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Stability-indicating HPLC method for the determination of artesunate. Application to tablets

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ABSTRACT

A simple, stability-indicating, reversed phase high performance liquid chromatographic method has been developed for the determination of artesunate (ART) in presence of two of its alkaline degradation products. The analysis was performed using a Nucleosil C18 column (150 x 4.6 mm i.d.) as a stationary phase at ambient temperature with UV- detection at 215 nm. A mobile phase consisting of of methanol: acetonitrile: 0.05 M potassium dihydrogen phosphate solution of pH 3.9 (50: 8: 42, v/ v) was pumped at a flow rate of 2 mL min^{-1}. The method showed good linearity over the range of 25-200 μg mL^{-1} with detection limit of 1.41 μg mL^{-1} and quantification limit of 4.28 μgmL^{-1} respectively. The proposed method was applied for the analysis of ART in pure form and commercial tablets with the mean % recoveries of 100.26 ± 1.34 and 100.30 ± 1.52 respectively. The results obtained were favorably compared to those obtained by the reference method.

KEYWORDS

Stability-indicating;
HPLC;
Artesunate;
Degradation;
Tablets.

INTRODUCTION

Artesunate (ART); butaanedioic acid mono-decahydro-3,6,9-trimethyl-3,12-epoxy-12-pyrano[3,4-j]-1,2-benzodioxepin-10-yl] ester.[1] (Figure 1) is used primarily for the treatment for malaria; but it has also been shown to be >90% efficacious at reducing egg production in *Schistosoma haematobium* infection.[2]

Few methods have been reported for the analysis of ART, these include: high performance liquid chromatography,[3-5] Gas Chromatography,[6] and capillary electrophoresis.[7]

The aim of the present work was to develop, validate and apply an efficient reversed phase liquid chromatographic method for the rapid and simultaneous determination of ART and its alkaline degradation products using a mobile phase consisting of methanol: acetonitrile: 0.05 M potassium dihydrogen phosphate solution of pH 3.9 (50: 8: 42, v/v) with UV detection at 215 nm. Further more, the developed method was utilized for the analysis of ART in its tablets.

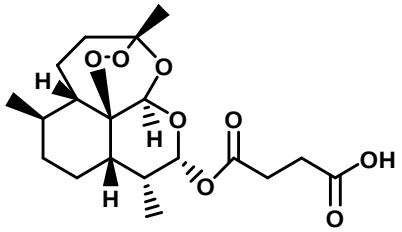

Figure 1 : Structural formula of artesunate

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EXPERIMENTAL

Apparatus

- Separation was performed with a Perkin Elmer tm Series 200 Chromatograph equipped with a Rheodyne injector valve with a 20 μL loop and a UV/VIS detector operated at 215nm. Total Chrom workstation was applied for data collecting and processing (MA, USA).
- A Shimadzu UV 1601 PC Spectrophotometer equipped with a pair of 1cm matched cells. Recording range: 0-2; wavelength: 215 nm; factor: 1; number of cells: 1; reaction time (min.); cycle time: 0.1 min.

Materials and Reagents

- Artesunate (ART) pure sample was purchased from Sigma (St. Louis, Mo, USA). Tablets containing the drug (labeled to contain 60 mg ART/ tablet) product of Multipharma, Cairo, Egypt) were obtained from the manufacturer.
- Cholroquin phosphate product of Naser Pharmaceutical Companey, Cairo Egypt, was used as internal standard (I.S.) and stock solution containing 200 μg/ mL was prepared in methanol and was further diluted with the same solvent.
- Methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany).
- Orthophosphoric acid (85 %) was obtained from Prolabo (Paris, France).
- 0.05 M aqueous solution of phosphate buffer of pH 3.9 was prepared.

