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Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Pyronaridine and Artesunate in Formulations

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ABSTRACT

A simple, accurate, precise, rapid, selective and reproducible reverse phase high performance liquid chromatographic method was developed for simultaneous estimation of artesunate and pyronaridine in the pharmaceutical dosage form. The separation was carried on Inertsil ODS 3V 250x4.6mm, 5micron column with a mobile phase containing acetonitrile and water in the ratio of 50:50 with a flow rate of 1 mL/min and UV detection at 220 nm. The retention time of artesunate and pyronaridine was found to be 4.0 min and 5.0 min. respectively. The correlation coefficients for the calibration curve of artesunate and pyronaridine was found to be 0.999 and 0.999 respectively. The developed methods were validated according to ICH guidelines.

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INTRODUCTION

Pyronaridine is an antimalarial drug. It was first made in 1970 and has been in clinical use in China since the 1980s[1]. Pyronaridine belongs to the family of Naphthyridines. These are compounds containing a naphthyridine moiety, naphthalene in which a carbon atom has been replaced by nitrogen in each of the two rings. The naphthyridine skeleton can also be described as an assembly two fused pyridine rings, which do not share their nitrogen atom.

IUPAC Name is4-[(7-chloro-2-methoxy-1,5-dihydrobenzo[b][1,5]naphthyridin-10-yl)imino]-2,6-bis(pyrrolidine-1-ylmethyl)cyclohexa-2,5-dien-1-one. Its molecular weight is 518.058 g/mol. A new Mannich base schizontocide originally developed in China and structurally related to the aminoacridine drug quinacrine is currently undergoing clinical testing. Pyronaridine targets hematin, as demonstrated by its ability to inhibit in vitro β-hematin formation (at a concentration equal to that of chloroquine), to form a complex with hematin with a stoichiometry of 1:2, to enhance hematin-induced red blood cell lysis (but at 1/100 of the chloroquine concentration), and to inhibit glutathione-dependent degradation of hematin. Our observations that pyronaridine exerted this mechanism of action in situ, based on growth studies of *Plasmodium falciparum* K1 in culture showing antagonism of pyronaridine in combination with antimalarials (chloroquine, mefloquine, and quinine) that inhibit β-hematin formation, were equivocal, effective in treating malaria-infected patients in regions of chloroquine resistance. However, more recent studies have shown that pyronaridine does not cause the formation of a protein-DNA complex in situ and thus does not appear to target the malaria parasite DNA topoisomerase II.

Artesunate (**AS**) is a medication used to treat malaria[2,3]. The intravenous form is preferred to quinidine for severe malaria.[2] Often it is used as part of combination therapy. It is not used for the prevention of malaria.[4] Artesunate can be given by injection into a vein, injection into a muscle, or taken by mouth.[4,5]

Artesunate is generally well tolerated.[5] Side effects may include a slow heartbeat, allergic reaction, dizziness, and low white blood cell levels.[4] During pregnancy, it appears to be a safer option, even though animal studies have found harm to the baby.[6] Use is likely okay during breastfeeding. It is in the artemisinin class of medication.[2]

Artesunate is a prodrug that is rapidly converted to its active form dihydroartemisinin (DHA). This process involves hydrolysis of the 4-carbon ester group via plasma esterase

enzyme.[7] It is hypothesized that the cleavage of the endoperoxide bridge in the pharmacophore of DHA generates reactive oxygen species (ROS), which increases oxidative stress and causes malarial protein damage via alkylation.[8] In addition, Artesunate potently inhibits the essential *Plasmodium falciparum* exported protein 1 (EXP1), a membrane glutathione S-transferase.[9]

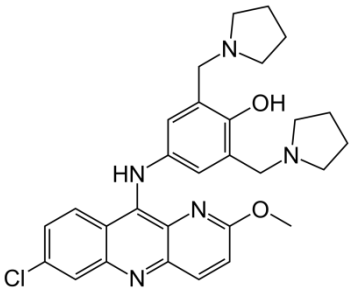
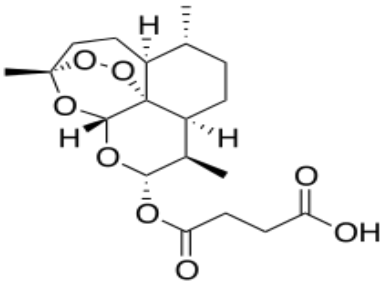


Fig. 1: Chemical structure of pyronaridine



Chemical structure of artesunate

MATERIALS AND METHODS:

MATERIALS: Pyronaridine, artesunate, Orthophosphoric acid, distilled water (HPLC grade) and acetonitrile was used in the study.

Instrument: A Liquid Chromatography is equipped with variable wavelength PDA-Detector and an Empower Software.

