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# Stability indicating method development and validation for the determination of piperaquine tetraphosphate and dihydro artemisinin by RP HPLC

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**Abstract**
On the basis of experimental results, the proposed method is suitable for the quantitative determination of piperaquine tetraphosphate and dihydro artemisinin in pharmaceutical dosage form. The method provides great sensitivity, adequate linearity and repeatability. The estimation of piperaquine tetraphosphate and dihydro artemisinin was done by RP-HPLC. The Phosphate buffer was pH 3.6 and the mobile phase was optimized which consists of Methanol: Phosphate buffer mixed in the ratio of 70:30% v/ v. A Symmetry C18 (4.6 x 150mm, 5µm, Make XTerra) column used as stationary phase. By using UV detector at 273 nm.

**Keywords:** RP-HPLC, Symmetry C18, piperaquine tetraphosphate and dihydro artemisinin

## Introduction

### Piperaquine Tetraphosphate

**Indication:** For the treatment of uncomplicated *Plasmodium falciparum* infection in adults, children, and infants aged 6 months and up weighing over 5 kg. Used in combination with Artenimol.

**Mechanism of action:** The mechanism of piperaquine inhibition of the haem detoxification pathway is unknown but is expected to be similar to that of Chloroquine.

**Drug:** Dihyroartemisinin

**Indication:** For the treatment of uncomplicated *Plasmodium falciparum* infection in adults, children, and infants aged 6 months and up weighing over 5 kg. Used in combination with Piperaquine.

**Mechanism of action:** The proposed mechanism of action of Dihyroartemisinin involves cleavage of endoperoxide bridges by iron, producing free radicals which damage biological macromolecules causing oxidative stress in the cells of the parasite. Malaria is caused by apicomplexans, primarily *Plasmodium falciparum*, which largely reside in red blood cells and itself contains iron-rich heme-groups (In the form of hemozoin). In 2015 artemisinin was shown to bind to a large number targets suggesting that it acts in a promiscuous manner. Recent mechanism research discovered that artemisinin targets a broad spectrum of proteins in the human cancer cell proteome through heme-activated radical alkylation.

## Literature Review

1. Uday A *et al.*, High Performance Liquid Chromatography (HPLC) methods are described for determination of drugs as a single or in combination in bulk or pharmaceutical formulation. The objective of the present study was to develop and validate novel, accurate, sensitive, precise, rapid and isocratic reverse Phase HPLC (RP-HPLC) method for the simultaneous determination of Piperaquine phosphate and Dihydroarte in bulk because no method is available for simultaneous estimation of these drugs.

2. Venkata Raveendra Babu Vemula *et al.*, A new reversed-phase HPLC method was developed and subsequently validated for simultaneous estimation of arterolane maleate and piperaquine phosphate in pharmaceutical dosage forms.) with aµChromatography is carried out at 30°C ± 0.5°C on Inertsil Silica C18 column (250 x 4.6 mm, 5 mobile phase composed of buffer and acetonitrile (25:75) at a flow rate of 1.0 mL/min. Detection was carried out using a PDA detector at 223nm. The retention times for Arterolane maleate and piperaquine phosphate are 3.1 min. and 7.2 min., respectively.
3. Joseph MD Fortunak *et al.*, to provide a robust, efficient synthesis of the malaria drug piperaquine for potential use in resource-poor settings. Methods: We used in-process analytical technologies (IPAT; HPLC) and a program of experiments to develop a synthesis of piperaquine that avoids the presence of a toxic impurity in the API and is optimized for overall yield and operational simplicity. Results: A green-chemical synthesis of piperaquine is described those proceeds in 92-93% overall yield.
4. Linda L Kjellin *et al.*, We report a sensitive LC–MS/MS. Method for quantitation of PQ with only 25 µI human plasma. Using a deuterated internal standard (PQ-$d_6$), an analytical PFP column, $APCI^+$ as the ion source and MRM (535/288 for PQ and 541/294 for the IS) for detection, the method has a linear calibration range of 1.5-250 ng/ml with a runtime of 3.0 min per sample. The method was applied to plasma samples from children.
5. Sam Salman *et al.*, Pharmacokinetic differences between piperaquine (PQ) base and PQ tetraphosphate were investigated in 34 Papua New Guinean children aged 5 to 10 years treated for uncomplicated malaria with artemisinin-PQ (ART-PQ) base or dihydroarte-PQ (DHA-PQ) tetraphosphate. Twelve children received ART-PQ base (two daily doses of 3 mg of ART and 18 mg of PQ base as granules/kg of body weight) as recommended by the manufacturer, with regular clinical assessment and blood sampling over 56 days.

## Materials and Methods

**Materials:** The list of instruments used in the course of experimental work is as follows.

**Table 1:** List of Instruments

| S. No. | Instrument | Model No. | Software | Manufacturer's name |
|---|---|---|---|---|
| 1 | HPLC Alliance<br>PDA Detector | Waters 2695<br>Waters 996 | Empower | Waters |
| 2 | UV double beam spectrophotometer | UV 3000 | UV Win 5 | Lab India |
| 3 | Digital weighing balance | BSA224SCW | - | Satorius |
| 4 | pH meter | AD102U | - | Lab India |
| 5 | Ultra sonicator | SE60US | - | - |
| 6 | Suction pump | VE115N | - | - |

**The experimental work involves several chemicals**

**Table 2:** List of Chemicals

| S. No. | Chemicals | Manufacturer | Grade |
|---|---|---|---|
| 1 | Water | Merck | HPLC Grade |
| 2 | Methanol | Merck | HPLC Grade |
| 3 | Acetonitrile | Merck | HPLC Grade |
| 4 | Potassium dihydrogen orthophosphate | Merck | A. R |
| 5 | Piperaqu tetraphosphate & dihydroart | - | - |

**Preparations and procedures**

**Preparation of Phosphate buffer: (PH: 4.6):** Weighed 6.8 grams of KH2PO4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 4.6 with ortho phosphoric acid.

**Preparation of mobile phase:** A mixture of pH 4.6 Phosphate buffer 300 mL (30%), 700 mL of MEOH (70%) are taken and degassed in ultrasonic water bath for 5 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration.

**Diluant Preparation:** Mobile phase is used as Diluant.

**Preparation of the individual Piperaquine tetraphosphate standard preparation:** 10mg of Piperaquine tetraphosphate working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of diluent is added.

**Preparation of the individual Dihydroarte standard preparation:** 10mg of Dihydroarte working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of diluent is added.

**Preparation of Sample Solution:** Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Dihydroarte and Piperaquine tetraphosphate (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution).

**Procedure:** 10µL of the standard, sample are injected into the chromatographic system and the areas for Dihydroarte and Piperaquine tetraphosphate peaks are measured and the % Assay are calculated by using the formulae.
Theoretical plates for the Dihydroarte and Piperaquine tetraphosphate peaks in Standard solution should not be less than 2000

**Assay calculation**

$$Assay\,\% = \frac{sample\ area}{Standard\ area} \times \frac{dilution\ sample}{dilution\ of\ standard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100$$

Where,
P = Percentage purity of working standard
Lc = Label claim of drug in mg/ml.

**Linearity**
The linearity range was found to lie from 25% to 125% and chromatograms are shown below.

**Area of different concentration of Piperaquine tetraphosphate and Dihyroartemisinin**

| Concentration (µg/ml) | Peak area of Piperaquine tetraphosphate | Peak area of Dihyroartemisinin |
|---|---|---|
| 25 | 296800 | 179891 |
| 50 | 653819 | 387781 |
| 75 | 983775 | 599708 |
| 100 | 1342535 | 799619 |
| 125 | 1694286 | 1019614 |