Chromatographic conditions

Columns and mobile phases

- Separation was achieved on a Nucleosil RP-18 (5 μm) (150 mm × 4.6 mm) combined with a guard column (Merck, Darmstadt, Germany). The columns were operated at ambient temperature. The analytical system was washed daily with 60 mL of 1:1 mixture of water and methanol to eliminate the mobile phase and this did not cause any change in the column performance.
- The mobile phase consists of mixture of methanol: acetonitrile: 0.05 M phosphate buffer of pH 3.9 (50: 8: 42, v/v). The mobile phase was filtered through a 0.45-μm membrane filter (Millipore, Ireland).
- The flow rate was 2mL/ min.
- The reference method was performed on a Nucleosil RP-18 (5 μm) (150 mm × 4.6 mm) combined with a guard column (Merck, Darmstadt, Germany) and using a moble phase consisting of acetonitrile: phosphate buffer (0.01 M of pH 2.9) (40: 60, v/v) was used with UV detection at 220 nm and the flow rate was 1mL/ min.

Stock solutions

Stock solution containing 1.0 mg/ mL of ART was prepared in methanol and further diluted with 2 M NaOH for the spectrophotometric measurement and mixed with Cholroquin phosphate (IS) (200 μg mL^{-1}) and diluted with the mobile phase for the HPLC measurement. This solution was found to be stable for at least one week without alteration when kept in the refrigerator.

Preparation of alkaline degradation products

Aliquot volumes of methanolic ART (1000 μg mL^{-1}) were transferred into a series of 10 mL volumetric flasks and diluted with the 2 M NaOH to the mark. These solutions were heated in a boiling water bath for about one hour and further diluted with the same solvent before measuring the absorbance at 237 nm.

For HPLC measurements, Aliquot volumes of methanolic ART (1000 μg mL^{-1}) were transferred into a series of 10 mL volumetric flasks and diluted with the 2 M NaOH to the mark. These solutions were heated in a boiling water bath for about one hour. Then, aliquots equivalent to 400 μg mL^{-1} were transferred to 10 ml volumetric flasks diluted with 1mL of distilled water to obtain clear solution, neutralized with 2 M HCl and mixed with 1ml of Cholroquin phosphate (IS) (200 μg mL^{-1}) and diluted with the mobile phase to the mark. Then the peak area ration was determined at 215 nm.

Construction of the calibration curve

Transfer aliquots of the standard solution containing 25 -200 μg mL^{-1} of ART prepared by serial dilution of aliqots of the sotck solution together with an aliquot of IS containing 20 μg mL^{-1} of Cholroquin phosphate and complete to the 10 mL with the mobile phase. Inject twenty μL aliquots (in triplicate) and elute with the mobile phase under the reported chromatographic conditions. Construct the calibration curve by plotting the peak area ratio against the final concentration of the drug (μg mL^{-1}). Alternatively, derive the corresponding regression equation.

Analysis of tablets

Weigh, pulverize, and mix well twenty tablets. Transfer an accurately weighed quantity of the powdered tablets equivalent to 100.0 mg of ART into a small conical flask, extract with 3 x30 mL of methanol and sonicate for 30 min. Filter the extract into a 50 mL volumetric flask and complete to volume with methanol. Follow the procedure as described under "Construction of the Calibration Curve". Determine the nominal contents of the tablets using either the calibration graph or the corresponding regression equation.

RESULTS AND DISCUSSION

A simple reversed phased liquid chromatographic method has been utilized for the separation of ART and its two degradation products. Figure 2 shows the chromatogram obtained for a mixture of the standard solution of ART and its two degradation products (A and B), chloroquine phosphate was used as internal standard under the described chromatographic conditions. The chromatogram revealed that ART was well separated from its alkaline degradation products. The mobile phase was chosen after several trials with different ratio of methanol, actonitrile, phosphate buffer and at different pH values. The chromatographic system described above, allows complete base line separation with good resolution factor (3.77) between ART and degradation product B. The proposed method was assessed for specificity, linearity, precision, accuracy, stability and recovery.

