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Analytical Method Development and Validation of Combination of "Piperaquine Phosphate" And "Arterolane Maleate" By Using "Rp-Hplc" Technique

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ABSTRACT: -
A New, Rapid, Accurate, Precise,reproducible reverse phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the simultaneous estimation of Piperaquine Phosphate and Arterolane Maleate in Combined dosage form (tablet).The chromatographic separation was achieved on C18 Column (4.6mm×150mm) particle packing size is 5µm. as a Mobile phase Methanol and Water (0.1% with acidic acid 50:50%) at flow rate of 0.9ml/min and UV detection wavelength used was 223nm.The Linearity over the concentration range of 75-375µ/mL and 15-75µ/mL for Piperaquine phosphate and Arterolane maleate. Correlation coefficient value was found to be (r^2= 0.998) for Piperaquine Phosphate and (r^2=0.999) for Arterolane maleate. The % recovery of the method was found to be within 98-101% Respectively. The Limit of detection and Limit of quantification were 0.624 µ/mL and 1.891 µ/mL for Arterolane maleate and 2.79 µ/mL and 8.45 µ/mL for Piperaquine phosphate. The present successfully validated and developed method was perform as per the ICH guidelines.

KEYWORDS: -Validation, RP-HPLC, Arterolane Maleate, Piperaquine Phosphate, Simultaneous Estimation.

I. INTRODUCTION: - PIPERAQUINE PHOSPHATE

Piperaquine phosphate is an Antiparasitic Drug used in combination with dihydroartemisinic to treat Malaria. Piperaquine kills the parasites by disrupting the detoxification of host heme. Piperaquine has a slow and longer schizontocidal activity against erythrocytic Stages of both Plasmodium Vivax and Plasmodium Falciparum and chloroquine-resistant plasmodium strains. Piperaquine phosphate it is highly soluble in chloroform, slightly soluble in ethanol and almost insoluble in water. Piperaquine phosphate (PQP) is chemically known as 1, 3-bis [4-(7-chloroquinoline-4-yl) piperazin-1-yl] propane. it is characterized as white or yellow crystalline power odourless and tasteless.

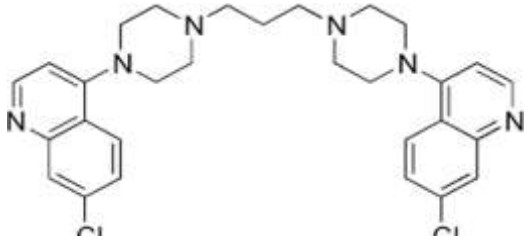

Fig No 1: - Chemical Structure of Piperaquine Phosphate

ARTEROLANE MALEATE: -Arterolane Maleate + Piperaquine Phosphate belongs to the class of medications called 'Anti- Malarial Primary used to treat Malaria by acting as a blood schizonticide against allblood stages of P. falciparum without having effect on liver stages.Arterolane maleate + Piperaquine Phosphate gives a degree of protection (prophylaxis) against malaria when people travel to areas where material cases exist. Arterolane maleate is chemically known as [(N-(2-amino-2-methylpropyl)-2-cis-dispiro (adamantane-2,3'- [1,2,4] trioxolane-5',1"-cyclohexan)-4"-yl] acetamide. Arterolane Maleate is one of first fully synthetic trioxolane peroxide, non - artemisinin antimalarial compound. It has rapid schizonticidal activity against all erythrocytic

International Journal of Pharmaceutical Research and Applications
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stages of Plasmodium falciparum without any effect on hepatic stages. This action of arterolane is attributed to inhibition of heme detoxification and PF- encoded sarcoplasmic endoplasmic reticulum calcium ATPase.