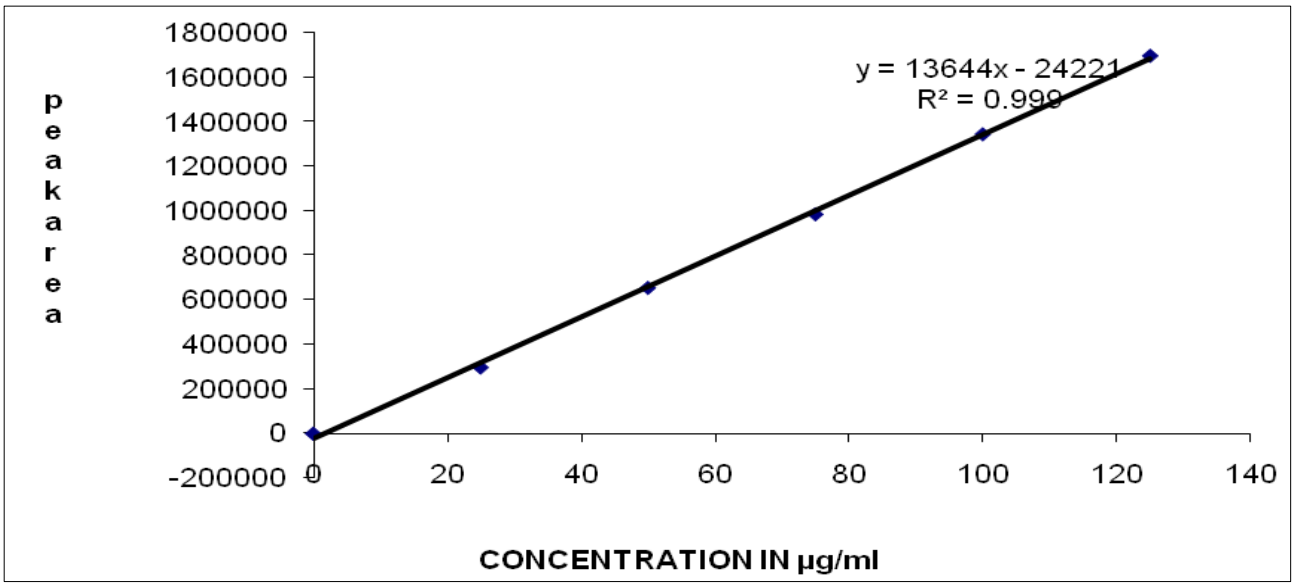


**Fig 1:** Calibration graph for Piperaquine tetraphosphate at 273 nm

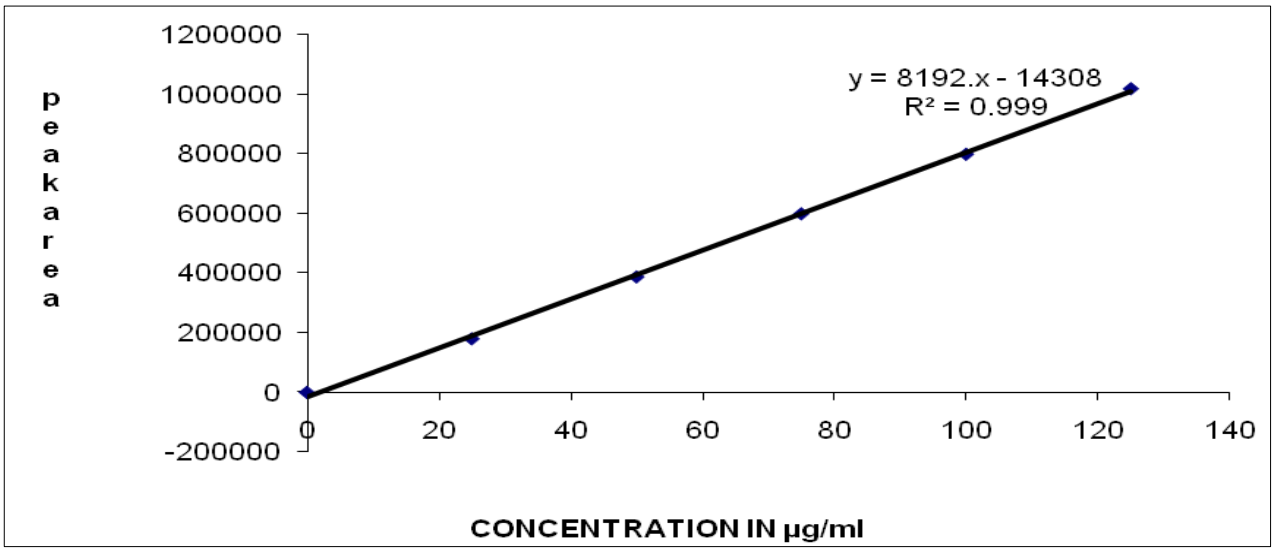


**Fig 2:** Calibration graph for Dihyroartemisinin at 273 nm

**Table 3:** Analytical performance parameters of Piperaquine tetraphosphate and Dihyroartemisinin

| Parameters | Piperaquine tetraphosphate | Dihyroartemisinin |
|---|---|---|
| Slope (m) | 13644 | 8192 |
| Intercept (c) | 24221 | 14308 |
| Correlation coefficient ($R^2$) | 0.999 | 0.999 |

**Acceptance criteria**
**Correlation coefficient ($R^2$) should not be less than 0.999**

The correlation coefficient obtained was 0.999 which is in the acceptance limit. The linearity was established in the range of 25 to 150µg/ml.