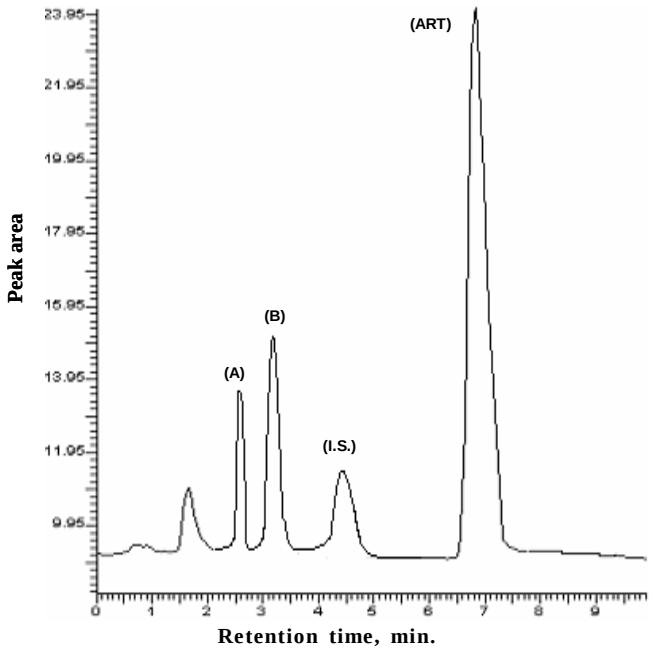

Figure 2 : Typical chromatogram of artesunate (200 µg mL^{-1}) and its degradation products (40 µg mL^{-1}) under the described chromatographic conditions. (A): Degradation product A, (B): Degradation product B, ART: Artesunate, I.S.: Chloroquine phosphate (20 µg mL^{-1})

The different experimental parameters affecting the separation selectivity have been investigated and optimized. Hence, the method was applied to the determination of ART in its tablets.

Chromatographic performance

A mobile phase consisting of mixture of methanol: acetonitrile: 0.05 M phosphate buffer of pH 3.9 (50: 8:42, v/v) achieved an optimum separation of the drug from its dgradation products, resolution factor of (3.77), in a reasonable time less than 10 min. with maximum detector response.

Choice of column

Artesunate was determined in pure form and tablets mainly on reversed-phase c18 column. Reversed phase chromatography was conducted using a phenyl (250 x 4.6 mm id) stainless steel column instead of c18 column, The two degradation products were eluted rapidly at retention time t_R 2 and 2.7 min, respectively. While ART was eluted at 5.3 min with broad and non symmetrical peak.

Choice of appropriate wavelength

Artesunate exhibits low absorbance at 224 nm with $A^{1\%}_{1cm}$ of about 100, while its alkaline degradation product shows maximum absorbance at 237 nm with high $A^{1\%}_{1cm}$ of about 425 nm (Figure 3). Using reversed – phase HPLC method revealed good separation of the drug from its degradation products at 215 nm regarding the sensitivity of the method, while at 200-210 nm the sensitivity was greatly decreased.

Choice of internal standard

Different drugs were investigated for the choice of a suitable internal standard. These drugs include: Chloroquine phosphate, flumequine, artemether and lumifantrine. Artemether and lumifantrine are not detected. On the other hand, flumequine interfered and greatly overlapped with either the peak of the degradation product or with that of the drug.

Chloroquine phosphate was the best internal standard producing a well resolved peak from each of the drug and its degradation products (Figure 2).

