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Original Article

Novel Stability Indicating RP-HPLC Coupled with PDA Detection for the Simultaneous Quantification of Artesunate and Amodiaquine in Bulk and its Tablet

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ABSTRACT

Background: The present research paper reports the development of novel, stable and validated RP HPLC method for the simultaneous estimation of Artesunate (ATS) and Amodiaquine (AMDQ) in bulk and tablet dosage form. **Methods:** Good chromatographic separation was achieved by isocratic mode with a mixture of Phosphate Buffer: Methanol in the ratio of 60: 40 as mobile phase with waters symmetry Shield Rp18Column (250 mm x 4.6mm) and 5 micron particle size as stationary phase at flow rate of 1.0 mL/min. The detection was performed at 223 nm. Retention times for ATS and AMDQ were found 3.6 and 1.5 min respectively. **Results:** The proposed method showed good intra-day precisions (%RSD=0.36 for ATS, 0.50 for AMDQ), highly accurate (recovery for both ATS and AMDQ>99%) and satisfactory correlation coefficient (R^2= 0.9914 for ATS and 99.38 for AMDQ). The detection and Quantitation limit were 0.53 µg/mL and 0.48 µg/mL for ATS, 1.23 µg/mL and 1.78 µg/mL for AMDQ. The percentage recovery in forced degradation study using acid, base, oxidation, photolytic, thermal and neutral conditions shows satisfactory and indicates well separation of both the drugs with other degradation products and the developed method also found good solution stability. **Conclusion:** Therefore, the present method was found stability indicating untroubled method ever developed, useful for the routine analysis of both the mentioned drugs in bulk as well as tablet dosage form.

Key words: Artesunate, Amodiaquine, RP-HPLC, Stability, Validation, ICH.

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INTRODUCTION

The treatment of malaria in current practice is based on the concept of combination therapy, because this offers several advantages, reduced risk of developing resistance, including reduced risk of treatment failure enhanced convenience and reduced side-effects.[1] Artemisinin Combination Therapy (ACT) is increasingly being advocated as promising treatment and are the best anti-malarial drugs available now.[2] The basis of ACT is the use of two drugs with different modes of action: an artemisinin-derivative (eg. artesunate) that causes rapid and effective reduction of parasite gametocyte carriage and biomass and a partner drug that has a longer duration of action.[3]

It is the foremost for an analyst to develop stable, accurate, easy, validated economic method for the analysis of APIs as well as marketed formulations. Comprehensive validated analytical methods are always accepted and which finally reduces the cost of marketed formulations and can easily affords by the common people. The above statement will be more precious for widely using formulations.[4] Here we considered such kind of formulation i.e Artesunate (ATS), which is chemically 1,2-benzodioxepin-10-yl hydrogen butanedioate, shows in Figure 1(A) and Amodiaquine (AMDQ) which is chemically -4-[(7-chloroquinolin-4-yl)amino]-2 [(diethylamino)methyl]phenol, shows in Figure 1(B). ATS and AMDQ was not official in any pharmacopoeia and also the increasing association of ATS and AMDQ as an effective treatment for the resistant malaria it is necessary to develop easy economic, stable validated method for its routine analysis. The detailed literature review revels that still now no method was officially published in any pharmacopoeia, for ATS and AMDQ. Also, there was very few literatures where this simultaneous analysis of both drugs were reported. One reported method,[5] used gradient elution technique, which is not considered simple. The reported method utilized large amount of acetonitrile,[6] which is considered costly and peak intensity was very low with narrow linearity range. In existing methods, the retention time of artesunate was reported 10 min[7] which was consider too long for HPLC analysis. All reported method shown the double UV detection and high limit of detection, which creates some doubts in sensitivity issue. Till now no stability indicating method was reported to observe the degradation pattern in various stressed conditions. Therefore it is very much important to develop easy, reliable and stability indicating method of analysis for artesunate and amodiaquine in bulk and tablet dosage form and to validate as per ICHQ2B guidelines.[8]