**Accuracy**

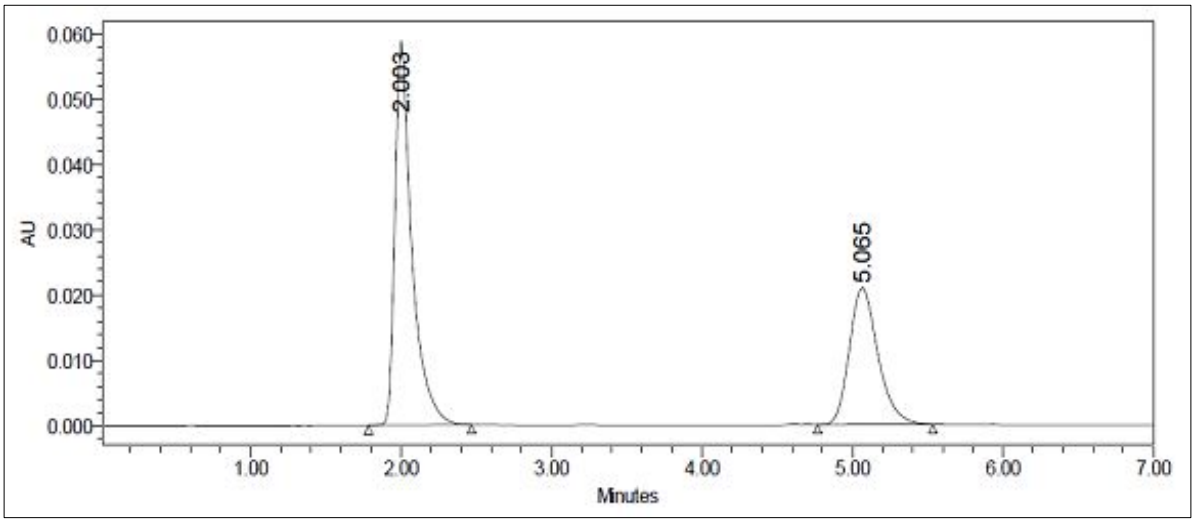


**Fig 3:** Chromatogram for sample concentration-50%

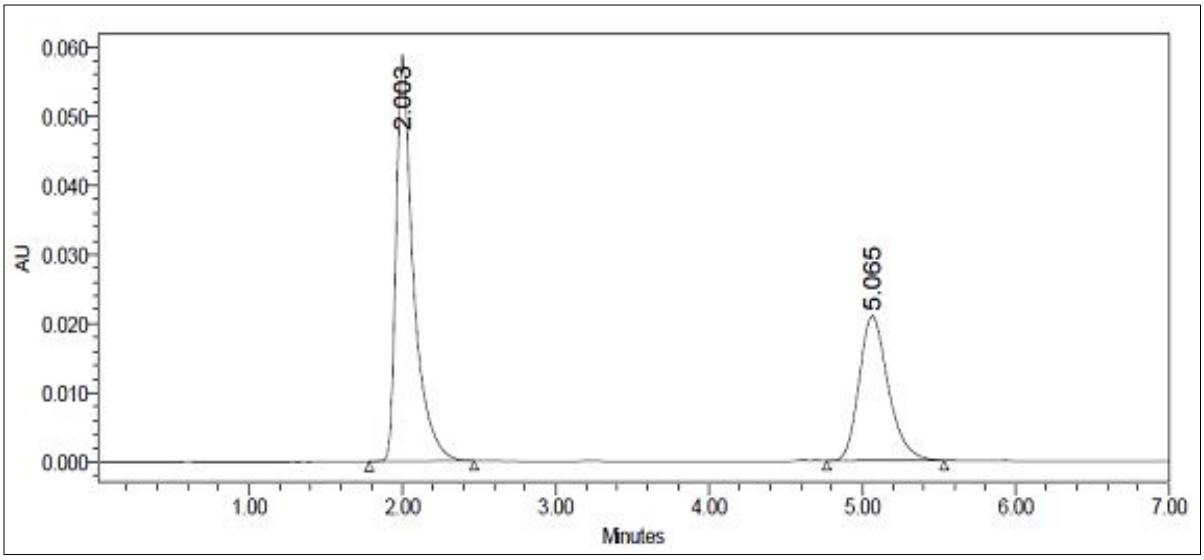


**Fig 4:** Chromatogram for sample concentration-50%

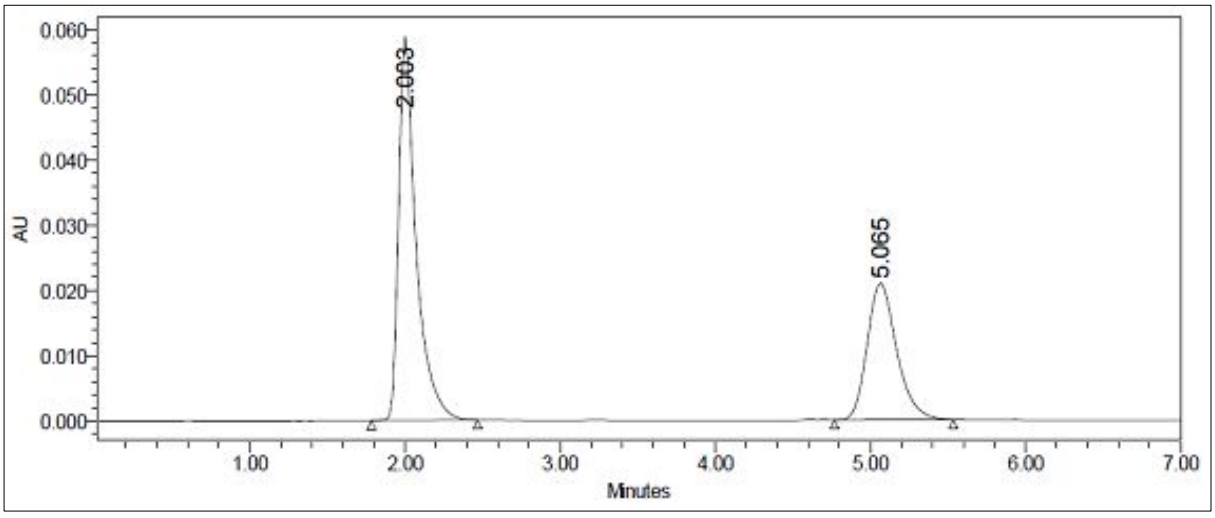


**Fig 5:** Chromatogram for sample concentration-50%

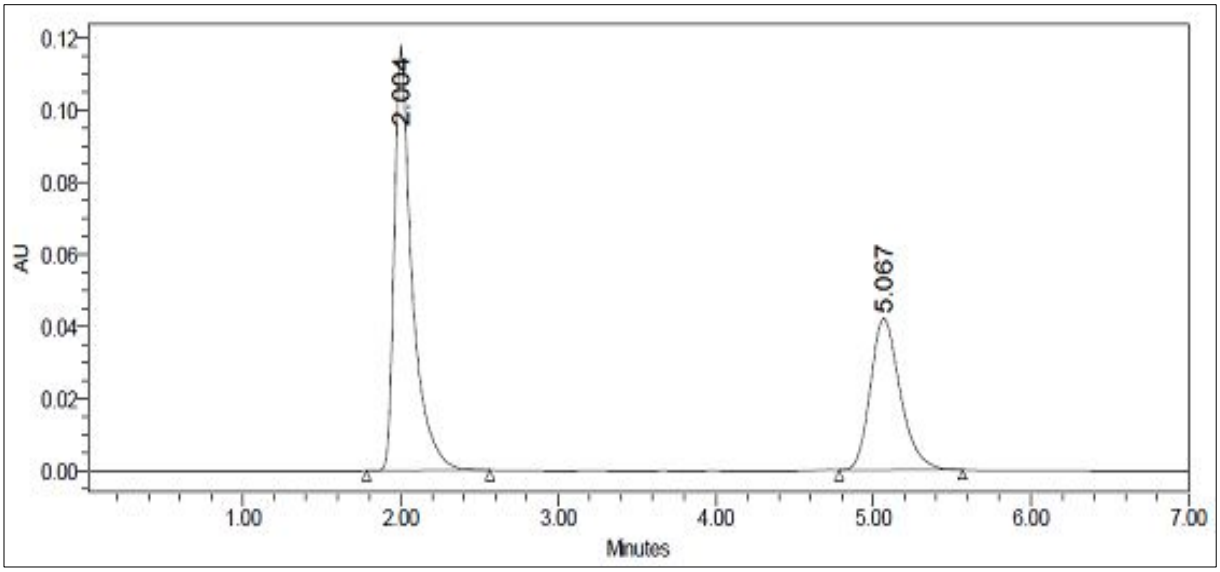


**Fig 6:** Chromatogram for sample concentration-100%

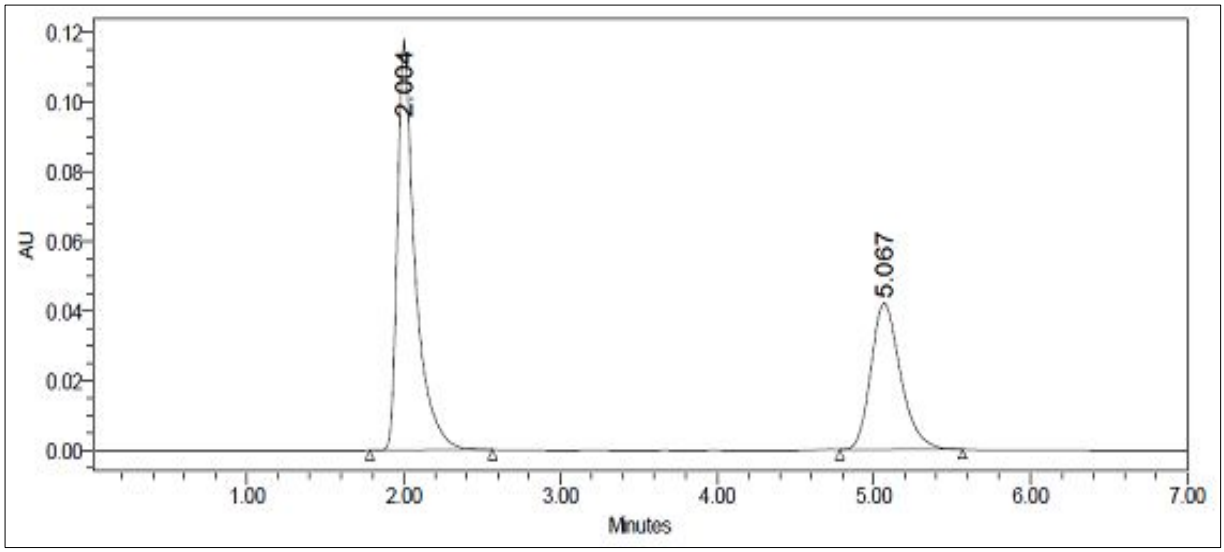


**Fig 7:** Chromatogram for sample concentration-100%

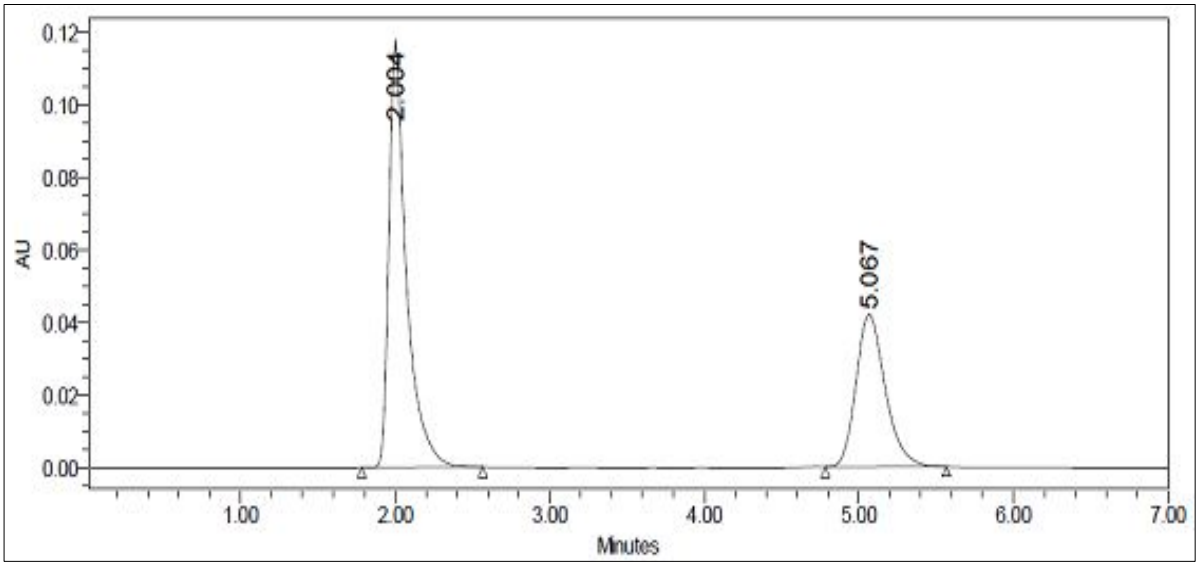


**Fig 8:** Chromatogram for sample concentration-100%

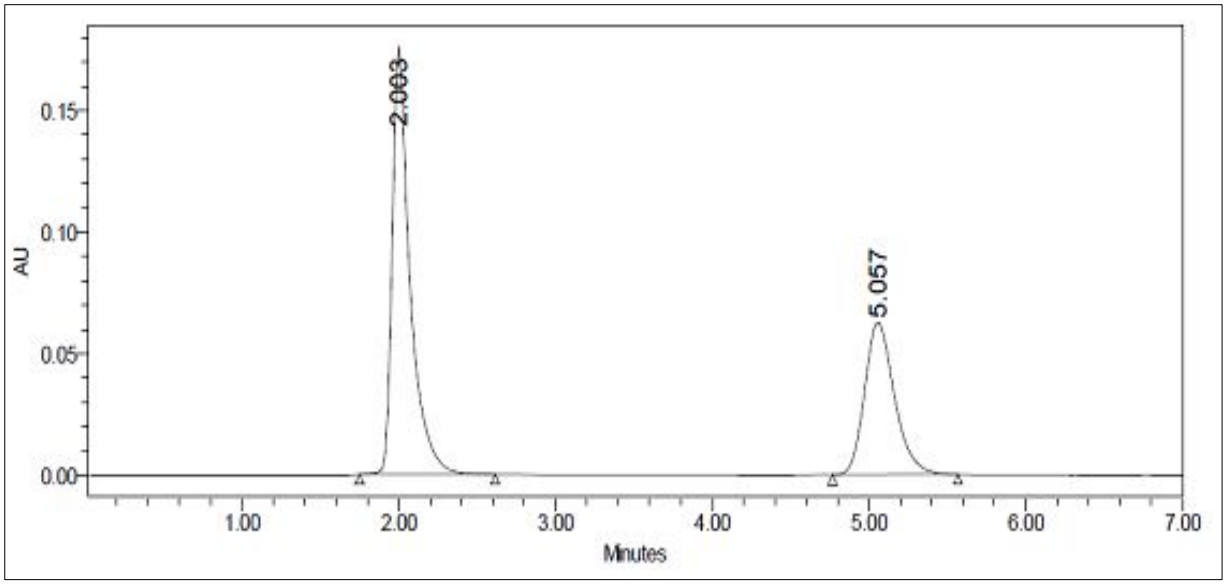


**Fig 9:** Chromatogram for sample concentration 150%

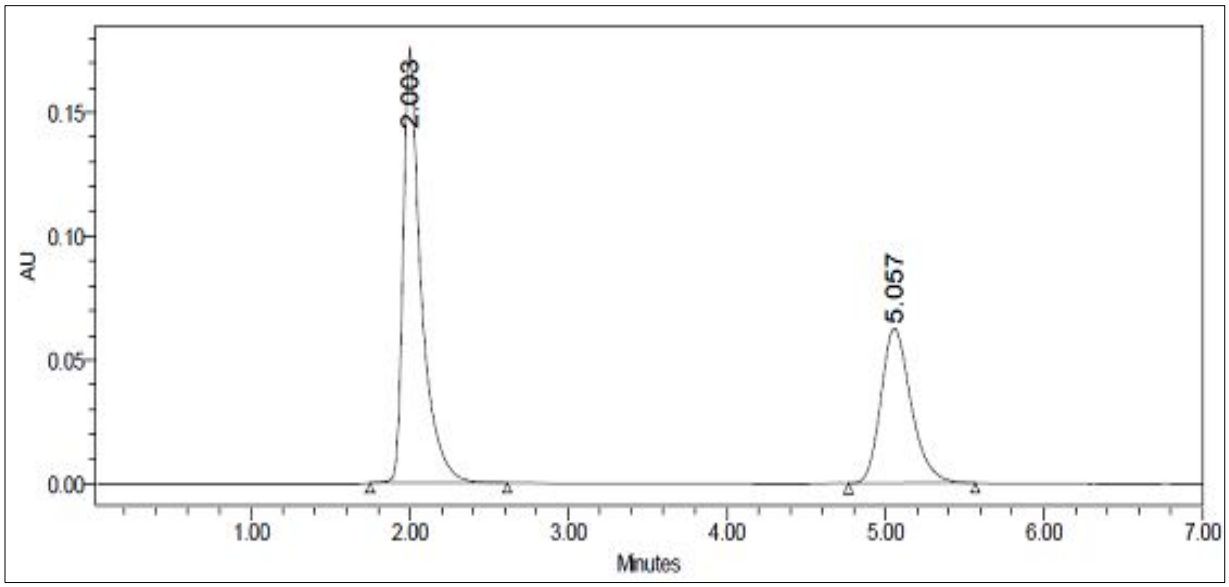


**Fig 10:** Chromatogram for sample concentration 150%

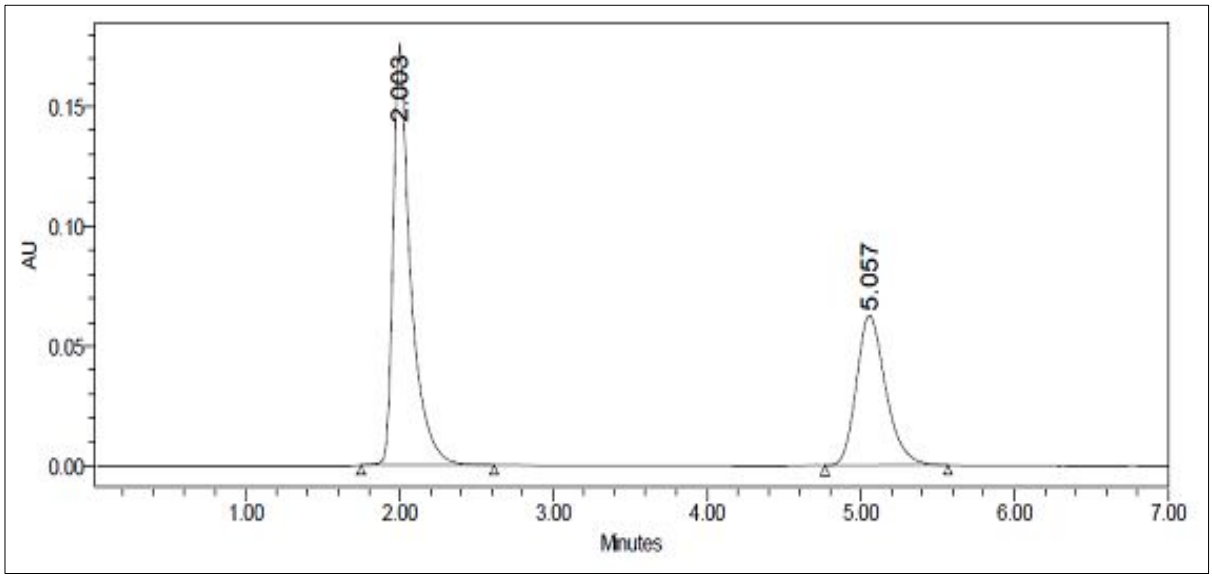


**Fig 11:** Chromatogram for sample concentration-150%

**Table 4:** Results of Accuracy

| Sample concentration | Sample set no | Sample area | | Assay | | % Recovery | |
|---|---|---|---|---|---|---|---|