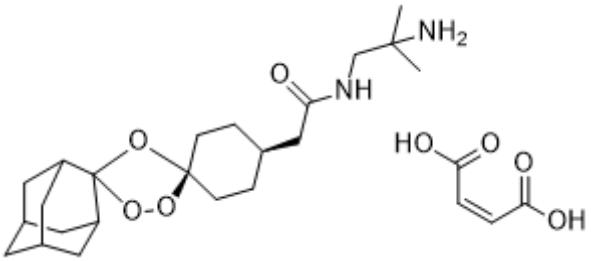


Fig No2: - Chemical Structure of Arterolane Maleate

II. MATERIALS AND METHODS : -

Selection and Procurement of Drug

Drug sample supplier

Table 1: Drug and Drug Supplier

Sr.No.	Name of Drug	Grade	Drug Supplier
1.	Piperaquine Phosphate	API	Swapnaroop drug and pharmaceutical
2.	Arterolane Maleate	API	Swapnaroop drug and pharmaceutical

List of reagents & chemicals used

Table 2: List of Reagents and Chemicals used

Sr. No.	Name of chemicals	Grade	Manufacturer.
1.	Methanol	HPLC	Merck Specialties Pvt. Ltd. Shiv Sagar Estate 'A' Worli, Mumbai
2.	Acetic Acid	HPLC	Avantor Performance Material India Ltd. Thane, Maharashtra
3.	Water	HPLC	Merck Specialties Pvt. Ltd. Shiv Sagar Estate 'A' Worli, Mumbai

Instruments and Equipment's

Table No.3.: Instrument (HPLC) Details used during Method Development

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector (Chemstation 10.1 software)
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C_8)	Waters C_{18} (150mmX 4.6mm,5µm)

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

4	pH meter	VSI pH meter (VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.
6	Sonicator	Ultrasonics' electronic instrument

a) Chromatographic Conditions:

Table No.4.: Chromatographic conditions (HPLC) details used during method Development

1.	HPLC	Agilent Tech. Gradient System with Auto injector
2.	Software	Chemstation 10.1 software
3.	Column	(Waters) C18 column (4.6mm x 150mm)
4.	Particle size packing	5 µm
5.	Stationary phase	C-18 (Waters)
6.	Mobile Phase	Methanol: Water (0.1% with Acetic Acid) 50:50 %
7.	Detection Wavelength	223 nm
8.	Flow rate	0.9 ml/min
9.	Temperature	Ambient
10.	Sample size	20 µl
11.	pH	3.0
12.	Run Time	15 min
13.	Filter paper	0.45 µm

METHOD DEVELOPMENT OF HPLC:

Table No.5: Selection of mobile Phase.

Sr.No.	Mobile Phase
1.	Methanol+ Water Acetic Acid 0.05% (90:10 % v/v)0.7ml 223 nm.
2.	Methanol+ Water Acetic Acid 0.05% (90:10 % v/v) 1 ml 223 nm sample in methanol.
3	Methanol+ Water Acetic Acid 0.05% (90:10 % v/v) 0.7 ml 223 nm sample in mobile phase.
4	Methanol+ Water Acetic Acid 0.05% (80:20 % v/v) 0.7 ml 223 nm
5	Methanol+ Water Acetic Acid 0.05% (60:40 % v/v) 0.7 ml 223 nm sample in Mobile phase
6	Methanol+ Water Acetic Acid 0.05% (50:50% v/v) 0.7ml 223 nm sample in Mobile phase
7	Methanol+ Water Acetic Acid 0.05% (38:62% v/v) 0.7ml 223 nm sample in mobile phase
8	Methanol+ Water Acetic Acid 0.05% (50:50% v/v) 0.7ml 223 nm column 150mmX 4.6 (Water)

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

Selection of wavelength by UV-Visible Spectrophotometry: -

Preparation of standard stock solution: -

- **Piperaquine standard stock solution: (Stock I)**

An accurately weighed quantity, 75 mg of Piperaquine (PIPERA) was dissolved in methanol in a 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 7500 ug/ml.

- **Arterolane standard stock solution: (Stock II)**

An accurately weighed quantity, 15 mg of Arterolane (ARTE) was dissolved in methanol in 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 1500 ug/ml.

- **Preparation of Stock Standard Combination Solution :(Stock III) [PIPERA + ARTE]**

Accurately weight and transfer 75 mg Piperaquine and Arterolane 15 mg working standard into 10 ml volumetric flask as about diluent methanol completely and make volume up to the mark with the same solvent to get 7500 µg/ml standard for Piperaquine and 1500 µg/ml for Arterolane (stock solution) and 15 min sonicate to dissolve it and remove the unwanted gas, further an aliquots portion of Piperaquine and Arterolane stock solution in ratio of 1:5 were mixed in volumetric flask in 10 ml and volume was adjusted up to mark with mobile phase from the resulting solution 0.1 ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Methanol :Water (0.1% Acetic Acid), prepared in (5 ml Methanol: 5 ml Water (0.1% Acetic Acid) solvent .