EXPERIMENTAL

Instrumentation and Reagents

Pure ATS (99.95%) and AMDQ (99.89%) standards were received from Alkem Laboratories Limited (Nalagarh road, Solan district, Baddi, India) as gift sample. Tablets (Larimal, from Ipca Laboratories) were purchased from the local Pharmacy retail shop, Hyderabad, India. Ultra pure water for HPLC obtained from Millipore system, methanol and water (HPLC grade) was obtained from Sigma-Aldrich, Hyderabad, India. Potassium dihydrogen orthophosphoric acid purchased from Merck (Mumbai, India). The chromatographic separation was achieved on symmetry Shield RP18 HPLC system (Milford, MA, USA) composed of 2998 PDA detector, quaternary pump with empower-3 software. Detector wavelength was selected at 223 nm. The elution was performed at a flow rate

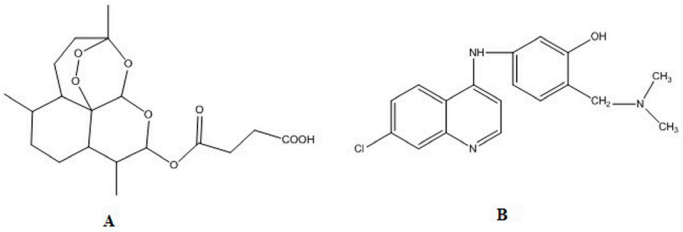

Figure 1: Chemical structure of ATS (A) and Chemical structure of AMDQ (B).

of 1ml/min and injection volume was 10µl. The mobile phase used was a mixture of potassium dihydrogen phosphate buffer (A): methanol (B) to pH 3.8 was adjusted with acetic acid. The volume ratio of solvent A against solvent B was (60:40). Column temperature was maintained at 30°C. Validation study, force degradation and solubility study were carried out using same optimised condition with suitable preparation of standard and sample solutions.

Standard and sample solution preparations

Preparation of standard solution

100 mg of both the APIs (ATS and AMDQ) was dissolved separately in 5 ml of methanol and volume was made up to 100 ml methanol (primary stock, 1000 µg/ml). 10 ml of the aliquot was withdrawn and transferred in a 100 ml volumetric flask and volume was made with mobile phase (methanol: Phosphate buffer) to obtained secondary stock solution (100 µg/ml). Both the APIs solution was prepared separately.

Combined working solution

Accurately 1 ml of aliquot of ATS and AMDQ was withdrawn from their secondary stock solution and transferred in a10 ml of volumetric solution. The volume was made up to the mark with mobile phase to achieve 10 µg/ml of combined solution.

Analysis of fixed dose tablet

Twenty Larimal tablets were taken in mortar and triturated into powder. 500 mg the tablet powder was taken which is equivalent to 70.88 mg of ATS and 213 mg of AMDQ was dissolved in 100 ml of methanol in a volumetric flask. Transfer 0.5 ml from the above solution in a 100 ml of volumetric flask and volume was made up to the mark with sufficient mobile phase, which finally produce 3.54 µg/ml of ATS and 10.65 µg/ml of AMDQ. 20 µl of the above solution was injected into the chromatographic system.

Validation

Accuracy

Accuracy of the method was studied by considering the recovery study. To the marketed formulation, the reference standards of the ATS and AMDQ were added at the level of 50%, 100% and 150%, injected three times. Formulation product (Tablet) conc. is kept constant (ATS – 3.54 mcg and AMDQ– 10.65 mcg) and standard drugs were spiked. Three 10 mL vol. flasks were taken and 1 mL of the test stock solution was added to each flask and 0.5 mL (50%), 1mL (100%) and 1.5 mL (150%) of the standard stock solution (spike) were added to different vol. flask and the volume was made up to 10mL with mobile phase to obtain concentrations of ATS– 8.54 µg and AMDQ-15.65 µg (50%), ATS– 13.54 µg and AMDQ– 15.65 µg (100%), ATS-18.54 µg and AMDQ– 25.65 µg (150%) respectively. 20 µL volume of the sample was injected three times and chromatograms were collected were recorded. The percentage recovery and % mean recovery were calculated.