| | | ARTE | PIPE | ARTE | PIPE | ARTE | PIPE |
| 50% | 1 | 460064 | 276931 | 24.9 | 25.0 | 99.8 | 100 |
| | 2 | 460124 | 276694 | 24.6 | 24.9 | 99.6 | 99.6 |
| | 3 | 460216 | 276891 | 24.8 | 24.9 | 99.8 | 99.6 |
| | Average Recovery | | | | | 99.7% | 99.7% |
| 100% | 1 | 923429 | 554156 | 49.9 | 50.0 | 99.8 | 100 |
| | 2 | 923654 | 554897 | 49.8 | 49.9 | 99.6 | 99.8 |
| | 3 | 923742 | 556371 | 49.8 | 49.9 | 99.6 | 99.8 |
| | Average recovery | | | | | 99.6% | 99.8% |
| 150% | 1 | 1387901 | 828113 | 74.8 | 75.0 | 99.8 | 100 |
| | 2 | 1385360 | 828794 | 74.9 | 74.9 | 99.8 | 99.8 |
| | 3 | 1386984 | 828349 | 74.6 | 74.8 | 99.6 | 99.8 |
| | Average recovery | | | | | 99.7% | 99.8% |

**Acceptance criteria**

**The percentage recovery at each level should be between (97-103%)**

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

**Precision**

Precision of the method was carried out for both sample and standard solutions as described under experimental work. The corresponding chromatograms and results are shown below.

