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Development and validation of stability indicating UPLC method for determination of related impurities in Artesunate and Amodiaquine fixed dose tablets

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ABSTRACT

The HPLC gradient method can be succefully transferred on UPLC. Transfer of the method was accomplished by geometrically scaling flow rate, injection volume, and gradient profile. Analysis time was reduced by four fold with an improvement of 64% in the resolution of critical pair of artesunate and artemisinin. Sensitivity of UPLC over HPLC was evaluated by comparison of LOQ values of dihydroartemisinin and glycan obtained in gradient HPLC for both systems. The almost ten times lower LOQ values with higher precision are attributed towards better sensitivity of UPLC method. The present work demonstrated the ease of HPLC to UPLC method transferability, and the benefits that can be obtained in any time and resource. It proved that UPLC can significantly increase throughput with quality results. With variety of column dimensions, scientists have the flexibility to tailor their UPLC separations to the goals at hand.

Development of HPLC/UPLC method

Key words- UPLC, gradient, sub-micron particles, resolution, LOQ, HPLC

INTRODUCTION

Streamlining Development

HPLC is ubiquitous in the pharmaceutical industry and is employed throughout the whole drug analysis process, including drug discovery screening, raw material analysis, impurity profiling, stability studies, pharmacokinetic studies and final product testing.

However, despite HPLC's popularity, by the end of the 1990s it had reached its technical performance limitations with existing technology. In an increasingly competitive environment, pharmaceutical scientists were looking for a next-generation solution that would not only increase productivity and efficiency, but also improve the quality of the information output. Whether employed in a standalone manner in high volumes to address the complicated task of method development, in conjunction with capital-intense mass spectrometers to improve assay

sensitivity and maximize performance, or in process analytical technology (PAT) analysis to facilitate real-time quality testing, UPLC has become the technique – and investment – of choice.

The process of developing a reversed-phase HPLC method can take anywhere from weeks to months, incurring significant expense. Scientists have found that by utilizing UPLC technology for methods development, a six-fold improvement in throughput can be realized. This, in turn, reduces cost per sample and time of analysis considerably, while maintaining or improving separation integrity.

By developing rapid, high-resolution analytical methods, products can be brought to market faster, therefore improving the overall profitability of the assay. In one case, a leading pharmaceutical company was able to reduce its regulatory filing time by 50 percent by adopting this technology.

New method development calls for a well thought-out experimental design. A systematic screening protocol that explores selectivity factors such as pH, organic modifier and column chemistry allows chromatographers to quickly determine which experimental parameters are most effective in manipulating the selectivity of a separation. By employing this type of strategy, the total number of steps necessary to develop a method is reduced, providing an efficient and cost-effective approach.

Productivity improvements associated with employing UPLC technology for methods development are shown in the table. When we compare the UPLC methods development strategy to one directly scaled to conventional HPLC, we see a five-fold improvement in time. This significantly reduces the overall instrument time required to develop chromatographic methods to one work day, as opposed to one work week with conventional HPLC.

Extending performance

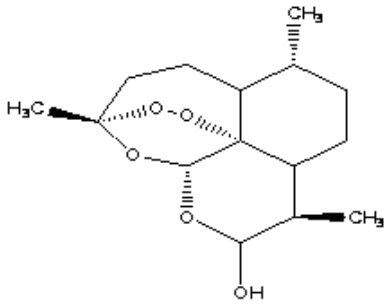
UPLC has been instrumental in facilitating unprecedented improvements in all fields of analytical chemistry; none more so than in the area of liquid chromatography/mass spectrometry (LC/MS). In countless examples, novel particle technology, low carryover and reduced cycle times have enabled extremely high-efficiency separations for improved resolution and sensitivity and increased throughput.

To the best of our knowledge, no reports are available on stability indicating analytical method for artesunate and amodiaquine tablets and each of pharmaceutical ingredients (API). It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination and quantitative estimation of known and unknown impurities. As artesunate is known for low solution stability, it was necessary to develop a faster chromatographic technique and hence ultra-performance liquid chromatography (UPLC) was selected for the present study.