HPLC used for chromatographic condition applies on the Preparation of standard solution: -

- **Preparation of std. Piperaquine solution: (StockI)**

From the freshly prepared standard stock solution (7500 ug/ml), 0.1-0.5 ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 75-375 ug/ml.

- **Preparation of std. Arterolane solution: (StockII)**

From the freshly prepared standard stock solution (1500ug/ml), 0.1-0.5 ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 15-75 ug/ml.

- **Preparation of std. Piperaquine and Arterolane solution :(Stock III)**

From the freshly prepared standard stock solution (7500 ug/ml) Piperaquine, and (1500 ug/ml) Arterolane, 0.1-0.5 ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 75-375 ug/ml for Piperaquine and Arterolane 15-75 ug/ml respectively.

Selection of mobile phase:-Various mobile phases tried, mobile phase containing Methanol and Water (0.1% Acetic Acid) was selected since it gave sharp.

Studies of Calibration plot: -

Optimization of Chromatographic condition:

Column	: C18 (150 mm× 4.6mm)
Particle size packing	: 5µm
Detection wavelength	: 223 nm nm
Flow rate	: 0.9 ml/min
Temperature	: Ambient
Sample size	: 20 µl
Mobile phase	:Methanol: Water (0.1% Acetic Acid) (50: 50)

Calibration Experiment:

- **RP-HPLC Method:**

a) Preparation of Calibration curve standard:

The above standard stock solution (7500:1500µg/ml) of Piperaquine and Arterolane was diluted with mobile phase to yield Five calibration curve (cc) standards with concentrations of 75,150,225,300 and 375 µg/ml of Piperaquine and 15,30,45,60 and 75 µg/ml of Arterolane The calibration curve of Piperaquine and Arterolane is depicted.

- **UV- Spectrophotometric Method:**

a) Selection of detection Wavelength:

Standard solutions range of 200-400nm, against 10 ml Methanol volume make with water solvent system as reference Piperaquine and Arterolane were showed absorbance maxima (lambda max) at 248nm and 284nm respectively.If Two Piperaquine and Arterolane sample Interact with this point is called Isosbestic point Then detection of wavelength in Isosbestic point in 223 nm where selection wavelength is HPLC Method can be used.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

b) Calibration standard drug and regression equation data:

From the standard stock solution of Piperaquine and Arterolane, different concentration were prepared respectively in the range of 75-375 µg/ml for Piperaquine and 15-75 µg/ml for Arterolane and measured at 248 nm and 284 nm. The calibration curves were plotted and Regression equation data presented.

c) Calibration runs and regression analysis:

These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column : Waters C18 Column (150mm x 4.6mm, 5µm particle size).
- Injection volume : 20µl.
- Flow rate : 0.9 ml/min.
- Mobile phase : Methanol: Water (0.01% ACETIC ACID) (50: 50 % V/V).
- Detection : 223 nm

Validation of method for analysis of Piperaquine and Arterolane

- The developed method was validated as per ICH guidelines.
- **Linearity:**

Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

- **Acceptance Criteria:**

Correlation Coefficient should not be less than 0.999.

- **Preparation of standard stock solution for linearity:**

Average weight of tablet sample (equivalent to 75 mg of Piperaquine and 15 mg of Arterolane) were weighed and transferred to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted up to 10 ml volumetric flask with diluents was added to make up the volume.

- **Preparation of linearity solution:**

A series of standard preparations of working standard of were prepared.

Table No.6: Table of linearity for RP -HPLC Method

Concentration (µg/mL)	
Piperaquine	Arterolane
75	15
150	30
225	45
300	60
375	75

- **Accuracy (recovery): -**

The accuracy of an analytical method is determined by applying the method to analysed samples, to which known amounts of analyte have been added. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay.