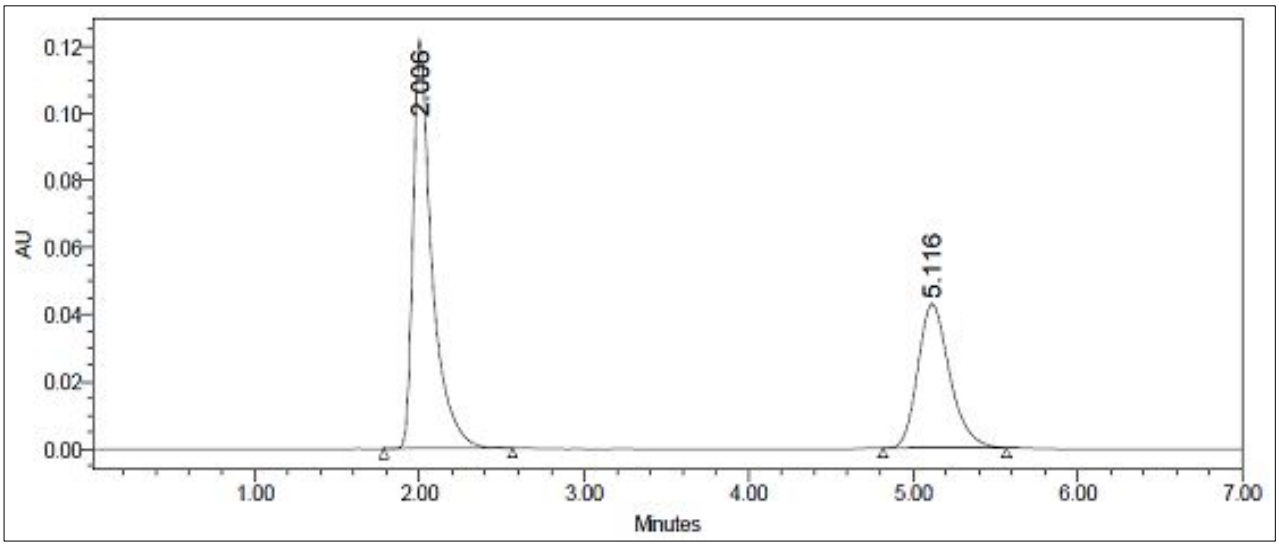


**Fig 12:** Chromatogram for sample injection-1

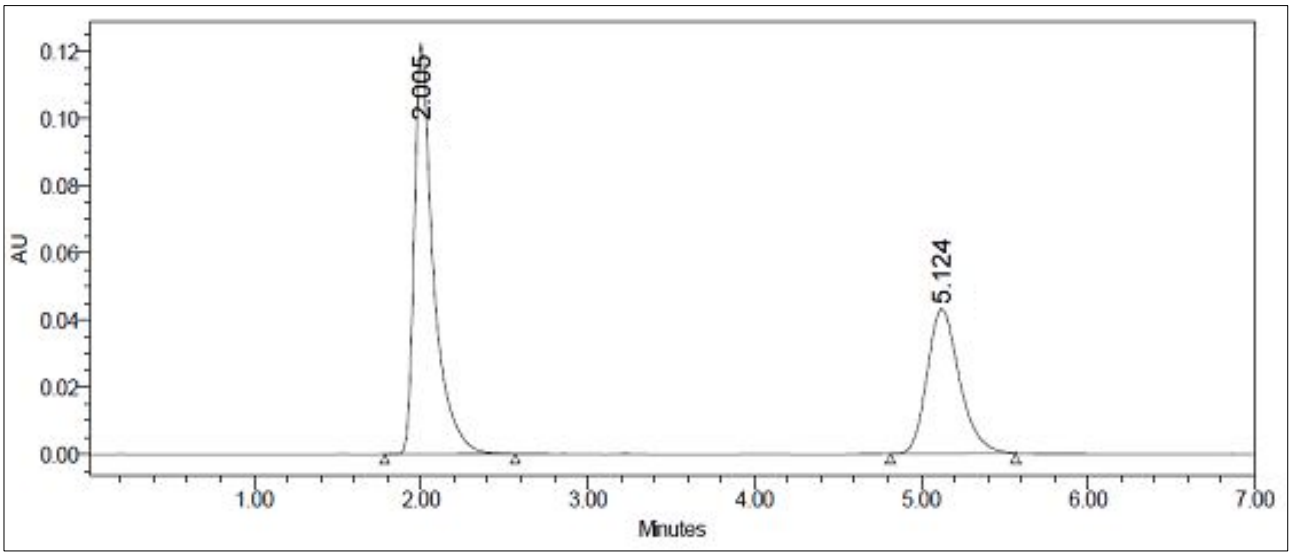


**Fig 13:** Chromatogram for sample injection-2

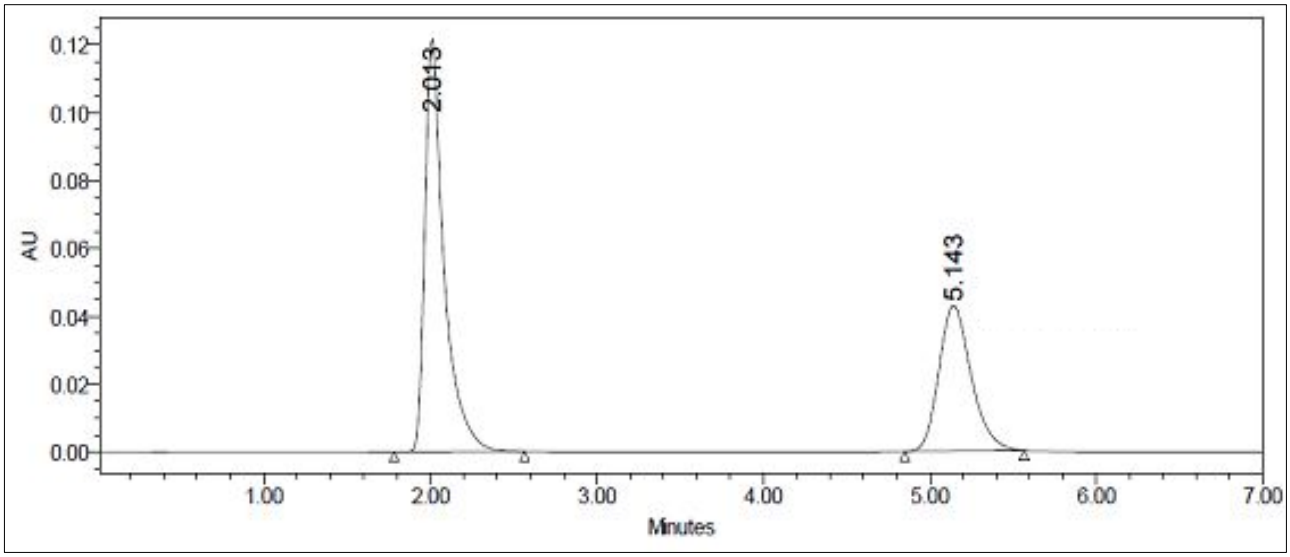


**Fig 14:** Chromatogram for sample injection-3

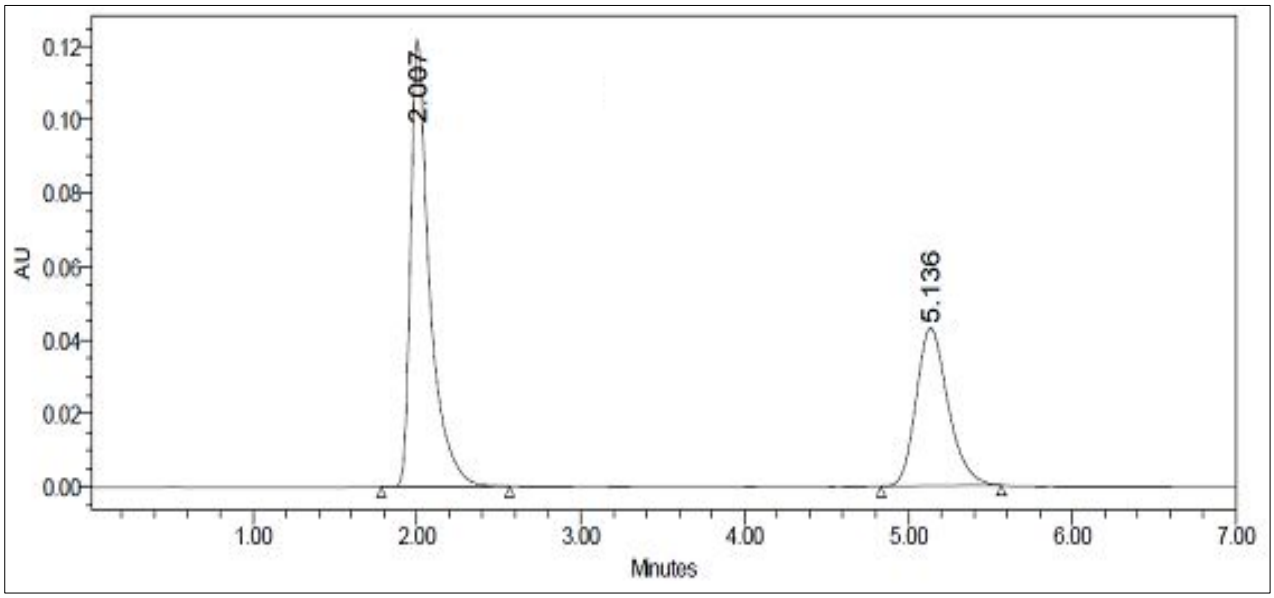


**Fig 15:** Chromatogram for sample injection-4

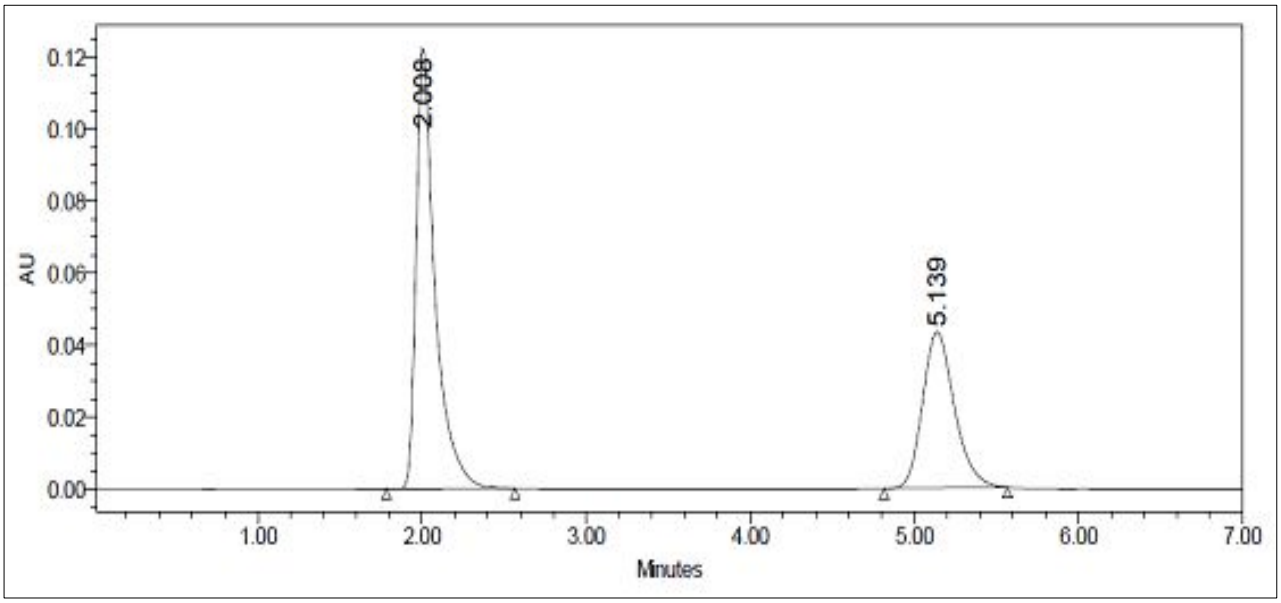


**Fig 16:** Chromatogram for sample injection-5

**Table 5:** Results of method precision for Piperaquine tetraphosphate

| S. No | Sample area | Standard area | Percentage purity |
|---|---|---|---|
| 1 | 983375 | 971536 | 101.04 |
| 2 | 985049 | 973007 | 101.03 |
| 3 | 982956 | 975717 | 100.54 |
| 4 | 985219 | 978909 | 100.44 |
| 5 | 994145 | 981422 | 101.09 |
| Average | | | 100.84 |
| %RSD | | | 0.304 |

**Table 6:** Results of method precision for Dihyroartemisinin

| S. No | Sample area | Standard area | Percentage purity |
|---|---|---|---|
| 1 | 592403 | 577531 | 101.36 |
| 2 | 592352 | 580381 | 101.85 |
| 3 | 592357 | 577723 | 102.32 |
| 4 | 592323 | 582190 | 101.44 |
| 5 | 596525 | 583378 | 101.09 |
| Average | | | 101.24 |
| %RSD | | | 0.46 |

**Acceptance criteria: %** RSD for sample should be NMT 2

- The % RSD for the standard solution is below 2, which is within the limits hence the method is precise.