METHOD DEVELOPMENT:

Wavelength selection: 220 nm

Column: Inertsil ODS 3V 250x4.6mm, 5micron

Flow Rate: 1.0ml/min.

Table 1: Results of Gradient Programme.

Time	Mobile phase-A	Mobile phase-B
0	80	20
6	80	20
10	30	70
15	30	70
17	80	20
20	80	20

Wavelength: 220 nm.

Temperature: Ambient.

Injection Volume: 20microlitrs.

Mobile Phase-A: 1ml of Orthophosphoric acid in 1000 ml of water (0.1%)

Mobile Phase-B: Acetonitrile

Diluent: Acetonitrile and Water in the ratio of 50:50

Procedure:

Standard preparation: Transfer 180 mg of standard pyronaridine and 60.0 mg of standard artesunate into a 100 ml Volumetric flask. Dissolve and dilute it with acetonitrile and water in equal amount. Take 5 ml of the above solution into 50 ml volumetric flask and dilute it with diluent.

Test solution: Transfer 361.2 mg of pyramax formulation into a 100 ml Volumetric flask, dissolve and dilute to100ml with diluent take 5 ml of the solution and dilute it with diluents in 50 ml volumetric flask.

Specificity: Inject the diluent as blank and ensure a steady baseline. Inject the 1.8mg/ml of Pyronaridine, 0.6mg /ml of Artesunate of standard solution and sample record the response.

Linearity:

Table 2: Results of linearity of pyronaridine and artesunate

Concentration (mcg/ml)	Pyronaridine Area	Concentration (mcg/ml)	Artesunate Area
40	2577965	40	444585
60	3881757	60	657392
80	5129301	80	867040
100	6359816	100	1059821
120	42769439	120	1293872

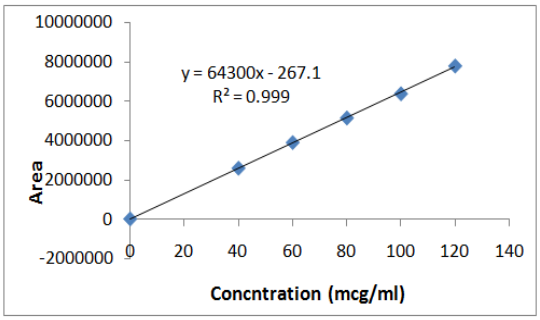


Fig. 2: Chromatogram of linearity of pyronaridine

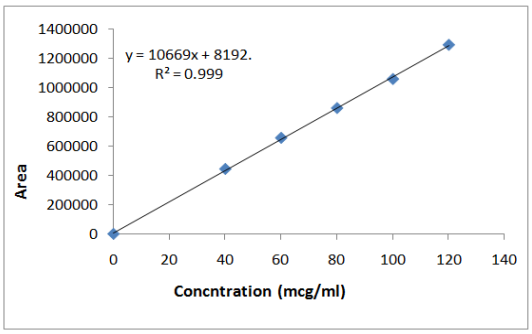


Fig 3: Chromatogram of linearity of artesunate

Precision: Inject the 1.8mg/ml of pyronaridine, 0.6mg/ ml of artesunate of standard solution six times and record the response. Inject the pyramax formulation sample six times and record the response.

Table 3: Results of precision values of pyronaridine and artesunate

Injection	Pyronaridine		Artesunate	
	Standard Area	Sample Area	Standard Area	Sample Area
1	6389446	6339546	1059663	1061493
2	6400198	6371184	1070018	1055854
3	6297132	6379015	1060947	1080058
4	6379835	6363764	1064519	1059913
5	6379934	6380275	1051787	1061272
6	6387712	6348638	1070300	1060394
Mean	6372376.2	6363737.0	1062872.3	1063164.0
Std. Dev	37616.6	16586.5	7011.9	8525.9
% RSD	0.6	0.3	0.7	0.8

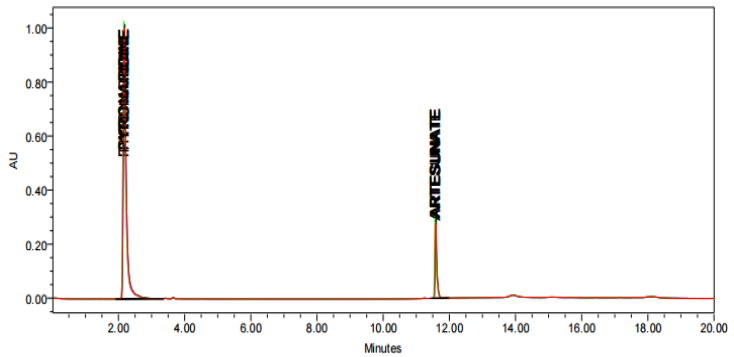


Fig. 4: Chromatogram of sample and standard area of pyronaridine & artesunate

Accuracy:

Inject 80%, 100 %, 120% respectively of sample and 10%, 10%, 12% respectively of Pyramax standard solution spiked 3 times and record the response.