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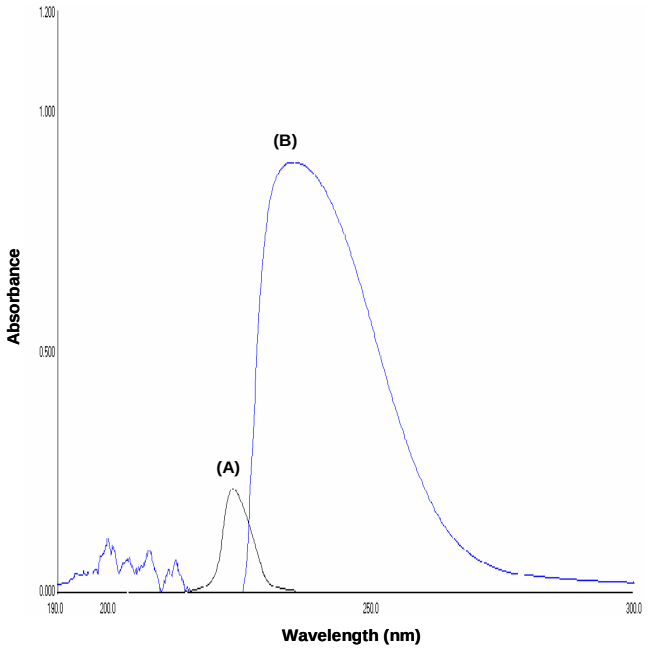


Figure 3 : UV spectra of artesunate (20 µg mL^{-1}) and its degradation product obtained after boiling with 2 M NaOH at diffused day light for one hour: (A) Artesunate at 224 nm, (B) Degradation product at 237 nm

Mobile phase composition

Several modifications in the mobile phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. The modifications include, the type and ratio of the organic modifier, the pH, the strength of phosphate buffer and flow rate.

Type of organic modifier

Methanol only and acetonitrile only did not give good resolved peaks. While, mixture of methanol and actonitrile produced well resolved and symmetrical peaks. So mixture of methanol and actonitrile was the organic modifier of choice used through out the study.

Ratio of organic modifier

The effect of changing the ratio of organic modifier on the selectivity and retention times of the studied drug and its degradation products was investigated. A satisfactory separation was achieved upon using a mobile phase consisting of methanol: acetonitrile: 0.05 M phosphate buffer of pH 3.9 (50: 8: 42, v/v) at ambient temperature (Figure 4). Increasing methanol ratio and decreasing the acetonitrile ratio resulted in a gradual increase in the resolution factor up to ratio (50: 8: 42, v/v) for methanol: acetonitrile: 0.05 M phosphate buffer of pH 3.9 respectively. Then any further increase in this ratio leads to poor separation and an increase in the retention time of the drug and its degradation product. (Figure 4).

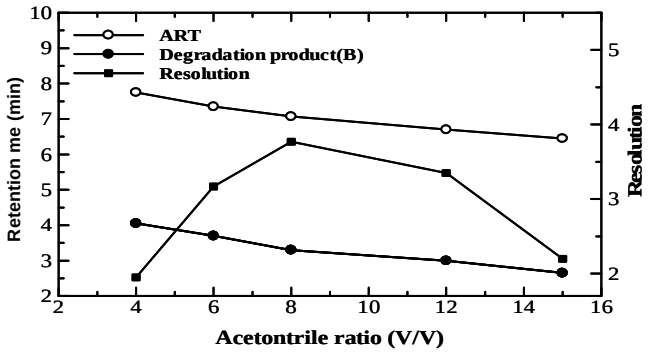


Figure 4 : Effect of acetonitrile: phosphate buffer ratio (pH 3.9) on the resolution of ART (200 µg mL^{-1}) form its degradation product using the proposed HPLC method.

pH

The effect of changing pH of the mobile phase on the selectivity and retention times of ART and its degradation products was investigated using mobile phase of pH ranging from 2.7-7.0. Figure 5 illustrates that pH 3.9 was the most appropriate giving well resolved peaks