**Intermediate Precession (Ruggedness)**

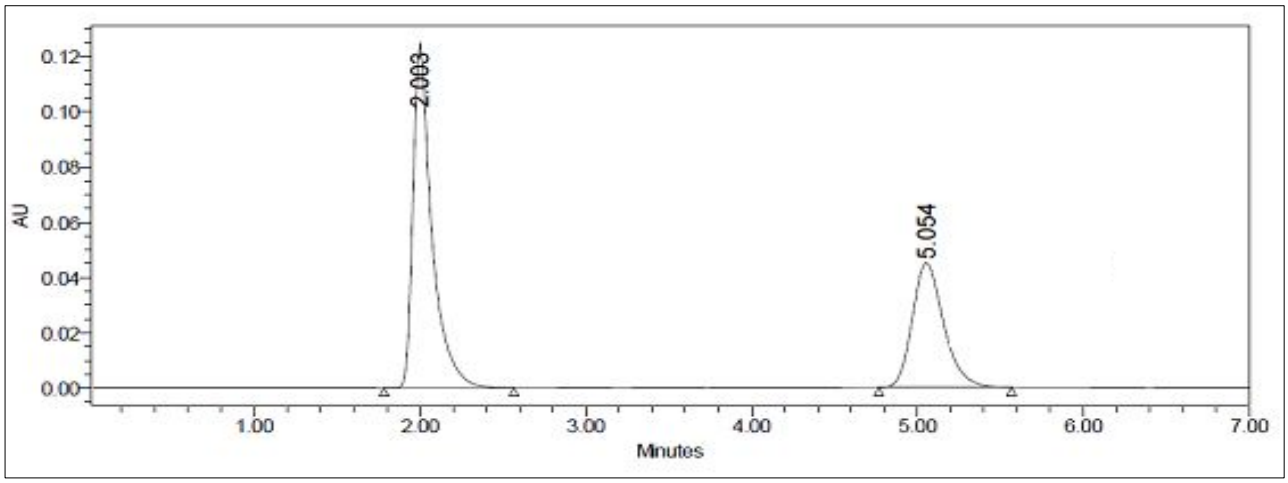


**Fig 17:** Chromatogram for sample injection-1

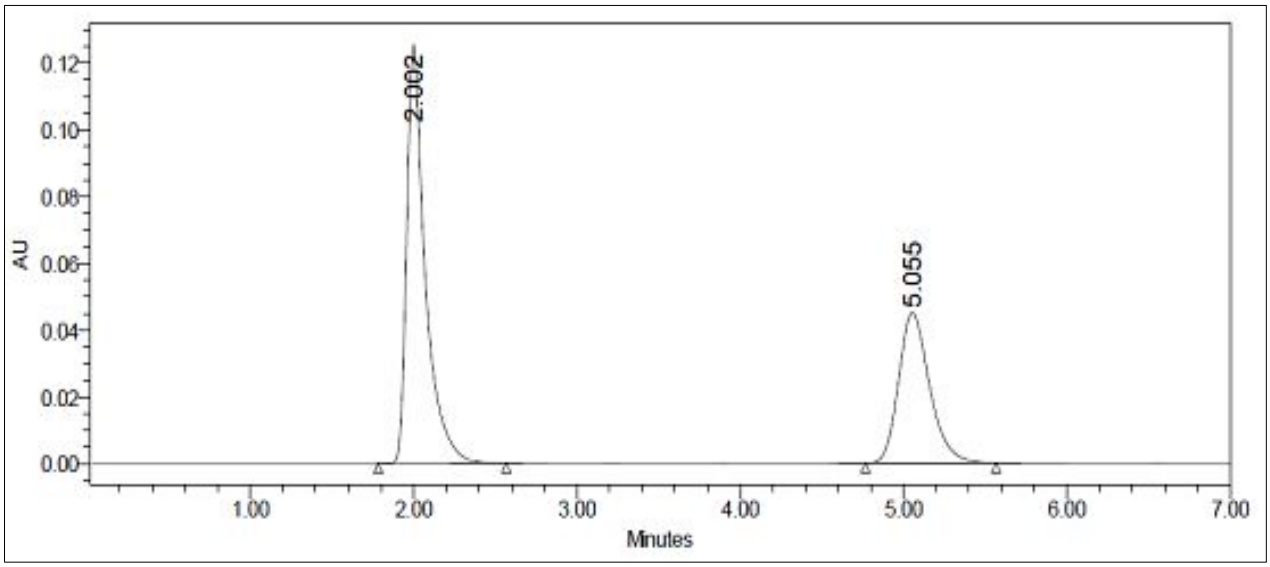


**Fig 18:** Chromatogram for sample injection-2

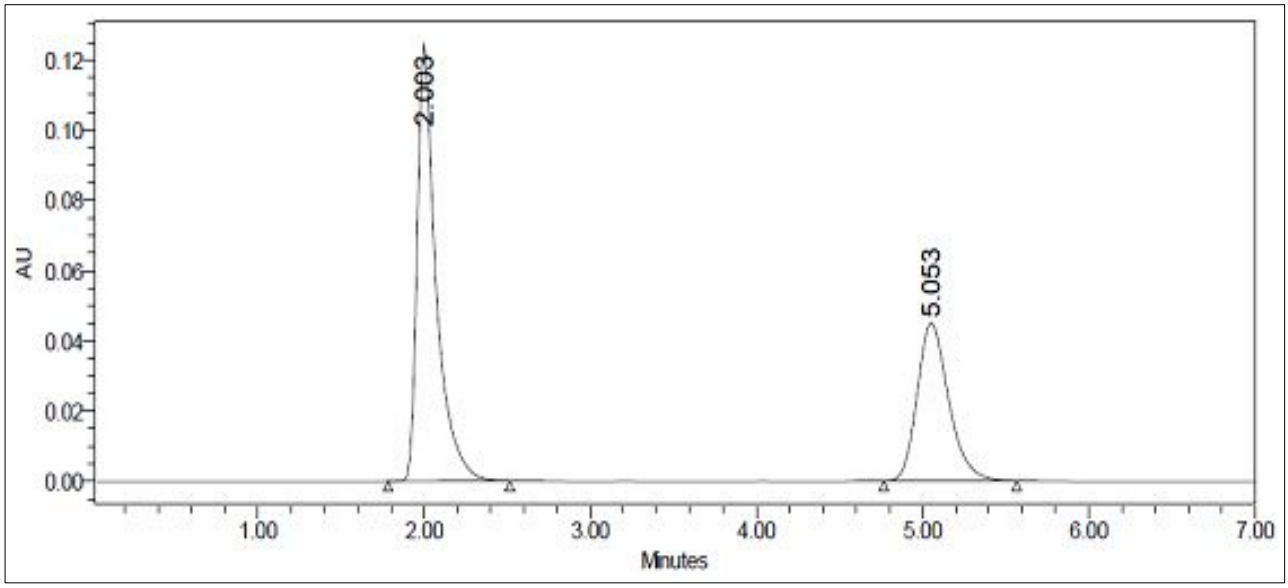


**Fig 19:** Chromatogram for sample injection-3

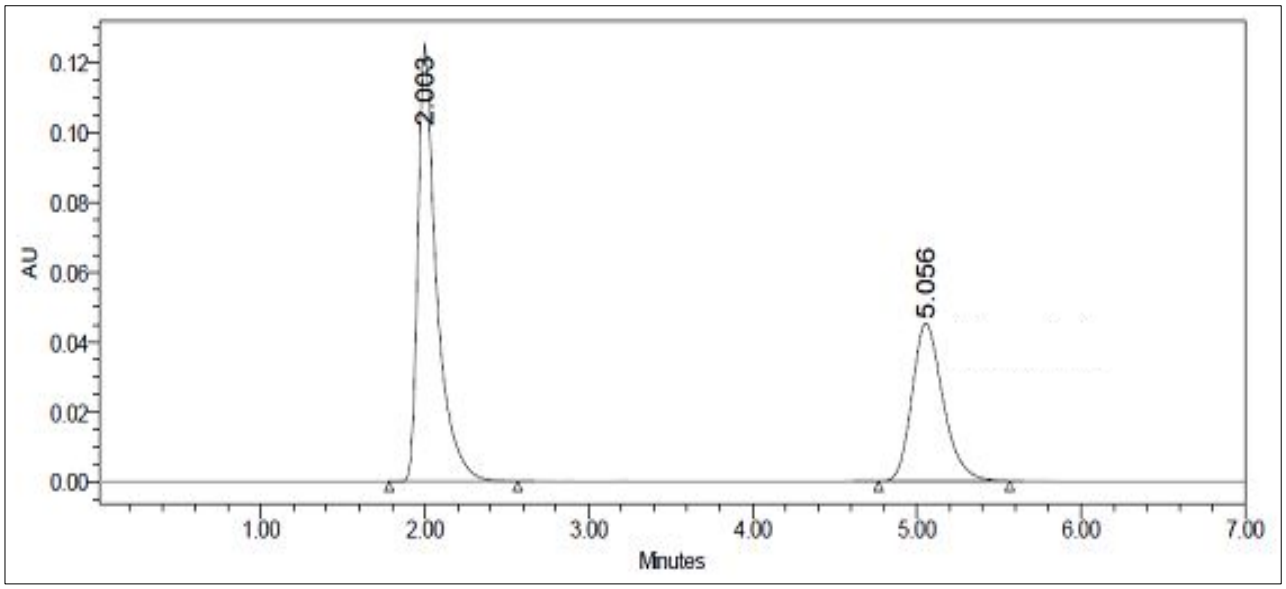


**Fig 20:** Chromatogram for sample injection-4

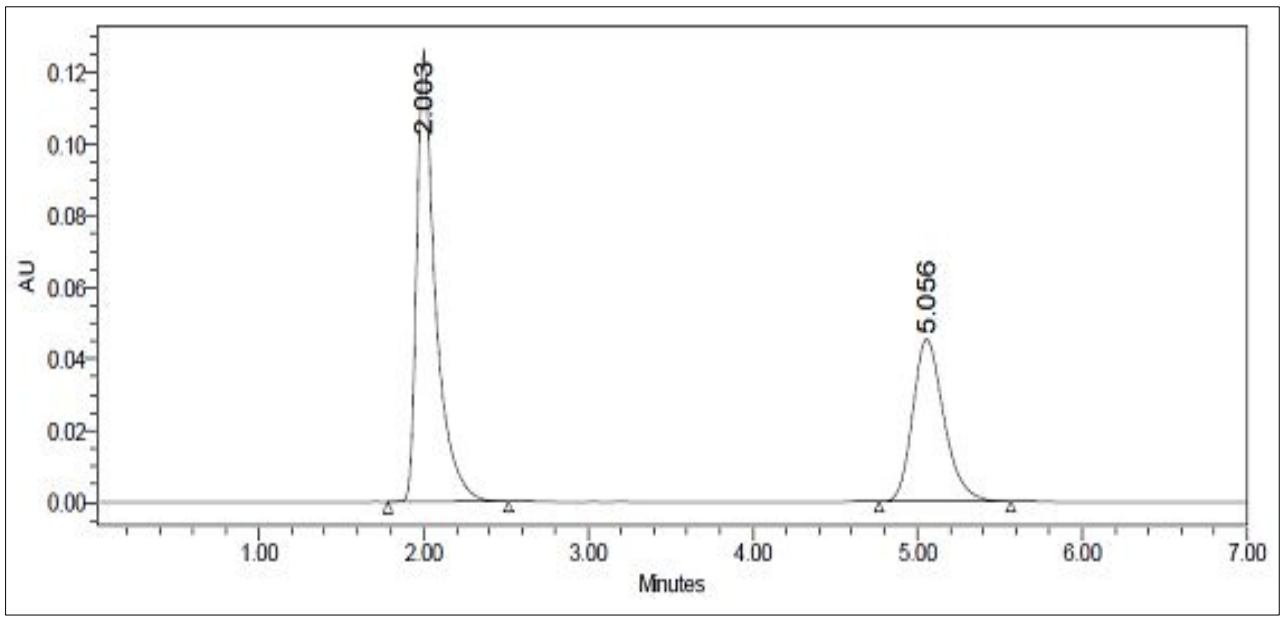


**Fig 21:** Chromatogram for sample injection-5

**Table 7:** Results of Intermediate precision for Piperaquine tetraphosphate

| S. No | Sample area | Standard area | Percentage purity |
|---|---|---|---|
| 1 | 979556 | 984395 | 99.30 |
| 2 | 982467 | 984039 | 99.64 |
| 3 | 979717 | 983976 | 99.36 |
| 4 | 978909 | 984278 | 99.28 |
| 5 | 981432 | 973915 | 100.57 |
| Average | | | 99.63 |
| %RSD | | | 0.54 |

**Table 8:** Results of Intermediate precision for Dihyroartemisinin

| S. No | Sample area | Standard area | Percentage purity |
|---|---|---|---|
| 1 | 583416 | 593403 | 99.12 |
| 2 | 583657 | 594352 | 99.01 |
| 3 | 584731 | 593357 | 99.52 |
| 4 | 583594 | 592673 | 99.61 |
| 5 | 597649 | 593671 | 99.12 |
| Average | | | 99.27 |
| %RSD | | | 0.27 |

**Acceptance criteria: %** RSD of five different sample solutions should not be more than 2

- The % RSD obtained is within the limit, hence the method is rugged.