Precision

Preparations of ATS (5 and 10 µg/mL) and AMDQ (5 and 10µg/mL) for six times the intra- and inter-day precision was determined by analysing for six times on same day (intra-day study) and repeated on the second day (inter-day study). The chromatograms were recorded. The peak area and retention time of both the drugs under study was determined and relative standard deviation (RSD) was calculated.

Linearity

Working standard solutions of ATS and AMDQ were prepared as described earlier, aliquots from these solutions were diluted with mobile phase in five different concentrations to obtain 5-40 µg/ml of ATS and 50-500 µg/ml of AMDQ. Calibration curves were plotted for both the drugs under study by considering the concentration and peak area. The obtained data was used for regression analysis.

System suitability

To verify whether analytical system is working properly, this study was evaluated by injecting the standard drugs of ATS and AMDQ six times. The RSD of the parameters like theoretical plates, peak area, retention time and asymmetric factor were calculated.

Specificity

The specificity study was carried out by placebo interference test of the sample solution using 283.88 mg of placebo equivalent to one tablet dissolved in 100ml of mobile phase and the placebo solution was treated like a standard solution. The solution was injected to the chromatographic system to evaluate the possible interfering peaks.

Robustness

This study was conducted by varying the various analytical conditions like flow rate, mobile phase ratio and detection wavelength at three different levels. One factor was changed at one time to estimate the effect on determination. The content of the ATS and AMDQ was determined along with other factors like retention time, tailing factor and peak area.

Detection and Quantitation limit

Standard solutions of ATS and AMDQ was prepared by sequential dilution and injected into the chromatographic system in decreasing order of concentration in the range of 0.5-10 µg/ml of ATS and 0.4-10 µg/ml of AMDQ.

Solution stability

Stability of the sample solution was conducted by storage of sample solution at 25°C for 24 hrs. Sample solution was reanalyzed after 12, 24, 36 and 48 hrs time intervals and content was determined using the developed assay method for the compound ATS and AMDQ and compared against the freshly prepared sample.

Force degradation study of ATS and AMDQ

Stress study was carried out in a environmental test chamber (by Acamus Technologies, India) at 60°C and relative humidity was maintained 75%, as per ICH prescribed stress condition such as acidic, basic, thermal, oxidative and photolytic stresses.

Acid degradation study was conducted in environmental test chamber (Acamus Technologies, India) at 60°C and 75% relative humidity with 1M HCL. 1 ml of stock solution was transferred in 10 ml of volumetric flask, 1 ml of 1 M HCL was added to the flask, kept in environmental test chamber for 16 hrs. After the suitable stress period, solution was neutralized using 1M NaOH and make up the volume with mobile phase.

Base degradation study was conducted at 60°C and 58% relative humidity using same environmental chamber. 1 ml of stock solution was transferred in 10 ml volumetric flask mixed with IM 1 ml of 1M NaOH and

kept for 16 hrs. After the suitable stress period the solution was neutralized with I M HCL and the volume was made with mobile phase.

Oxidative degradation study was performed in versatile environmental chamber at 40°C, 75% relative humidity using 6% H_2O_2. For this purpose, 1 ml of stock solution was taken in 10 ml volumetric flask and 1 ml of 6% H_2O_2 was added in to flask and kept at 60°C for 16 hr, finally make up the volume up to mark with mobile phase. Thermal degradation study also has been carried out using environmental chamber at 40°C, 75% relative humidity in oven at 105°C, 1 ml of stock solution was taken in 10 ml volumetric flask and kept in chamber for 144 hr. and for dry heat thermolysis, 1 mg of dry drug in solid form was placed in oven at 110°C for 2 days.

Photolytic degradation study was carried out in sunlight (60000- 70000 lux) during day time and in UV light at 254 nm for the period of 48 hr. 1 ml of stock solution was taken in 10 ml volumetric flask and make the volume up to mark with mobile phase was used for the study.

RESULTS

Method development and Optimisation

The selected mobile phase consisting of phosphate buffer and methanol in the volume ratio of 60:40(v/v) with apparent pH of the mixture was adjusted to 3.8 with acetic acid using Symmetry shield RP 18 (250nm × 4.6mm, 5µm) column. The retention time of ATS and AMDQ was found 1.519 and 3.643 min. The optimum working temperature was found to be 30°C. 10µl injection volume with PDA detection at 223 nm was found optimal as shown in Figure 2. The above condition was adopted for subsequent analysis.