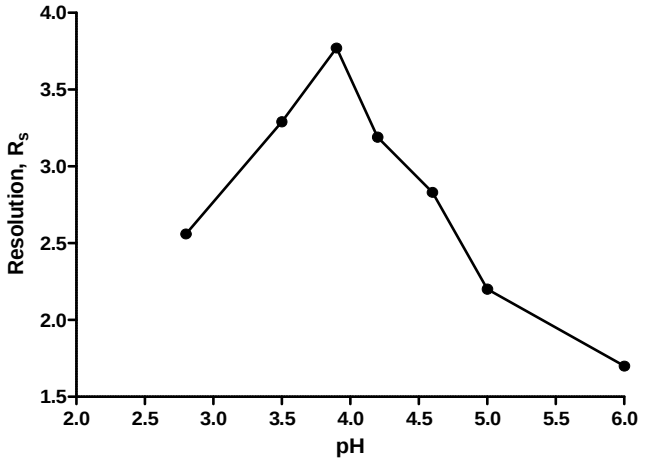


Figure 5 : Effect of pH on the resolution of ART (200 µg mL^{-1}) form its alkaline degradation product using the proposed HPLC method.

in a short reasonable time regarding the peak symmetry and sensitivity. Upon increasing the pH value ART and its degradation product B were considerably retained, with the increase in retention time of ART and its degradation product B. At lower pH less than 3.9 a decrease of the retention time was achieved. (Figure 5).

Ionic strength buffer

The effect of changing the ionic strengths of phosphate buffer (pH 3.9) on the selectivity and retention times

of ART and its degradation products was investigated. The study was performed over the range (0.02- 0.10 M) phosphate buffer (pH 3.9). It was found that 0.05 M phosphate buffer (pH 3.9) was the most suitable one giving the best resolution and the highest detector response. A gradual decrease in the peak area ratio was observed by increasing the molar strength up to 0.1 M buffer (Figure 6), while the retention times of the drug and its degradation products was slightly increased.

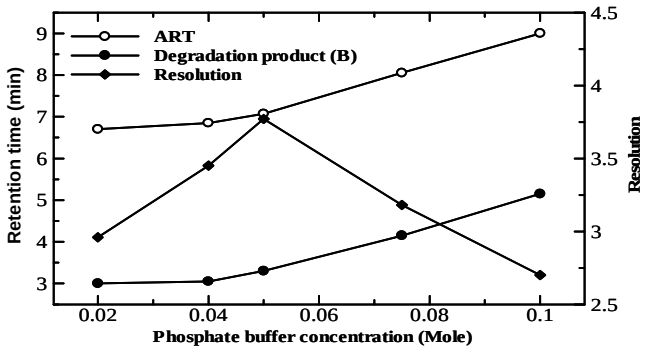


Figure 6 : Effect of molarity of phosphate buffer (pH 3.9) on the resolution of ART (200 µg mL^{-1}) form its degradation product using the proposed HPLC method.

Flow rate

The effect of flow rate on the separation of ART from its two degradation products was studied and a flow rate of 2 mL min^{-1} was optimal for good separation in a reasonable time.

Validation of the method

The proposed method was tested for linearity, specificity, accuracy and precision

Linearity

The peak area ratio of ART varied linearly with the concentration over the range (25-250 µg mL^{-1})

Linear regression analysis of the data gave the following equations:

P = -0.021 + 0.0141 C (r = 0.9999)

Where C is the concentration in µg/ mL and P is the peak area ratio.

Limit of quantitation (LOQ) and Limit of detection (LOD)

LOQ and LOD were calculated according to ICH Q2B recommendations[8] and they were found to be 4.28 and 1.41 µg mL^{-1} respectively.

LOQ and LOD were calculated according to the following equations:[8]

LOQ = 10 σ /S

LOD = 3.3 σ /S

where σ is the standard deviation of the intercept of regression line and S is the Slope of the calibration curve.

A plot of peak area ratio versus the concentration of ART was rectilinear over the ranges cited in TABLE 1.

The validity of the method was proved by statistical evaluation of the regression lines, using the standard deviation of the residuals ($S_{y/x}$), the standard deviation of the intercept (S_a) and standard deviation of the slope (S_b). The results are abridged in TABLE 1. The small values of the figures point out to the low scattering of the calibration points around the calibration curve and high precision.