- **Acceptance Criteria:**

Mean recovery 98-102%.
The Relative Standard DeviationNMT 2.0%.

- **Preparation of standard stock solution:**

75 mg of Piperaquine and 15 mg of Arterolane working standards were weighed and transferred to 10 mL volumetric flask & diluent

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

was added to make up the volume 0.1 ml of this solution diluted up to 10 ml with diluent.

- **Accuracy**

The accuracy was determined by Piperaquine and Arterolane (equivalent to 75 mg of Piperaquine and 15 mg of Arterolane (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed tablets. The % recovery of added drug was taken as a measure of accuracy**.**

Table No.7: Table of Accuracy for Rp-HPLC Method

Sample	**Amount Added (mg)**	
	Piperaquine	Arterolane
Accuracy 80%	60	12
Accuracy 100%	75	15
Accuracy 120%	90	18

- **Repeatability:**

Precision of the system was determined with the sample of RP-HPLC for. Six replicates of sample solution containing 75 mg of Piperaquine and 15 mg Arterolane were injected and peak areas were measured and %RSD was calculated it was repeated for five times.

- **Precision:**

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test.

- **Intra-day precision:**

Sample solutions containing 75 mg of Piperaquine and 15 mg of Arterolane three different concentration (75µg/ml, 225µg/ml, 375µg/ml Piperaquine and (15µg/ml, 45µg/ml, 75µg/ml) Arterolane. Piperaquine and Arterolane were analyzed three times on the same day and %R.S. D was calculated.

- **Inter-day precision:**

Sample solutions containing 75 mg of Piperaquine and 15 mg of Arterolane three different concentration (75µg/ml, 225µg/ml, 375µg/ml Piperaquine and (15µg/ml, 45µg/ml,75µg/ml) Arterolane.

- **Acceptance criteria:**

The Relative Standard Deviation should not be more than 2% for test.

- **Preparation of standard stock solution:**

75 mg of Piperaquine and 15 mg Arterolane working standards were weighed and transferred to 10 mL volumetric flask & diluent was added to make up the volume. 0.2 ml of this solution diluted up to 10 ml with diluent.

- **Robustness:**

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion of Methanol: Water (0.1 % ACETIC ACID)in the mobile phase composition and the flow rate was (±1ml/ min^{-1}**)**and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examinedit was performed using 150µg/ml and 15µg/ml solution of Piperaquine and Arterolane in duplicate.

- **Detection Limit**

Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$$DL = \frac{3.3\sigma}{S}$$

Were,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

the y-intercepts of regression line in calibration curve.

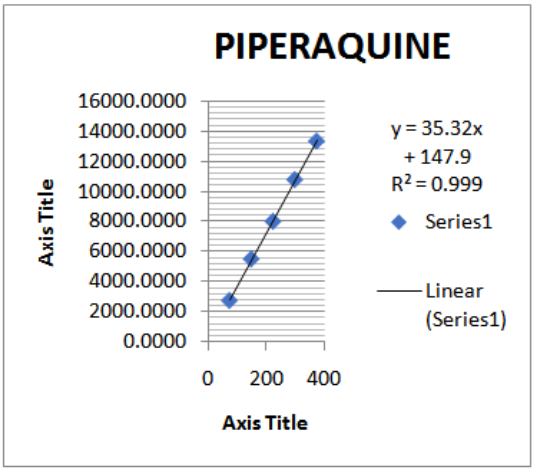
- **Quantitation Limit**

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$QL = \frac{10\sigma}{S}$

III. RESULT AND DISCUSSION: -

- **Linearity: -** Linearity of Piperaquine and Arterolane was observed in75-375µg/ml and 15-75µg /ml. Detection wavelength used was 223 nm. The plot should be linear passing through the origin; Correlation Coefficient should not be less than 0.999 that concluded.