Method Validation

The accuracy of the method was evaluated by the determination of recovery of spiked crude drugs at three levels, 50%, 100% and 150%. ATS mean recovery (n=6) was 98.12 and 99.34 for AMDQ indicates the accuracy of the method as shown in Table 1. Intra-day precision analysis (n=6) for ATS and AMDQ shows %RSD value of 0.36 and 0.48 respectively. Whereas the mean content of the inter-day precision was found %RSD value of 0.44 for ATS and 0.50 for AMDQ. Shown in Table 2. The regression coefficient R^2 was found 0.991 for ATS and 0.993 for AMDQ clams the linearity of the developed method. The regression analysis data was presented in Table 2. The result of system suitability study using standard drugs of ATS and AMDQ six times shows that % RSD of several parameters is less than 2. like theoretical plate, retention time, peak area, asymmetric factor were within limit i.e. less than 2 and results are shown in Table 2, which indicates the suitability of the analytical system to carry out the method.

In the study of specificity, no peak was detected at the retention time corresponding to both ATS and AMDQ. According to the standard signal to noise ratio formula, the LOD value was obtained 0.53 µg/mL for Artesunate and 1.23 µg/mL for Amodiaquine. The LOQ value was found to be 0.48 µg/mL for ATS and 1.78 µg/mL for AMDQ shown in Table 2 and chromatograms were depicted in Figure 4. Robustness study was performed by changing in flow rate from 0.8, 1, 1.2 ml/min, buffer and methanol ratio from (62: 38, 60: 40 and 38: 42), detection wavelength of 222, 223 and 224. The %RSD of the retention time and peak area in all modified conditions were found within acceptance limit. Therefore, the developed method was found robust. The details of the study results were shown in Table 3. Fixed dose combination tablet Larimal, which contains 50 mg of ATS and 153 mg of AMDQ were analysed. Result was presented in Table 4 and the chromatogram was shown in Figure 3. The ATS content of the tablet was found 97.98 mg and AMDQ content was found 100.88 mg. Solution stability study was performed up to 48 hr (every 12hr interval) of prepared marketed fixed dose sample of ATS and AMDQ. The amount of ATS was found 52.223, 48.013, 50.611, 48.902 mg, similarly AMDQ was found as 152.32, 153.30 and 152.77, 152.21 mg at 12th, 24th, 36th and 48th hour. Results were summarized in Table 5.

Force degradation study was conducted using developed optimised condition and further assay values of the stressed samples were calculated. ATS which shown 12.45% degradation and 11.49% for AMDQ at peroxide degradation and 11.36% at thermal degradation study. ATS also shown 10.63% degradation at thermal degradation. The details of

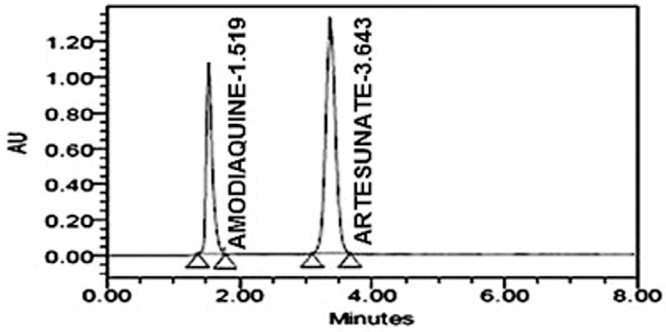

Figure 2: Chromatogram of optimised condition.

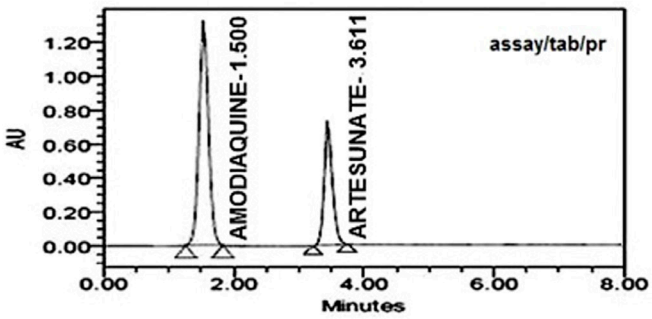

Figure 3: Chromatogram of tablet assay.