TABLE 1 : Performance data of the proposed method

Parameter	Proposed method
Concentration range (µg mL^{-1})	25-200
LOD (µg mL^{-1})	1.41
LOQ (µg mL^{-1})	4.28
Correlation coefficient (r)	0.9999
Slope	0.0141
Intercept	-0.021
$S_{y/x}$ = standard deviation of the residuals.	6.9 x 10^{-3}
S_a = standard deviation of the intercept of regression line.	6.04 x10^{-3}
S_b = standard deviation of the slope of regression line	4.82 x10^{-5}
% Error = RSD% / √ n	0.60

Accuracy

To test the validity of the proposed method it was applied to the determination of authentic sample of ART over the working concentration range. The results obtained were in good agreement with those obtained using reference method.[5] Using Student's t-test and variance ratio F-test[9] revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (TABLE 2). An HPLC reference method was adopted using a mixture of acetonitrile -phosphate buffer pH 3 (40:60, v/v) as a mobile phase, at flow rate of 1ml min^{-1}. The detection was carried at 220 nm.[5]

Precision

Repeatability

The repeatability was evaluated through the replicate analysis of three different concentrations of ART samples, either in pure or in tablets, the mean percent-

age recoveries based on the average of six separate determinations for pure and dosage forms are abridged in TABLE 3.

Intermediate precision

It was performed through replicate analysis of three different concentrations of ART samples, either in pure or tablets on six successive days. The percentage recoveries are based on the average of six separate determinations. The results are abridged in TABLE 3.

Stability

The stability of the methanolic sample solutions at room temperature (25 °C) for 24 hours after preparation, was verified by reassaying them (after dilution with the mobile phase). There was no indication of any decomposition of ART in the samples.

Recovery

The recovery of the method was checked by adding known amounts of degraded ART to known amounts

TABLE 2 : Application of the proposed and reference methods to the determination of artesunate in pure form and tablets

Parameters	Proposed method	Reference Method[5]
1- ART in pure form		
No of experiments	5	3
Mean found, %	100.26	100.03
±SD	1.34	0.59
RSD, %	1.34	0.59
Variance	1.795	0.348
Student's t-value	0.28 (2.45)	
Variance ratio F-test	5.16 (6.94)	
2- ART in tablets containing 60 mg/tablet		
No of experiments	3	3
Mean found, %	100.30	98.93
±SD	1.52	1.36
RSD, %	1.52	1.36
Variance	2.31	1.845
Student's t-value	1.16 (2.78)	
Variance ratio F-test	1.25 (19.00)	

N. B.: Values between parenthesis are the tabulated t and F values respectively, at p = 0.05[9].

TABLE 3 : Validation of the proposed method for the determination of ART in pure form and tablets

Parameters	Intra-day precision		Inter-day precision		Parameters	Intra-day precision		Inter-day precision	
	Concentration taken (µg mL^{-1})	% Recovery	Concentration taken (µg mL^{-1})	% Recovery		Concentration taken (µg mL^{-1})	% Recovery	Concentration taken (µg mL^{-1})	% Recovery
Artesunate pure	50.0	101.99	50.0	95.78	ART 60 mg/ tablet	50.0	99.24	50.0	101.06
		101.17		95.17			100.90		96.66
		103.99		98.74			102.07		95.36
		97.82		98.82			101.27		98.60
		100.80		102.05			101.79		100.74
		98.74		98.22			105.66		101.65
x±S.D.		101.34±1.91		98.13±2.47	x±S.D.		101.82±2.13		99.01±2.58
%RSD		1.88		2.52	%RSD		2.09		2.61
%Er		0.77		1.03	%Er		0.85		1.07
	100.0	98.58	100.0	100.24	ART 60 mg/ tablet	100.0	103.83	100.0	103.17
		102.43		101.48			100.49		96.61
		98.21		99.18			99.02		100.49
		96.46		98.43			104.37		104.67
		9.7 97		99.19			98.96		97.66
		101.47		105.06			100.16		101.47
x ±S.D.		99.19±2.28		100.60±2.43	x±S.D.		101.14±2.38		100.68±3.11
%RSD		2.30		2.42	%RSD		2.35		3.09
%Er		0.94		0.99	%Er		0.96		1.26
	150.0	99.54	150.0	99.46	ART 60 mg/ tablet	150.0	97.47	150.0	99.59
		101.99		103.05			100.44		98.71
		104.76		100.46			95.39		106.52
		98.47		99.69			106.21		105.53
		99.86		101.70			98.79		98.07
		103.06		94.91					99.45
x±S.D.		101.28±2.40		99.88±2.78	x±S.D.		98.49±3.29		101.31±3.70
%RSD		2.37		2.78	%RSD		3.34		3.65
%Er		0.97		1.13	%Er		1.36		1.49