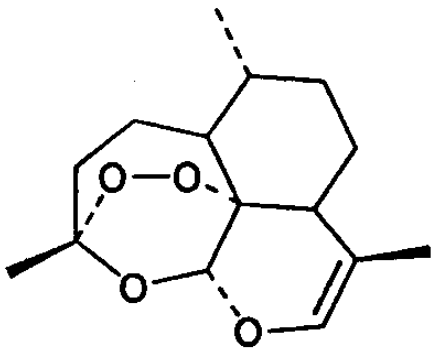
Ultra performance LC (UPLC) is a new category of separation science which builds upon well established principles of liquid chromatography, using sub 2 µm porous particles. These particles operate at elevated mobile phase velocities to produce rapid separations with increased sensitivity and increased resolution. Thus UPLC technology allows analysts time to be drastically reduced while still meeting assay acceptance criteria based on plate count, resolution and analyze retention.

Sub 2 µm packed columns offer advantages over the more traditional columns packed with 3 & 5 µm particles through shorter analysis time, improvement in resolving power, sensitivity and peak

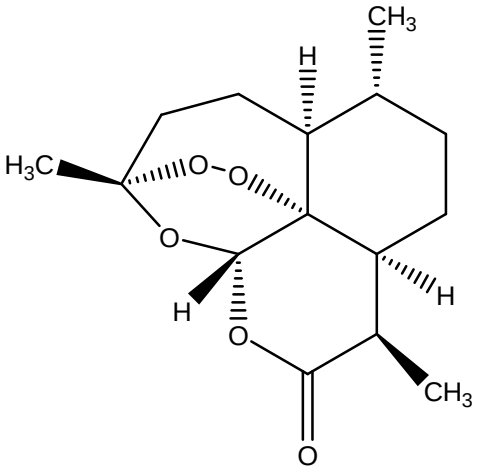
capacity when transferring methods from HPLC to UPLC [1]. Several approaches can be taken depending on the analytical needs. If column dimensions are maintained and only particle size is reduced then improvement in efficiency, resolution and peak capacity is obtained. 2nd approach is reducing not only particle size but also column dimension, which has a benefit of reducing analysis time. In both cases, care must be taken to ensure operating flow rate, gradient profiles and injection volumes are scaled approximately to obtain an equivalent or superior separation [2]. Literature survey showed that amodiaquine hydrochloride is very stable drug and artesunate is highly unstable drug component [3]. Hence focus of this study was to separate prominently the major degradents of artesunate .Hence initially the isocratic and gradient HPLC conditions were optimized for artesunate and its impurities in artesunate and amodiaquine tablet sample. The main target of the chromatographic method was to achieve separation of impurities and main component artesunate and amodiquine. Dihydroartemisinin–I and dihydroartemisinin –II are metabolites and major degradant of artesunate. Glycan, and artemisinin are some more potential degradent [4] impurities in artesunate and amodiquine dosage form. Structure and name of the impurities represented in figure.1.0



Dihydroartemisinin (DHA)



Glycan



Artemisinin

Figure 1 Structures of impurities of artesunate & amodiaquine tablets

During LC development study it was observed that dihydroartemisinin II and artesunate were eluting with a meager resolution while glycan impurity was eluting at higher retention time. The response of all these impurities and artesunate was found to be maximum at 210nm. The

chromatographic separation was achieved on a Base Deactivated column C18, 100mm×4.6 mm, 3µm column. In an isocratic mode using a mobile phase consisting of buffer (0.01M KH2PO4 pH adjusted to 3.0 with orthophosphoric acid) and acetonitrile in the ratio of 56:44 (v/v), there was a good separation between dihydroartemisinin-II and artesunate while glycan impurity was found to be eluting at higher retention time. Sample loading another challenge to achieve required LOQ, The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1%, the method LOQ should be less to ensure the results are accurate up to one decimal place. This requires looking further modification for in the developed SIM (section 3.3). TO achieve the required LOQ the test concentration needs to increase almost 20 times. Due to increased column loading the k' for amodiaquine reduces to less than 1 and k' for glycan impurity goes to >20 which is not the good sign for good chromatography. Also the resolution between artemisinin and artesunate < 1.2 which needs to retook in the method. To reduce the run time and achieve better peak shape it was decided to switchover to gradient HPLC mode. To start with initial linear gradient of 5-100% ACN was employed and observed late eluting glycan elutes at 8-10 minutes where as all other peaks elutes at near t0. The same gradient is run with 5-100% buffer where glycan impurity does not resolve and artesunate elutes very later stage. Peaks of the sample eluting during the gradient are not adequately separated.