**Limit of Detection for Piperaquine Tetraphosphate and Dihyroartemisinin**

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

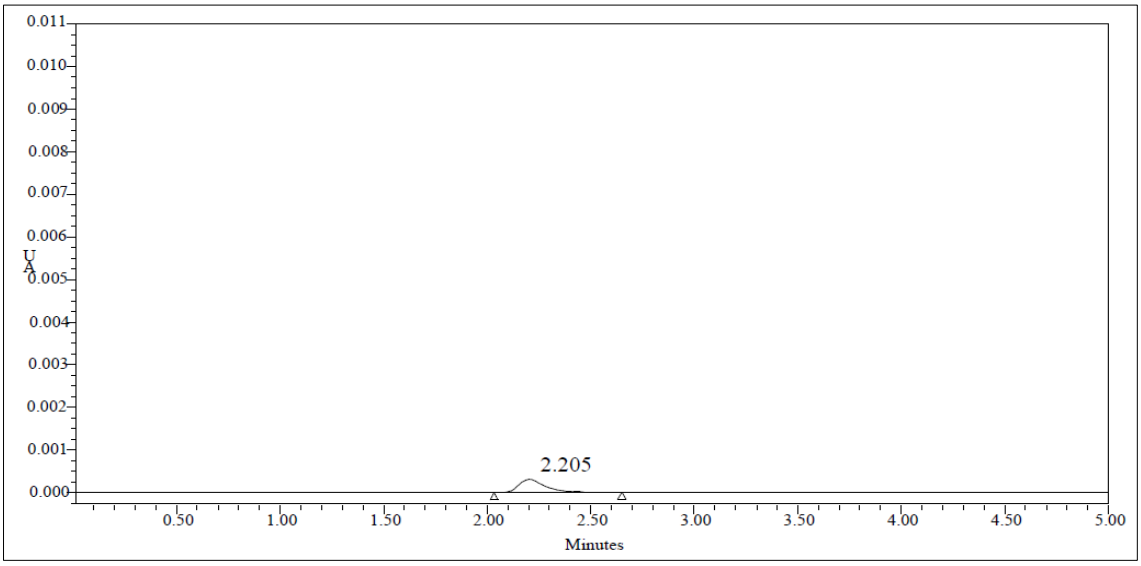


**Fig 22:** Chromatogram of Piperaquine tetraphosphate showing LOD

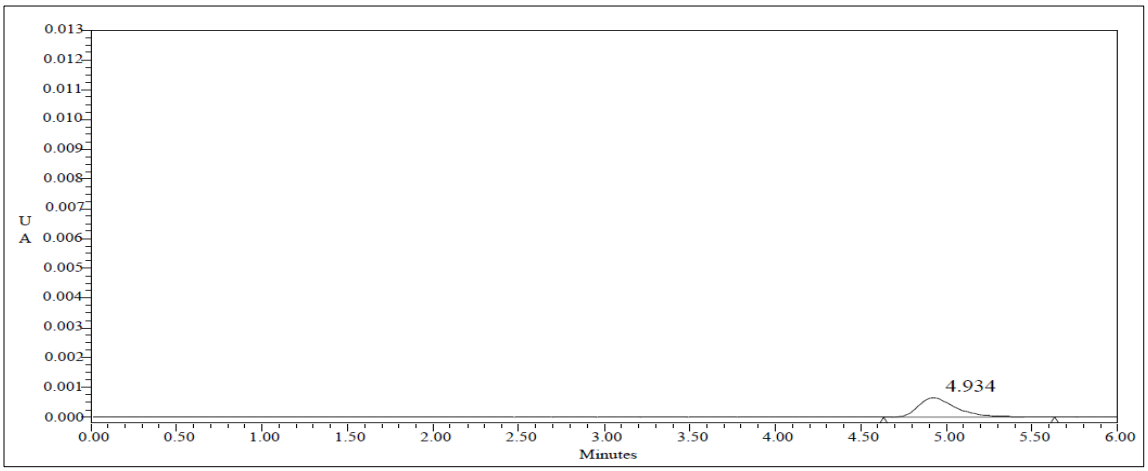


**Fig 23:** Chromatogram of Dihyroartemisinin showing LOD

**Table 9:** Results of LOD

| Drug name | Baseline noise(µV) | Signal obtained (µV) | S/N ratio |
|---|---|---|---|
| Piperaquine tetraphosphate | 56 | 176 | 3.14 |
| Dihyroartemisinin | 56 | 154 | 2.75 |

**Acceptance criteria:** Signal to noise ratio should be 3 for LOD solution

- The results obtained are within the limit.

### 7.3.6 Limit of Quantitation for Piperaquine Tetraphosphate and Dihyroartemisinin

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio

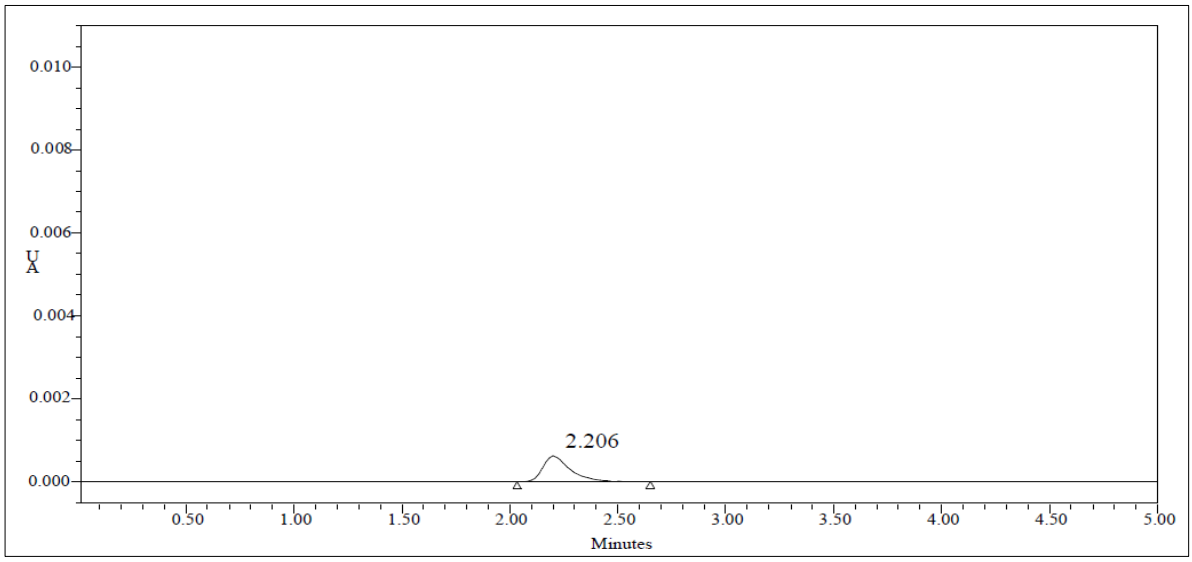


**Fig 24:** Chromatogram of Piperaquine tetraphosphate showing LOQ

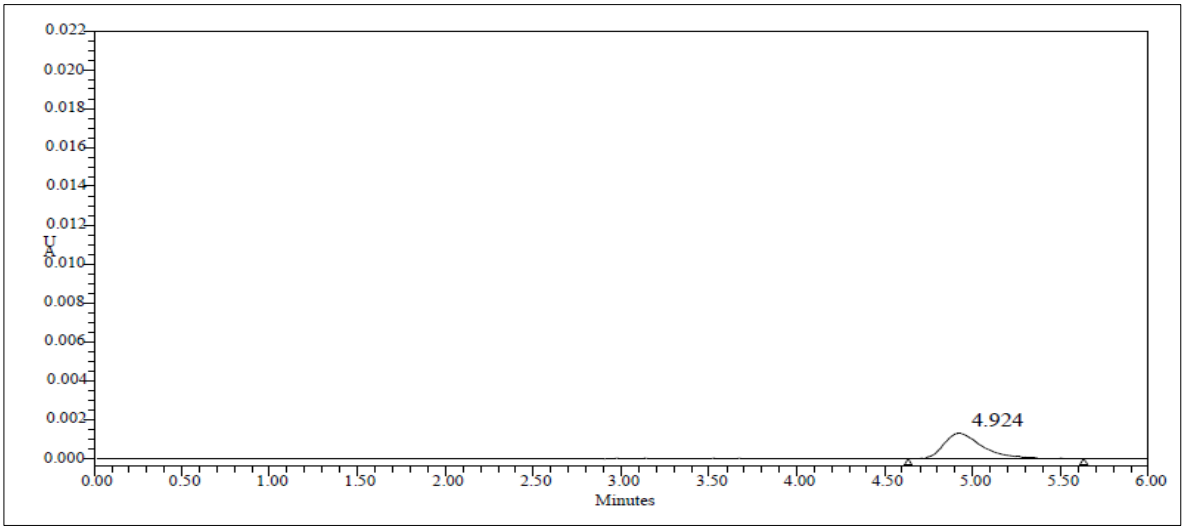


**Fig 25:** Chromatogram of Dihyroartemisinin showing LOQ

**Table 10:** Results of LOQ

| Drug name | Baseline noise(µV) | Signal obtained (µV) | S/N ratio |
|---|---|---|---|
| Piperaquine tetraphosphate | 56 | 563 | 10.05 |
| Dihyroartemisinin | 56 | 558 | 9.96 |

**Acceptance criteria:** Signal to noise ratio should be 10 for L.O.Q solution

- The results obtained are within the limit.

### 6.3.7 Robustness

The standard and samples of Piperaquine tetraphosphate and Dihyroartemisinin were injected by changing the conditions of chromatography.