of standard ART. The calculated recoveries were satisfactory indicating that no interference had been observed from the degradation products. The accuracy of the proposed method was evaluated by analyzing standard solutions of the studied drugs. The results obtained by the proposed method were compared with those given by the reference method..[5]

Application

Dosage form analysis

The proposed method was successfully applied to the determination of the drug in tablets. The percentage recoveries are abridged in TABLE 2. The results were compared with those obtained using the reference method.[5] Statistical analysis[9] of the results using student·s t test and varience ratio F- test showed no significance difference with those obtained using the two methods regarding accuracy and precision, respectively.

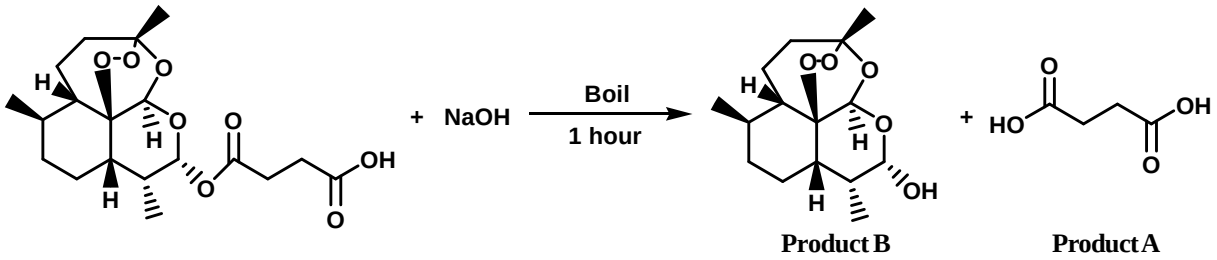
Chromatographic performance

Three well-defined symmetrical peaks were obtained upon measuring the *UV*-response of the eluate under the optimum experimental parameters (Figure 2), at retention time of 7.07 for ART and t_R 2.7 and 3.3 min for its two degradation products A and B respectively.

Pathway of degradation

The chromatogram of ART degradation showed 2 degradation peaks in addition to that of ART.

Degradation product A was eluted first at retention time of 2.7 since it is more polar than degradation product B which was eluted at t_R 3.3 min. photoinduced alkaline degradation of ART is suggested to proceed as shown in the following scheme.



Scheme 1 : Proposal mechanism for induced alkaline degradation of artesunate with 2 M NaOH after boiling for one hour

CONCLUSION

A rapid, precise, and selective HPLC method using a single isocratic system has been developed for the determination of ART either per se or in presence of its alkaline degradation products. The method is considered as a stability-indicating assay for the determination of ART in pharmaceutical preparations .A simple sample preparation enables the use of this method for routine quality control analysis of ART in its tablets, with good accuracy. The proposed method is characterized by high sensitivity for the analysis of ART with LOD of 1.41and LOQ of 4.28 μg mL^{-1}.

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