If the difference between the retention times of the first (t1) peak and the last peak (tt) of chromatogram divided by the gradient time TG >0.25 a gradient is needed [5]. In above case it is 0.425. The first step is to optimize gradient range by adjusting initial % of ACN for minimum separation time for initial peaks t1 and adequate spacing early eluting peaks dihydroartemisinin I and amodiquine. Next final % of ACN is selected to elute artesunate, artemisinin and glycan near to the end of gradient. Initial gradient was tried as presented in table 1.

Table 1 Gradient I

Time	Buffer	Acetonitrile	Flow Rate
0.0 min.	88	12	1.0 ml/min
12 min	88	12	1.0 ml/min
15 min	70	30	1.0 ml/min
15.1min.	50	50	1.0 ml/min
35.0 min.	50	50	1.0 ml/min
36.0 min.	88	12	1.0 ml/min
40.0 min.	88	12	1.0 ml/min

In this gradient all other peaks separated but artemisinin and artesunate peaks elutes at the same RT at about 22.0 mins and with Rs 0. Hence minor change in the gradient I to separate artemisinin is decided as described in table 2.

Table 2 Gradient II

Time	Buffer	Acetonitrile	Flow Rate
0.0 min.	90	10	0.8 ml/min
20 min	50	50	0.8 ml/min
30 min	50	50	0.8 ml/min
40 min.	30	70	0.8 ml/min
40.1 min.	90	10	0.8 ml/min
45.0 min.	90	10	0.8 ml/min

This gradient gave very good resolution between artesunate and artemisinin (3.20) and glycan resolved at around 40 mins. Total run time is 50 mins. Artesunate is highly unstable at room temperature and immediately gets converted to dihydroartemisinin and hence the solution need to be prepared freshly and injected immediately. This limits number of analysis and hence needs switching over to UPLC arise.

Method Transfer to UPLC [6-8]

The basic chromatographic conditions like stationary phase, solvents and UV detection, maintained in HPLC were taken into account while developing the new UPLC method.

The parameters: detection wavelength, column temperature, nature of buffer and solvent used in HPLC were maintained as such. The stationary phase of C18 was chosen in order to have similar chemistry to that used with the HPLC. A BEH C18, 50 mm x 2.1mm, 1.7 µm column was employed for the separation.

To transfer the method geometrically to the smaller column geometrics packed with 1.7 µm particles and, therefore ensure equivalent chromatography, it is necessary to scale the flow rate, injection volume and gradient.

Step 1: Adjust the flow rate
Calculate the flow rate by following equation
$F2= F1 \times (dc2^2/dc1^2) \times (dp1/dp2)$
Where F1- Original flow rate
F2-New flow rate
Dc1- Original column id (mm)
Dc2- New column id (mm)
Dp1- Original particle size
Dp2- new particle size.
Solving this equation we got the new flow rate as 0.208ml/min approximately 200 ul.

Step 2. Adjustment of injection volume
$Vi2= Vi1x (dc2^2 xL2/ dc1^2 xL1)$
Vi2-New injection volume
Vi1- Original injection volume
Dc1- Original column id (mm)
Dc2- New column id (mm)
L1- Original column length (mm)
L2- new column length (mm)
This equation gives 10 µL injection volumes

Step 3 Adjustment of gradient profile
Expected flow and injection volume shows scaling down of 5 times

At the beginning an isocratic mode was chosen with the same ratio of buffer to acetonitrile as used in isocratic HPLC mode (56: 44 v/v). The flow rate was scaled to 200 µL/min by above equation. Though by calculation 10 µL was the injection volume for better back pressure and sharp peaks 5 µL injection volume is selected. Using these conditions a satisfactory separation was achieved between artesunate and artemisinin while glycan impurity was eluting around 9-10 min giving a total run time of 12 minutes. Further increase in the flow rate increases the back pressure of the system to more than 13000psi which will make very difficult for system to run

for long term hence 200 µL was selected as the final flow. The optimized gradient parameters of the system is shown in Table 3.