Table 1: Result of recovery study of ATS and AMDQ.

Spiked level (%)	Amount Present (µg/mL)		Amount of standard added (µg/mL)		Amount found (µg/mL)		% of recovery		% of mean recovery	
	ATS	AMDQ	ATS	AMDQ	ATS	AMDQ	ATS	AMDQ	ATS	AMDQ
50	3.54	10.66	5	5	4.97	4.95	98.24	99.16		
100	3.54	10.66	10	10	9.78	10.29	97.85	100.29	98.12	99.34
150	3.54	10.66	15	15	14.74	14.78	98.27	98.59		

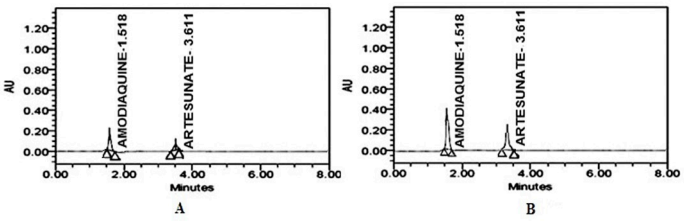

Figure 4: LOD (A) and LOQ (B) chromatograms.

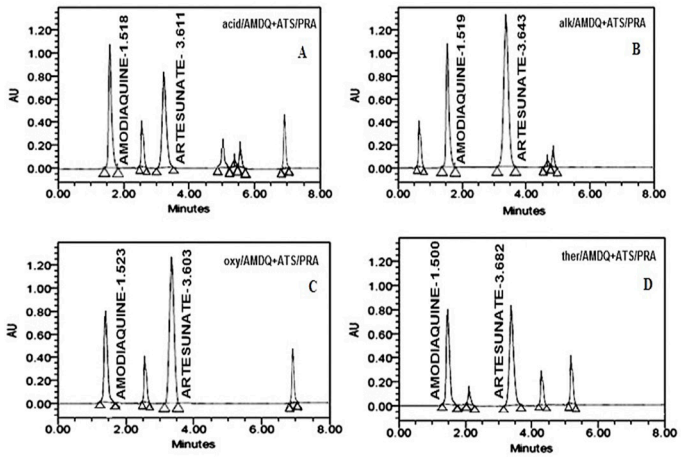

Figure 5: Chromatograms of force degradation study.

Table 2: Summary of validation parameters.

Validation Parameters	ATS	AMDQ
Linearity	0.991	0.993
Intraday precision (%RSD)	0.46	0.97
Interday precision (%RSD)	0.36	0.50
Recovery (%)	98.12	99.34
LOD (µg/mL)	0.53	1.23
LOQ (µg/mL)	0.48	1.78

% RSD= percentage relative standard deviation.

Table 3: Robustness study of ATS and AMDQ.

Parameter	Value	ATS (5µg/mL)		AMDQ (5µg/mL)	
		RT	Peak area	RT	Peak area
Flow-rate (mL/min)	0.8	3.610	235027	1.533	210207
	1	3.592	232128	1.524	214288
	1.2	3.689	228779	1.603	212823
Detection wavelength (nm)	222	3.630	228323	1.502	222492
	223	3.621	225238	1.513	218553
	224	3.678	232105	1.511	219705
M.P Variation	62:38	3.570	220237	1.500	208773
	60:40	3.622	230292	1.512	212335
	58:42	3.617	238033	1.511	223864
Mean		3.625	230018	1.512	215337
SD		0.037	5257	0.01068	5129.44
% RSD		1.04	1.29	1.71	1.93

Table 4: Result for assay of marketed formulation (Larimal).

Drug	Label claim (mg)	Amount found (mg)	% Assay
Artesunate	50	48.99	97.98
Amodiaquine	150	151.32	100.88

Table 5: Solution stability study of fixed dose combination (50 + 150 mg).