Table 4: Results of Accuracy -80% of Pyronaridine

Injection	Standard Area	Sample area	Standard Area	Sample Area
1	5167880	5820074	861186	978816
2	5158558	5783540	863315	979884
3	5170005	5818509	860546	974254
Mean	5165481.0	5807374.3	861682.3	977651.3
Std. dev	6088.9	20656.0	1449.7	2990.2
% RSD	0.1	0.4	0.2	0.3

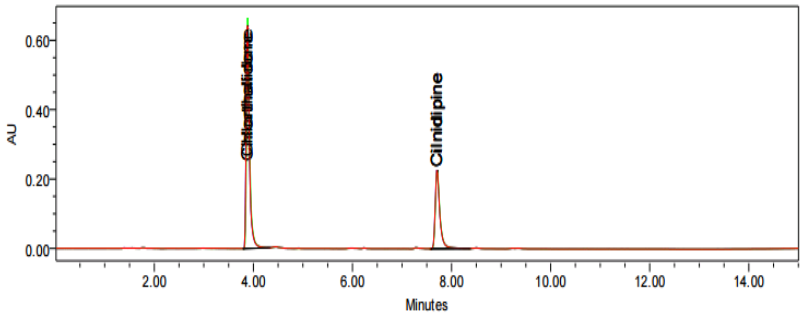


Fig. 5: Chromatogram of Accuracy of 80% Spiked.

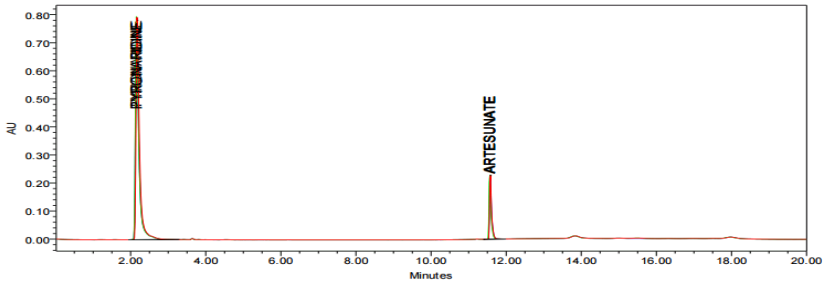


Fig. 6: Chromatogram of Accuracy of 80% Standard.

Table 5: Results of Accuracy -100% of pyronaridine

Injection	Standard Area	Sample Area	Standard Area	Sample Area
1	6362608	7057716	1059400	1178065
2	6364773	7088905	1056552	1178249
3	6339781	7078970	1055866	1176974
Mean	6355720.7	7075197.0	1057272.7	1177762.67
Std. Dev	13846.5	15933.1	1874.0	689.2
% RSD	0.2	0.2	0.2	0.1

Table 6: Results of Accuracy -120% of pyronaridine

Injection	Standard Area	Sample Area	Standard Area	Sample Area
1	7818782	8390970	1295700	1383350
2	7883772	8311382	1297600	1383456
3	7842034	8381721	1308114	1378767
Mean	7848196	8361358	1300471	1381857.67
Std. Dev	32930.3	43526.6	6686.6	2677.1
% RSD	0.4	0.5	0.5	0.2

LOD & LOQ: Starting with concentration of 20%, 10%, 5%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, 0.02%, 0.01%, 0.005% respectively and record the response.

Table 7: Results of LOD & LOQ of Pyronaridine and Artesunate.

S. No.	Injection	Pyronaridine Area	Artesunate Area
20%	1	1287214	222000
10%	1	510693	113291
5%	1	247497	62878
2.0%	1	50377	24643
1.0%	1	13314	12966
0.5%	1	ND	4943
0.2%	1	ND	1523
0.1%	1	ND	980
0.05%	1	ND	ND
LOD	-	1.0%	0.10%
LOQ	-	3.0%	0.30%

Robustness:

Different Column: Inject the 1.8mg/ml of pyronaridine, 0.6mg/ ml of artesunate standard solution 3 times record the response. Inject the pyramax formulation sample in 3 times and record the response

Table 8: Results of a different column of Pyronaridine.

Injection	Standard Area	Sample Area	Standard Area	Sample Area
1	5768546	5600937	958895	928514
2	5747571	5597876	957689	929953
3	5758009	5610327	955216	928768
Mean	5758042.0	5603046.7	957267	929078.3
Std. Dev	10487.5	6488.1	1875.5	768.1
% RSD	0.2	0.1	0.2	0.1

FLOW INCREASE (Flow: 1.1ml/min)

Table 9: Results of Flow increase of Pyronaridine.