Table 3 Optimized Gradient

Time	Buffer	Acetonitrile	Flow Rate ml/min
0.0 min.	95	5	0.2
2.40 min	75	25	0.2
2.5 min	56	44	0.2
7.5min.	56	44	0.2
10 min.	20	80	0.2
10.10 min.	95	5	0.2
12.0 min	95	5	0.2

Comparison Study of Chromatographic Performance

A comparative data on chromatographic performance of HPLC gradient and UPLC (gradient) has been obtained by injecting a solution of artesunate and impurities the performance parameters of both the systems are shown in Table 4.

Table 4. A Comparison of system performance of HPLC and UPLC

Component	Elution Time (min)		Resolution		Tailing Factor		LOQ (µg)	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Amodiaquine	5.45	**2.4**	NA	NA	1.52	**1.20**	0.01	**0.005**
DHA-I	18.7	**5.0**	NA	NA	1.01	**0.66**	0.25	**0.022**
DHA-II	21.0	**6.2**	NA	NA	0.77	**0.65**		
Artesunate	23.64	**6.8**	3.6	**4.8**	0.93	**0.54**	0.14	**0.013**
Artemisinin	24.4	**7.6**	3.20	**5.4**	1.17	**0.55**	0.13	**0.012**
Glycan	39.8	**10.1**	NA	- NA	1.04	**0.52**	0.05	**0.0065**

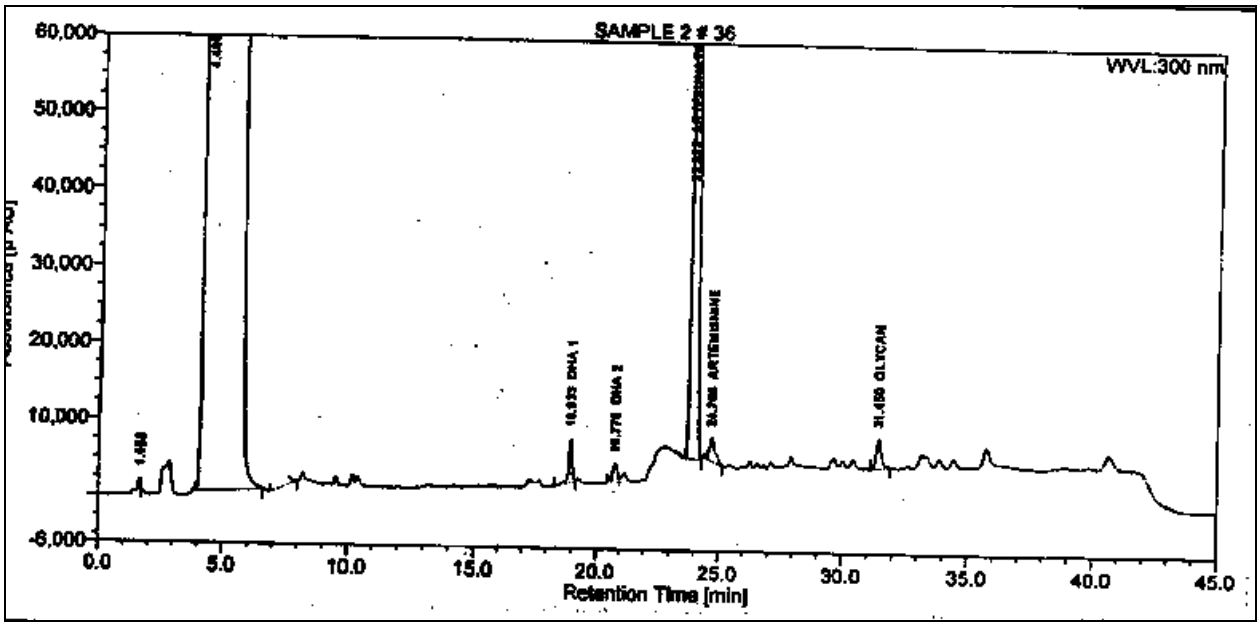


Figure 2 Typical chromatogram of HPLC Gradient method.