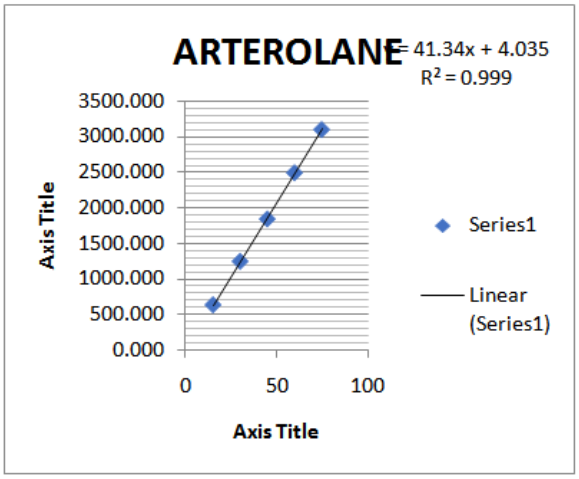


Fig No.3,4. Calibration graph of Piperaquine and Arterolane for HPLC method

Regression Equation Data Y=mx+c	
Slope(m)	35.32x
Intercept(c)	147.9
Correlation Coefficient	0.999

Table No.9 Equation data for Piperaquine

Regression Equation Data Y= mx+c	
Slope(m)	41.34 X
Intercept(c)	4.035
Correlation Coefficient	0.999

Table No.10 Equation data for Arterolane

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

Concentration µg/ml	Area Piperaquine
75	2761.0840
150	5523.2878
225	8022.9311
300	10804.6000
375	13366.6500

Table No 11. Linearity of Piperaquine

Concentration µg/ml	Area Arterolane
30	1244.682
45	1844.753
60	2495.979
75	3106.596
30	1244.682

Table No 12. Linearity of Arterolane

- **Accuracy: -** Accuracy of RP-HPLC method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 98-101%.

Table No.13. Result of Recovery data for Piperaquine and Arterolane

METHOD	Drug	Level (%)	Amt. taken (µg/ml	Amt. Added (µg/ml	Area Mean* ± S.D.	Amt. recovered Mean *±S.D.	%Recovery Mean *± S.D.
RP-HPLC Method	PIPERAQUINE	80%	75	60	135.5±0.073	60.50±0.073	100.84±0.12
		100%	75	75	150.1±0.076	75.23±0.076	100.31±0.10

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

		120%	75	90	165.8±0.126	90.83±0.126	100.93±0.14
	ARTEOLANE	80%	15	12	26.99±0.058	11.99±0.058	99.90±0.49
		100%	15	15	30.0±0.035	15.00±0.035	99.98±0.23
		120%	15	18	33.19±0.072	18.19±0.072	101.07±0.40

***Mean of each 3 reading for RP-HPLC method**

Table.14. Statistical Validation of Recovery Studies Piperaquine and Arterolane

METHOD	Level of Recovery (%)	Drug	Mean % Recovery	Standard Deviation*	% RSD
Rp-HPLC Method	80%	PIPERA	100.84	0.12	0.12
		ARTE	99.90	0.49	0.49
	100%	PIPERA	100.31	0.10	0.10
		ARTE	99.98	0.23	0.23
	120%	PIPERA	100.93	0.14	0.14
		ARTE	98.05	0.40	0.39

***Denotes average of three determinations for RP-HPLC.**

- The % recovery was found to be within 98-101%.
- **Repeatability: -**

Repeatability studies on RP-HPLC method for Piperaquine and Arterolane was found to be, The %RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded.

Table No.15: Repeatability studies on RP-HPLC forPiperaquine and Arterolane

Sr.No.	Concentration of Piperaquine (mg/ml)	Peak area	Amount found (mg)	% Amount found
1	150	5456.5737	143.54	100.81
2	150	5526.1476	155.14	100.86
		Mean	151.29	
		SD	49.20	
		%RSD	0.90	

Sr.No.	Concentration of Arterolane (mg/ml)	Peak area	Amount found (mg)	% Amount found

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

1	30	1263.108	30.11	100.68
2	30	1244.18	30.29	100.88
		Mean	30.23	100.76
		SD	13.38	13.38
		%RSD	1.07	1.07

- **Precision: -**

Intraday and Inter day Precision studies on RP-HPLC for Piperaquine and Arterolane which shows the high precision %amount in between 98% to 102% indicates to analytical method that concluded.