Condition	Amount * of ATS found (mg)	Amount * of AMDQ found (mg)
Initial (0hr)	50.182	150.65
12 hr	52.223	152.32
24 hr	48.013	151.10
36 hr	50.611	152.77
48 hr	48.902	152.21

*amount was found after performing present developed assay method.

Table 6: Results of stress degradation studies.

Condition	ATS (10µg/mL)			AMDQ (10µg/mL)		
	Peak area	% Assay	% DEG	Peak area	% Assay	% DEG
Acid (After stress period)	53225	85.53	12.45	98569	92.37	8.51
Acid (Before stress period)	62876	96.23	3.45	123647	98.64	1.56
Base (After stress period)	326887	90.05	7.20	88247	93.13	6.53
Base (Before stress period)	395462	96.46	3.29	95647	97.45	2.66
Peroxide (After stress period)	320259	94.22	3.78	68238	89.39	11.49
Peroxide (Before stress period)	375462	97.46	1.98	76234	98.21	1.54
Thermal (After stress period)	83279	87.35	10.63	59942	89.52	11.36
Thermal (Before stress period)	89564	97.48	2.79	76354	98.89	0.98
Photolytic (After stress period)	386934	99.23	-	187234	100.02	-
Photolytic (Before stress period)	384783	99.21	-	186392	100.01	-

the results were shown in Table 6. Degradation peaks were shown in chromatogram Figure 5.

DISCUSSION

Prior to select the optimised chromatographic condition, numbers of preliminary trials were conducted with different combination of solvents, various buffers, pH, flow rate, temperature and columns in order to justify the retention time, peak shape, resolution and other chromatographic parameters. The mobile phases used in the optimization procedure were prepared by mixing various buffer system with organic solvents. Several solvents (methanol, acetonitrile), buffers (orthophosphoric acid pH 2, 8, ammonium acetate pH 5 and 7, potassium dihydrogen orthophosphate pH 5, 6.8) used in different volume ratio. Four types of analytical column were initially tested *viz* Hypersil BDS C18 (250nm x 4.6mm, 5µm), Agilent Eclipse XBD C8 (150 x 4.6mm, 5µm), Purosper® Star (250 × 4.6 mm ID, 5 µm particle size) Symmetry shield RP 18 (250nm x 4.6mm, 5µm) with U.V detection at 223nm. The temperature of the column was varied between 20°C and 40°C. The PDA detector at 223 nm was also tried with the mobile phase (A) contains potassium dihydrogen phosphate buffer and methanol (B) (A: B 50:50 v/v). This mobile phase showed poor peak shape for both the drug. Further optimization was accomplished by investigating various pH and volume ratio of these two components. Finally, a selected mobile phase consisting of phosphate buffer and methanol in the volume ratio of 60:40(v/v) with apparent pH of the mixture was adjusted to 3.8 with acetic acid using Symmetry shield RP 18 (250nm x 4.6mm, 5µm) column. Under this adopted chromatographic conditions ATS and AMDQ were fully separated within 1.519-min retention time for ATS and 3.643 min with a better sensitivity and excellent peak shape with proper resolution. The use of PDA detector with selected wavelength of 223 nm greatly improves the sensitivity of both the drug under study. The values of both intra-and inter-day precision were presented as % RSD indicates that the developed method was precise. Linear correlation was found between the concentration and peak area in a specific range. Several parameters like theoretical plate, retention time, peak area, asymmetric factor were within limit for the system suitability study using standard drugs of ATS and AMDQ. The result of specificity study which clearly indicates there is no interference of excipients in the formulation and strongly supports the specificity of the developed method. LOD and LOQ values clearly indicates the sensitivity of the developed method. From the robustness study it was observed that not much variation in tailing factor was observed with deliberate changes in flow rate and temperature. The tailing factor was found within the limits for Artesunate and Amodiaquine. The amount of both the drug was found very close to the labeled claim amount, which indicates the efficiency of assay procedure for the determination of drug content. The result of solution stability study, the calculated assay results of stability study reveals that both the drugs shows approximately similar results to freshly prepared sample, which assures the solution stability of the present developed method.