## Variation in flow

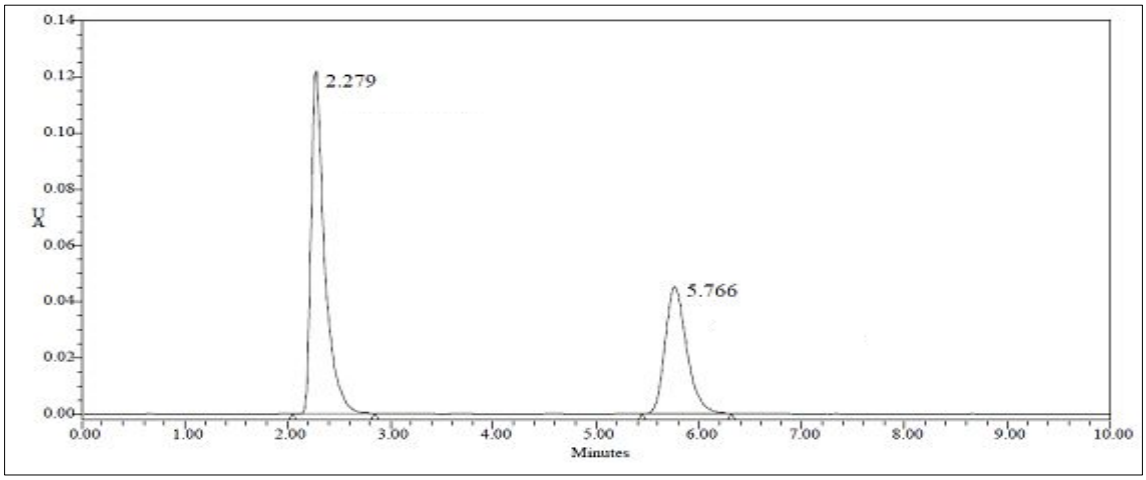


**Fig 26:** Chromatogram showing less flow of 0.7ml/min

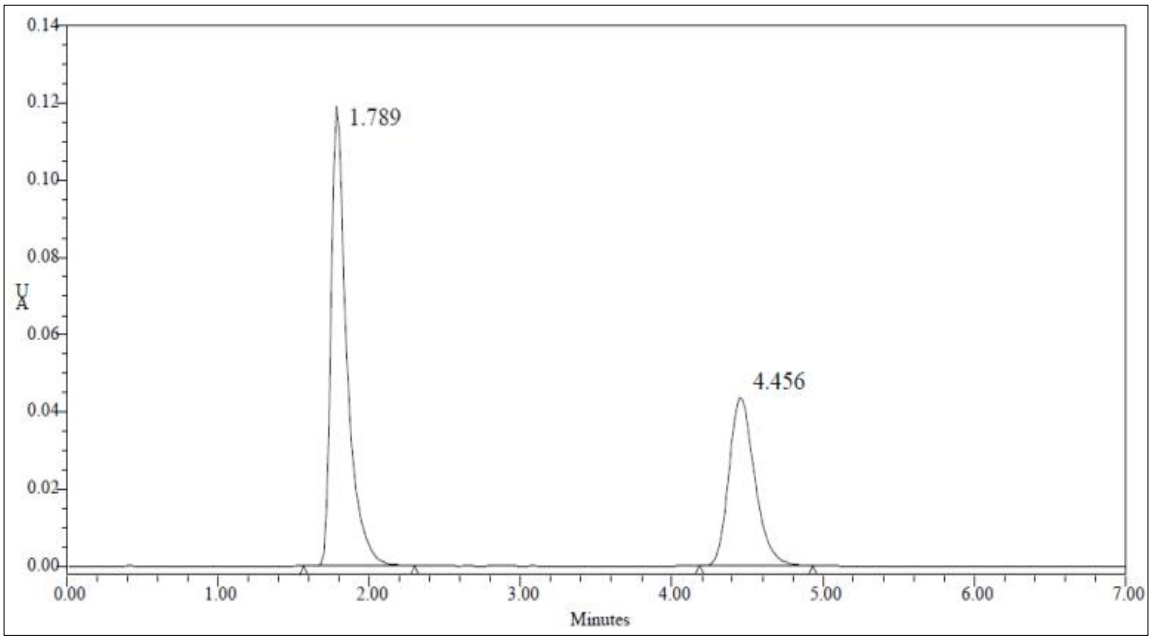


**Fig 27:** Chromatogram showing more flow of 0.9ml/min

## Variation of mobile phase composition

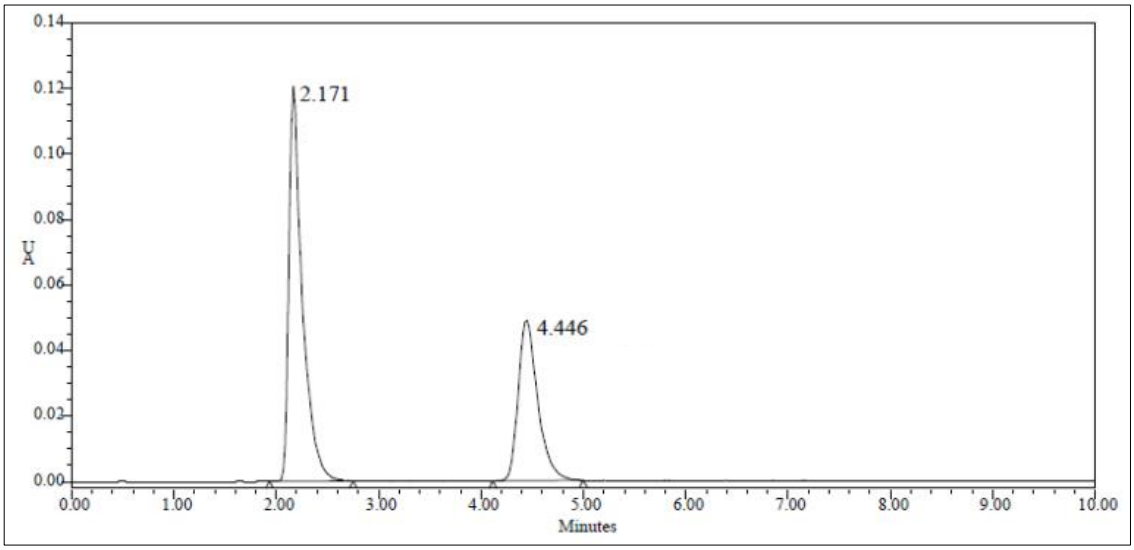


**Fig 28:** Chromatogram showing less organic composition

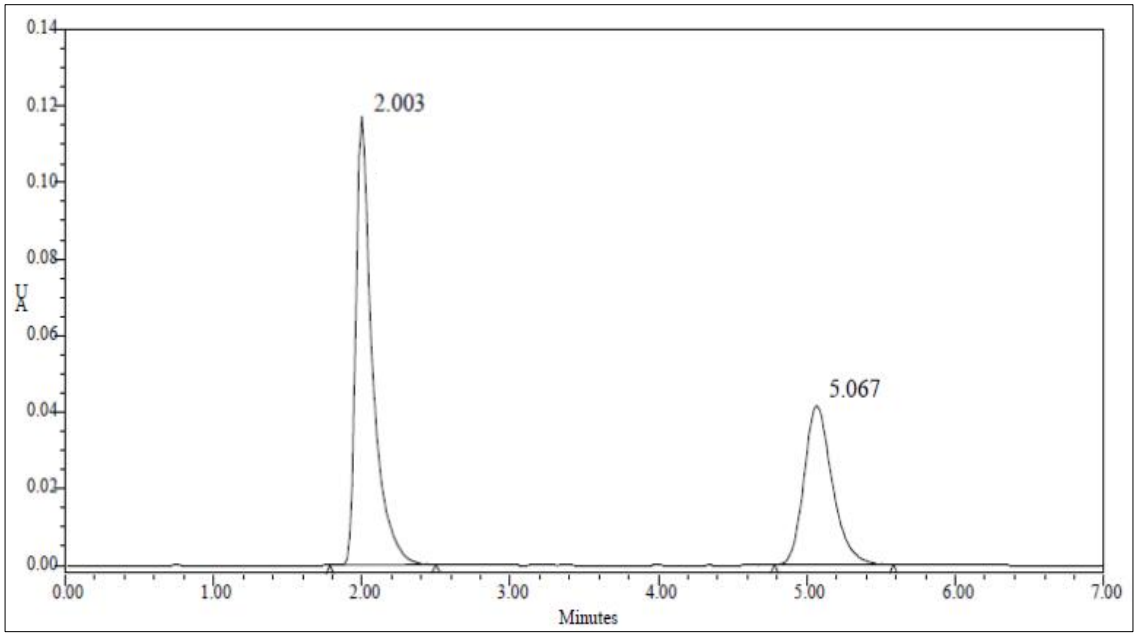


**Fig 29:** Chromatogram showing more organic composition

**Table 11:** Results for effect of variation in flow

| S. No | peak area for Less flow (0.7 ml/min) | | peak area for More flow (0.9 ml/min) | |
|---|---|---|---|---|
| | Piperaquine tetraphosphate | Dihyroartemisinin | Piperaquine tetraphosphate | Dihyroartemisinin |
| 1 | 983465 | 575351 | 971563 | 592641 |
| 2 | 985134 | 580381 | 973021 | 592352 |
| 3 | 983467 | 587724 | 975674 | 595471 |
| 4 | 985217 | 583190 | 978974 | 594416 |
| 5 | 994245 | 584468 | 984542 | 583453 |
| Mean | 986306 | 582223 | 976755 | 591667 |
| %RSD | 0.45 | 0.80 | 0.53 | 0.80 |

**Table 12:** Results for effect of variation in mobile phase composition

| S. No | Peak area for Less organic (70%) | | Peak area for More organic (90%) | |
|---|---|---|---|---|
| | Piperaquine tetraphosphate | Dihyroartemisinin | Piperaquine tetraphosphate | Dihyroartemisinin |
| 1 | 984565 | 574371 | 981565 | 593761 |
| 2 | 986134 | 585481 | 983527 | 592462 |
| 3 | 984268 | 587627 | 985489 | 594491 |
| 4 | 986216 | 585362 | 987954 | 596316 |
| 5 | 995247 | 585448 | 994672 | 587353 |
| Mean | 987286 | 583658 | 986641 | 592877 |
| %RSD | 0.45 | 0.90 | 0.51 | 0.57 |

**Acceptance criteria:** Percentage RSD should not be more than 2.

- The % RSD obtained for change of flow rate, variation in mobile phase was found to be below 2, which is within the acceptance criteria. Hence the method is robust.

## Summary and Conclusion

The estimation of Pipera Tetrapho and Dihydro artemisinin was done by RP-HPLC. The Phos buff was pH 4.6 and the mobile phas was optimized which consists of Methanol: Phosphate buffer mixed in the ratio of 70:30% v/ v. A Symmetry C18.1 (4.6 x 150mm, 5µm, Make XTerra) column used as stationary phase. The detection was carried out using UV detector at 273 nm. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of Pipera Tetrapho and Dihydro artemisinin were found to be from 25-125 µg/ml. Linear regression coefficient was not more than 0.999.

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