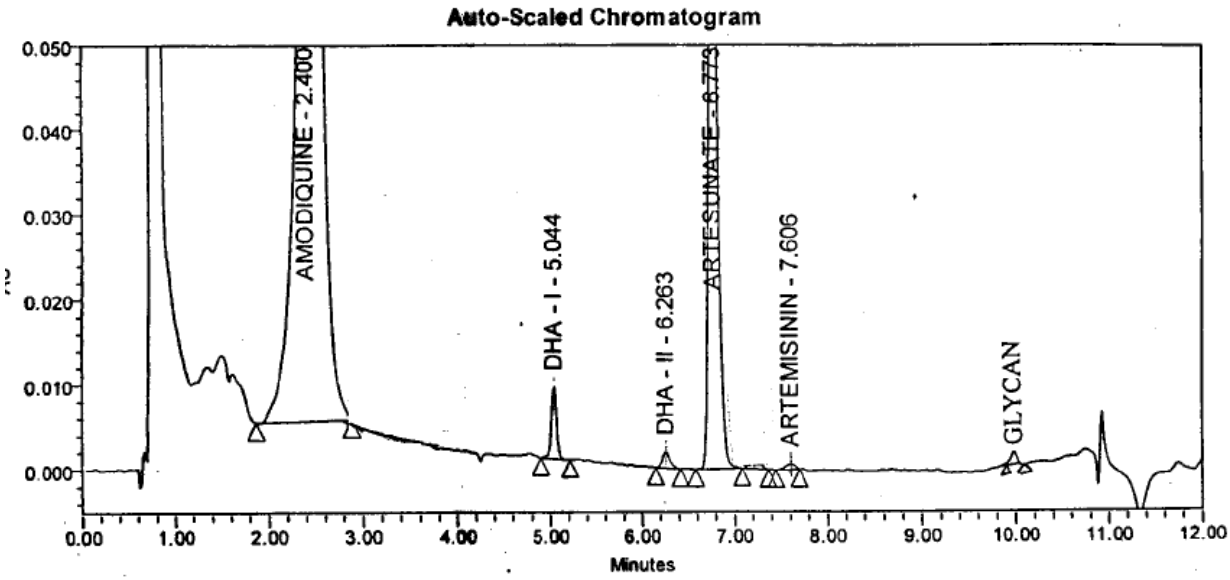


Figure 3 Typical Chromatograms of of artesunate and amodiaquine tablets using UPLC

It is observed that elution time of glycan in UPLC was reduced by four-fold to that of gradient mode HPLC .The resolution and asymmetry obtained for artesunate and DHA-II in UPLC showed comparatively better separation efficiency than HPLC. Theoretical plates obtained for all impurities in gradient mode HPLC were obviously higher than UPLC. The typical chromatograms of final HPLC and UPLC conditions are depicted in Fig. 2 and fig.3.

UPLC Method validation [9]

System Suitability

The validation study allowed the evaluation of method suitability for routine analysis. The tablets of artesunate and amodiaquine tablets showed presence of impurities like dihydroartemisinin and glycan up to level of 0.2% the system suitability parameters obtained for related substance method are given in table 5.

Table 5 System suitability report for UPLC related substance method

Component	Resolution (USP)	USP Tailing Factor	USP Plate count
Artesunate	1.0	0.54	6500
Artemisinin	3.20	0.55	5980

Specificity

It is the ability of analytical method to measure the analyte response in the presence of its potential impurities and degradents. The specificity of the UPLC method was determined by injecting individual impurity samples, wherein no interference was observed for any of the components. Samples obtained in forced degradation experiment were analysed by UPLC. Major degradation was observed in acid and base hydrolysis, oxidation of artesunate. The reduction of artesunate and amodiaquine content resulted in the formation of a major degradent dihydroartemisinin and glycan. Peak purity of artesunate and amodiquine in the degradent sample was verified by PDA. The peak purity spectra of obtained in reductive and oxidative degradation are pure and no co-elusion is observed confirming the peak purity.

