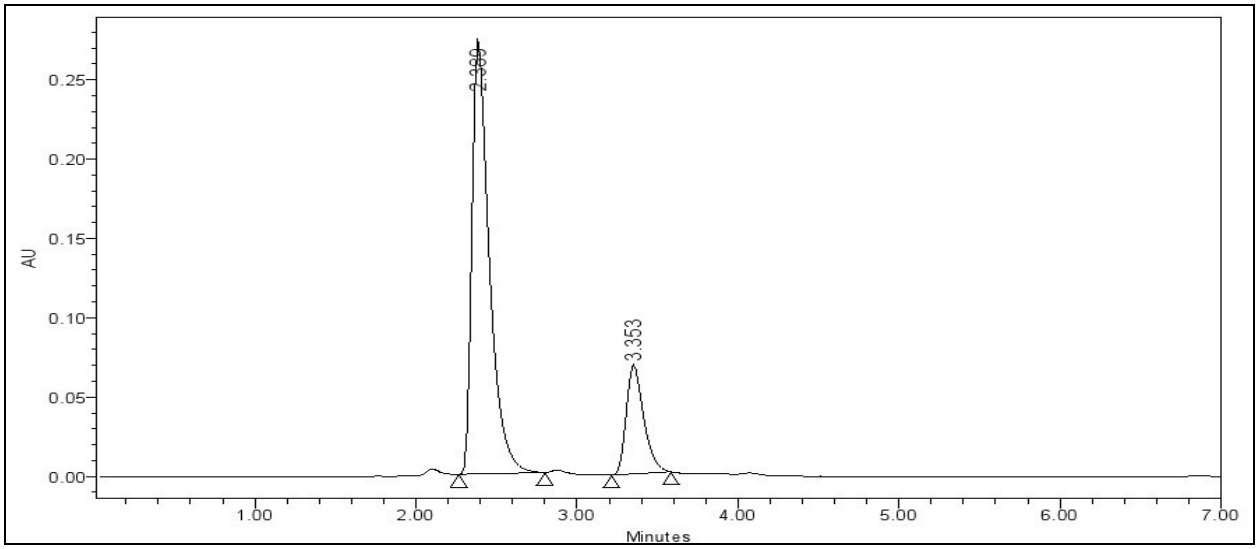

FIG. 5: THE CHROMATOGRAM REPRESENTINGTHEWELLRESOLVED PEAKS OF ART & PIP

RESULTS & DISCUSSION: The objective of the proposed work was to develop method for the determination of Arterolane maleate and Piperaquine phosphate and to validate the method according to USP and ICH guidelines and applying the same for its estimation in pharmaceutical formulations. Initially, various mobile phase compositions were tried to elute title ingredient. Mobile phase and flow rate selection was based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor, run time, resolution).

C-18 (250mm X4.6mm,5µ)column and mobile phase comprising of phosphate buffer, acetonitrile, methanol in proportion of 40:30:30 v/v. Different mobile phases were tried and mobile phase used was phosphate buffer: methanol: acetonitrile which satisfactorily gives symmetrical and well resolved Arterolane maleate and Piperaquine phosphate. The retention time for Arterolane maleate and Piperaquine phosphate were 3.353 min and 2.389 min respectively flow rate kept at 1ml/min and PDA detection performed at λmax 244 nm.

The method was validated as per ICH guidelines linearity for detector was observed in 20-70 µg/ml for ART and 100-350 µg/ml for PIP respectively. Percentage recovery of both drug was found in range 99.30 – 100.72 % & 99.30 -100.18 % indicating accuracy of proposed method the intra-day and inter day coefficient for Arterolane maleate and Piperaquine phosphate were found to be 0.38-0.48%, 0.52 -0.47% and 0.04- 0.43%, 0.0078%, the percentage RSD for both the tablet analysis and recovery studies is less than 2% indicating high degree of precision. The results of robustness study also indicates that the method is robust and is unaffected by small variation in chromatographic condition. It was observed that excipient present in formulation did not interfere with peaks of Arterolane maleate and Piperaquine phosphate.

CONCLUSION: From all results, it was concluded that the developed RP-HPLC method is simple, sensitive, accurate, precise, and selective. Percentage recovery shows that the method is free from interference of excipients used in the formulation.

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