Injection	Standard Area	Sample Area	Standard Area	Sample Area
1	5775365	5752617	954387	951577
2	5766718	5704790	952072	947960
3	5746149	5735559	956654	948779
Mean	5762744	5730988.7	954371.0	949439
Std. Dev	15007.9	24238.8	2291.0	1896.6
% RSD	0.3	0.4	0.2	0.2

FLOW DECREASE (Flow: 0.9ml/min)

Table 10: Results of Flow decrease of Pyronaridine.

Injection	Standard Area	Sample Area	Standard Area	Sample Area
1	7070556	7078460	1204738	1195229
2	7076139	7065140	1200062	1194744
3	7068094	70676739	1204501	1194945
Mean	7071596	28273446.3	1203100.3	1194972.7
Std. Dev	4122.2	36722329.3	2633.9	243.7
% RSD	0.1	129.9	0.2	0.0

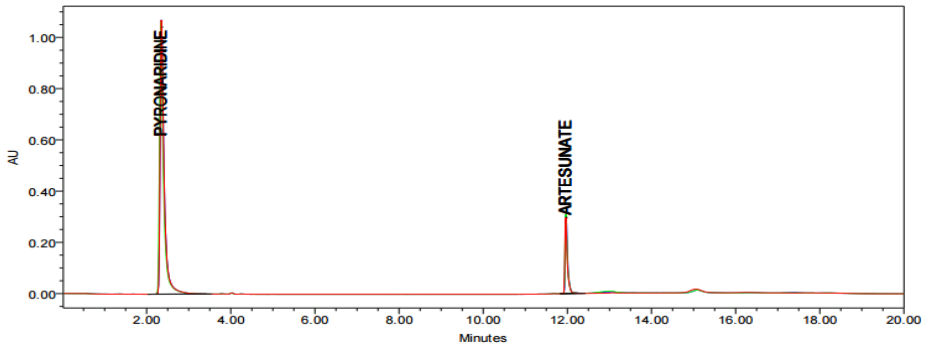


Fig. 7: Chromatogram of Standard of Flow Decrease.

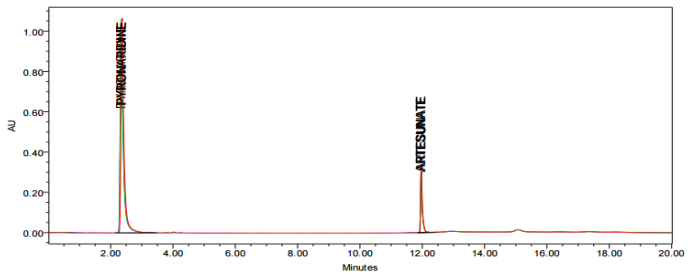


Fig. 8: Chromatogram of Sample of Flow Decrease

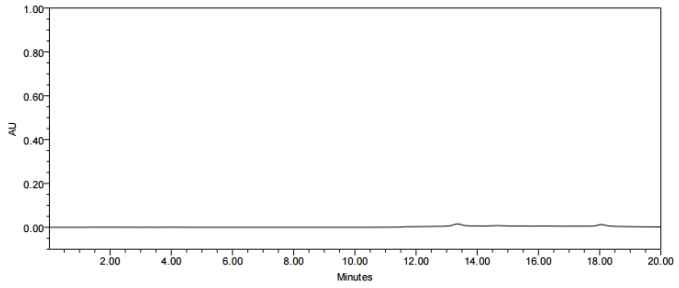


Fig. 9: Chromatogram of Blank of Flow Decrease.

RESULTS AND DISCUSSION

The estimation of Pyronaridine and Artesunate was done by RP-HPLC. The assay of Pyronaridine and Artesunate was performed with tablets and the % assay was found to be 100.83 and 100.23 which shows that the method is useful for routine analysis. The linearity of Pyronaridine and Artesunate was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision are RSD should be not more than 2.0% and the method show precision 0.6 and 0.5 for Pyronaridine and Artesunate which shows that the method is precise. The acceptance criteria of intermediate precision are RSD should be not more than 2.0% and the method show precision 0.6 and 0.2 for Pyronaridine and Artesunate which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.40% and 100.25% for Pyronaridine and Artesunate. The validation of the developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ is 3 and 10. The LOD and LOQ for Pyronaridine were found to be 0.1and 1 and LOD and LOQ for Artesunate was found to be 0.3 and 3.0. The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits, Which shows that the method is having good system suitability and precision under given set of conditions.

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