Forced degradation studies were also performed for artesunate and amodiaquine bulk drug samples to demonstrate the stability indicating power of the newly developed UPLC method.

During the forced degradation study a considerable degradation of drug substance was observed in oxidative and reductive conditions .The chromatograms were checked for the appearance of any extra peak. Peak purity of these samples under stressed condition was verified using a photodiode array (PDA) detector. The purity of the principle and other chromatographic peaks was found to be satisfactory. This study confirmed the stability indicating power of the UPLC method.

Precision

The precision of related substance method was examined using six replicate injections of standard impurity solution. The RSDs for diluted standard were found to be 1.98 %and 1.35% for amodiaquine and artesunate respectively. These values are well within the generally acceptable limit of 5%. RSD of responses for impurities dihydroartemisinin, artemisinin and glycan were well within 10%, confirming good precision of the related substance method.

Accuracy

The accuracy of the method was determined for the related substance by spiking known amount of impurities in artesunate and amodiaquine tablet (test preparation) in triplicate at levels 80%, 100% and 120% of the specified limit. The recoveries of impurities were calculated. The percentage recovery of dihydroartemisinin-I, dihydroartemisinin -II, artemisinin and glycan drug product samples ranged from 98.78 to 102.95 %

Limit of Quantification

The limit of quantification (LOQ) values for, amodiaquine, dihydroartemisinin, artesunate, artemisinin and glycan were found to be 0.005 µg, 0.022 µg, 0.013 µg, 0.012 µg and 0.0065 µg respectively each of analyte concentration (1000µg/mL).

Linearity

Linear calibration plots for the related substance method were obtained over the calibration range (LOQ to150%) at six concentration levels in triplicate. For amodiaquine dihydroartemisinin, artesunate, artemisinin and glycan corresponding correlation coefficient (R) are 0.999, 0.9991, 0.9990, 0.999 and 0.9991 greater than 0.998. Respectively with the correlation coefficient (R) greater than 0.999. The results showed excellent correlation between the peak area and concentration of impurities.

The intermediate precision or ruggedness

It was determined on six separate sample solutions prepared from same batch by spiking the related substances at the specification level by a different analyst using different mobile phase and diluents preparation and instrument on a different day with different lot of same brand column. The overall RSD was evaluated and were 3.37%, 2.21%, 2.26%, 7.19% and 1.58% for dihydroartemisinin, artemisinin, glycan, individual unknown impurity and total impurities respectively. The results are within the acceptance criterion of NMT 10.0% RSD.

Robustness

In all the deliberately varied chromatographic conditions the chromatogram for system suitability solution for related substance showed satisfactory resolution between artesunate and artemisinin.

CONCLUSION

The HPLC gradient method can be succefully transferred on UPLC. Transfer of the method was accomplished by geometrically scaling flow rate, injection volume, and gradient profile.

Analysis time was reduced by four fold with an improvement of 64% in the resolution of critical pair of artesunate and artemisinin. Sensitivity of UPLC over HPLC was evaluated by comparison of LOQ values of dihydroartemisinin and glycan obtained in gradient HPLC for both systems. The LOQ concentration for UPLC was found to be 0.005 µg, 0.022 µg , 0.013 µg, 0.012 µg and 0.0065 µg for amodiaquine , dihydroartemisinin ,artesunate ,artemisinin and glycan respectively with RSD 6.89 %,2.71%, 3.98% ,1.36%,and 1.2% at injection volume 5 µL. The LOQ concentration for HPLC was found to be 0. 05 µg, 0.25 µg , 0.14 µg, 0.12µg and 0.055 µg for amodiaquine , dihydroartemisinin ,artesunate ,artemisinin and glycan respectively with RSD 6.97.% 7.2% ,5.3% ,and 2.3% at injection volume 50 µL. The almost ten times lower LOQ values with higher precision are attributed towards better sensitivity of UPLC method.

The present work demonstrated the ease of HPLC to UPLC method transferability, and the benefits that can be obtained in any time and resource. It proved that UPLC can significantly increase throughput with quality results. With variety of column dimensions, scientists have the flexibility to tailor their UPLC separations to the goals at hand.

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