Force degradation study indicates that the peaks of both the analytes were well separated and found as very specific from the degradation peaks which were shown in the chromatogram. Stressed conditions significantly decreased the peak area of both the ATS and the AMDQ. The additional peaks due to degradation of analytes in different conditions has shown that both the drugs were degraded greater extent in acidic stressed condition, slightly less extent in alkaline stressed and lesser extent in oxidative stressed conditions. changes were not observed in the peak areas of both the ATS and AMDQ, also no degraded peaks were obtained in photolytic and thermolysis stressed conditions. The chromatogram was like a fresh sample before stressed, indicates both the drugs are stable in above two stressed condition.

CONCLUSION

Based on the empirical evidences, the present method was strongly clammed about the novelty of the present developed method over the reported methods. This is the first stability indicating method, which is 'rapid' because it significantly reduced the total analysis time i.e 3.6 min, which is the lowest analysis time required. It was considered "economic" because present method replaced costly acetonitrile with methanol. The method justifies "easy", because the proposed method does not involved use of dual wavelength, gradient techniques. The present method is "stability indicating" as this has been shown less degradation pattern in stressed conditions and good separation of both the drugs among the other degraded peaks. The method considers "validated" because all the results of validation parameters were found within the limits as per the ICH Q2B guidelines. Hence the present developed method can be designate as a reliable, validated and stable, highly useful for the routine analytical and quality control study of the ATS and AMDQ in the tablet dosage form.

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CONFLICT OF INTEREST

The authors declare conflict of interest.

ABBREVIATIONS

ATS: Artesunate; **AMDQ:** Amodiaquine; **RP HPLC:** Reverse phase High performance liquid chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantitation; **RSD:** Relative standard deviation; **PDA:** Photo diode array; **ICH:** International conference on Harmonization.

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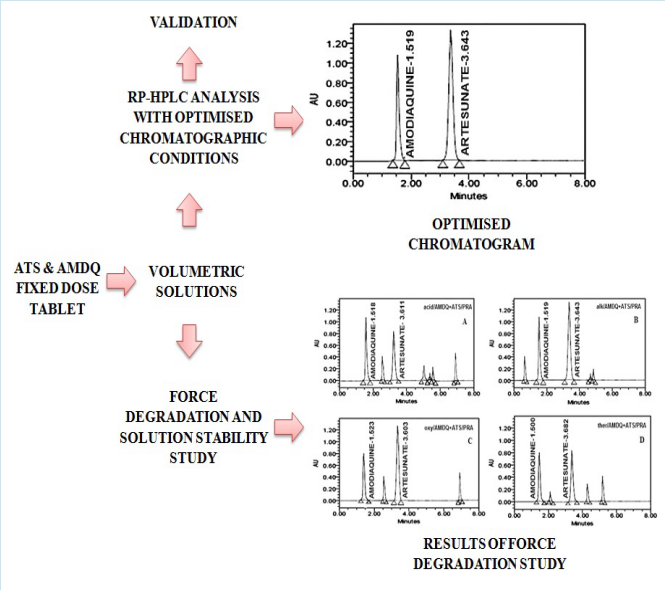
PICTORIAL ABSTRACT



SUMMARY

The present research paper reports the development of novel, stable and validated RP HPLC method for the simultaneous estimation of Artesunate (ATS) and Amodiaquine (AMDQ) in bulk and tablet dosage form. Good chromatographic separation was achieved by isocratic mode with a mixture of Phosphate Buffer: Methanol in the ratio of 60: 40 as mobile phase with waters symmetry Shield Rp18Column (250 mm x 4.6mm) and 5 micron particle size as stationary phase at flow rate of 1.0 mL/min.

The proposed method showed good intra-day precisions, highly accurate and satisfactory correlation coefficient. The detection and Quantitation limit were 0.53 µg/mL and 0.48 µg/mL for ATS, 1.23 µg/mL and 1.78 µg/mL for AMDQ. The present method was found stability indicating untroubled method ever developed, useful for the routine analysis of both the mentioned drugs in bulk as well as tablet dosage form.

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