Table No .16 Result of Intraday and Inter day Precision studies on RP-HPLC for Piperaquine and Arterolane

METHOD	**Drug**	**Conc^{n} (µg/ml)**	**Intraday Precision**		**Interday Precision**	
			Mean± SD	**%Amt Found**	**Mean± SD**	**%Amt Found**
Rp-HPLC METHOD	**Piperaquine**	20	74.27± 10.98	99.03	74.25±5.65	99.01
		30	223.29±2.26	99.24	223.03±11.73	99.12
		40	377.30±0.42	100.61	377.03±6.29	100.54
	Arterolane	15	15.16± 6.38	101.08	15.04±7.80	100.29
		45	44.66±0.53	99.25	44.66±9.48	99.24
		75	75.01±0.08	100.01	75.12±1.64	100.16

***Mean of each 2 reading for RP-HPLC method**

- **Robustness: -**

Robustness Study of Piperaquine:

The changes were did flow rate (±1 ml/ min^{-1}), PH of mobile phase composition (±1 ml/ min^{-1}),and Wavelength (±1 ml/ min^{-1}) .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded.

Table No.17 Result of Robustness Study of Piperaquine

Parameters	**Conc.(µg/ml)**	**Amount of detected (mean ±SD)**	**%RSD**
Chromatogram of flow change 0.8ml	150	6136.88±2.94	0.05
Chromatogram of flow change 1.0 ml	150	4993.67±2.60	0.05
Chromatogram of comp change 51 Methanol +49 Water	150	5496.4±2.07	0.04

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

Chromatogram of comp change 49 Methanol+ 51 Water	150	5487.35±4.11	0.07
Chromatogram of comp change Wavelength change 222 nm	150	5241.7±0.62	0.01
Chromatogram of comp change Wavelength change 224 nm	150	5714.40±1.68	0.03

Robustness Study of Arterolane:

The changes were doing flow rate (±1 ml/ min^{-1}), PH of mobile phase composition (±1 ml/ min^{-1}), and Wavelength (±1 ml/ min^{-1}). %RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded**.**

Table No.18. Result of Robustness Study of Arterolane

Parameters	**Conc.(µg/ml)**	**Amount of detected(mean ±SD)**	**%RSD**
Chromatogram of flow change 0.8 ml	30	1407.35±1.25	0.09
Chromatogram of flow change 1.0 ml	30	1141.88±1.92	0.17
Chromatogram of comp change 51 Methanol +49 WATER	30	1261.4±1.64	0.13
Chromatogram of comp change 49 Methanol+ 51 WATER	30	1254.38±2.87	0.23
Chromatogram of comp change wavelength change 222 nm	30	1281.6±1.96	0.15
Chromatogram of comp change wavelength change 224 nm	30	1204.09±1.47	0.12

- **Limitof Detection**

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

LOD = 3.3 (SD)/S

Where, SD = Standard deviation of Y intercept
S = Slope

Limit of detection = 3.3 X 19.54/35.32= 1.82(µg/mL)

Limit of Quantitation = 10 X 19.54/35.32= 5.532 (µg/mL)

The LOD and LOQ of Piperaquine was found to be 1.82 (µg/mL) and 5.532 (µg/mL), analytical method that concluded

- **Limit of Quantification**

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,

The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S

Where, SD = Standard deviation Y intercept
S = Slope

Limit of Detection = 3.3X7.82/41.34=0.624 (µg/mL)

Limit of Quantitation = 10 X7.82/ 41.34=1.891 (µg/mL)

The LOD and LOQ of Arterolane was found to be 0.624 (µg/mL) and 1.891 (µg/mL), analytical method that concluded.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 3 May-June 2023, pp: 1469-1480 www.ijprajournal.com ISSN: 2249-7781

IV. CONCLUSION: -

Development and Validation of methods to achieve the final goal of ensuring the quantity of drug substances and drug products. Piperaquine phosphate and Arterolane maleate is an antimalarial drug. Few analytical methods were appeared in the literature survey for the determination of Piperaquine phosphate and Arterolane Maleate, which includes UV Spectrophotometric, RP-HPLC methods. In view of the above fact, some simple analytical methods were planned to develop New, simple, sensitive, accurate, precise, robust, economical and specific for the estimation of piperaquine phosphate and arterolane maleate in combined tablet dosage form.

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