Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1019–1024
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Stability of artesunate in pharmaceutical solvents

Karen Gaudin [a,*], Marie-Hélène Langlois [a], Anne Barbaud [a], Chantal Boyer [a], Pascal Millet [b], Fawaz Fawaz [c], Jean-Pierre Dubost [a]

[a] *Laboratoire de Chimie Analytique UFR Pharmacie, EA 3677, Bases Thérapeutiques des Inflammations et Infections, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*
[b] *TropiVal, EA 3677, Bases Thérapeutiques des Inflammations et Infections, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*
[c] *Laboratoire de Galénique, UFR Pharmacie, EA 3677, Bases Thérapeutiques des Inflammations et Infections, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*

Received 8 June 2006; received in revised form 15 September 2006; accepted 24 September 2006
Available online 3 November 2006

Abstract

Stability of artesunate (ART) was established in three pharmaceutical solvents. The chromatographic conditions developed for this study were acetonitrile:potassium phosphate buffer 10 mM (40:60, v:v; pH 2.9) at 0.7 mL min^{-1} with UV detection at 220 nm using a short X-Terra RP C18 column (50 mm × 3 mm, 3.5 μm). This isocratic condition led to the separation between ART and its main degradation products (i.e. α-DHA and β-DHA) with analysis time of less than 4 min. The retention factors are 1.49, 2.26 and 2.79 min for α-DHA, β-DHA and ART, respectively. This method was proved linear ($r^2 = 0.9995$), accurate (R.S.D. = 0.20), precise (R.S.D. = 0.74) and robust. The system performance remained unaffected by pH variation from 2.6 to 3.2 and variation of acetonitrile percentage from 38 to 42. Stability of ART was assessed in ethanol, propylene glycol (PG) and polyethylene glycol 400 (PEG 400). Unfortunately none of these solvents prevented ART from degradation longer than 3 months. In ethanol, significant degradation of ART occurred after 3 months at room temperature and this degradation was characterised by numerous degradation products. In PEG 400, significant degradation was observed after only 1 month, however DHA was the unique degradation product, which is also an efficient anti-malarial drug.

Keywords: Artesunate; Degradation; Ethanol; Polyethylene glycol 400; Propylene glycol; Validation

1. Introduction

Artesunate (ART) is the most widely available and used among artemisinin derivatives for the treatment of malaria. ART belongs to a group of compounds, which has proven to be particularly effective in the treatment of severe and multi-drug resistant malaria caused by *Plasmodium falciparum* [1,2].

ART is rapidly hydrolysed to DHA in presence of water (Fig. 1). DHA is both an *in vivo* metabolite and an artemisinin derivative therapeutically active against malaria. Therefore ART acts as a pro-drug [3]. This essential feature must be taken into account for pharmaceutical formulation development. As ART is more soluble in organic solvents such as ethanol, the use of pharmaceutical solvents can contribute to delay ART degradation. However stability of ART in such solvents is not well established.

For this study, an analytical method was developed. The main methods for quantitative determination of ART were performed by RP-HPLC. As ART is not stable in gas chromatography analysis, this technique is not convenient for characterizing the intact structure of this sesquiterpene lactone. ART is poorly detected by standard spectrophotometric detection. Therefore the reported detection systems were electrochemical detection [4–6], derivatisation [7] and post-column degradation before UV detection [8,9], evaporative light scattering detection (ELSD) [10] and more recently MS [11,12]. However most of them were required for pharmacokinetic assay, whereas quality control needed more simple method and specific detection may constitute a drawback to observe degradation

* Corresponding author. Tel.: +33 5 5757 4686; fax: +33 5 5757 4684.
E-mail address: karen.gaudin@u-bordeaux2.fr (K. Gaudin).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.09.039

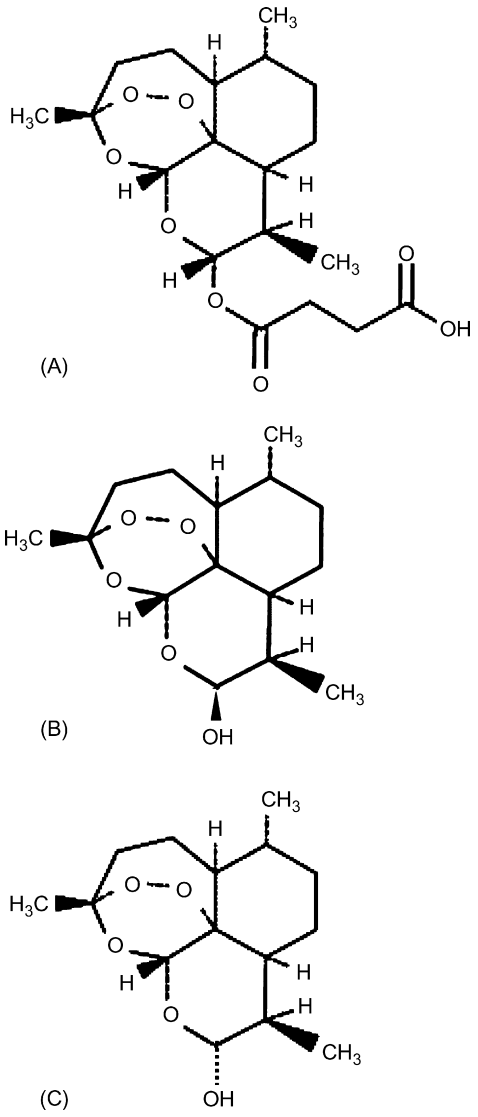

Fig. 1. Chemical structures: (A) ART; (B) β-DHA; (C) α-DHA.

products. Therefore this work described a method with direct UV detection in RP-HPLC. This method was validated and allowed the stability study of ART in different pharmaceutical solvents.

2. Experimental

2.1. Chemicals and reagents

ART was purchased from Knoll BASF Pharma (Liestal, Switzerland) by DNDi (Drug for Neglected Disease initiative) (Geneva, Switzerland). β-DHA was synthesized by COMIPSO (Bordeaux, France). Acetonitrile isocratic HPLC grade and HCl 37% were purchased from Prolabo VWR (Fontenay-sous-Bois, France). Absolute ethanol, polyethylene glycol 400 and propylene glycol were purchased from Cooper (Melun, France). H_3PO_4 and KH_2PO_4 were purchased from JT Baker Chemicals (Deventer, Holland) and Merck (Darmstadt, Germany), respectively.

2.2. Instrumentation and chromatographic conditions

The HPLC system was a Hewlett Packard HPLC 1050 series which consisted of a quaternary pump, a DAD detector set at 220 nm, an online degasser, an Agilent Chemstation LC 3D and a Rheodyne 7125 injection valve with a 20 μL sample loop (Cotati, California, USA).

Column lichrospher 100 RP 8 EC 5 μm, 150 mm × 4 mm (CIL Cluzeau, Saint Foy La Grande, France) was previously used. The final chromatographic separation was performed using a X-Terra RP C18 (50 mm × 3 mm i.d., 3.5 μm particle size) (Waters, USA). The mobile phase consisted of acetonitrile:potassium phosphate buffer 0.01 M (40:60, v:v; pH 2.9), and was filtered under vacuum through a 0.45 μm nylon membrane filter. The flow rate was set at 0.7 mL min^{-1}.

2.3. Validation procedure

2.3.1. Robustness

The effect of the factors on different responses was evaluated with a 2^2 experimental design (Table 1). The effects *Ex* of a given factor *X* on experimental response were calculated as:

$$Ex = \frac{\sum Y(+)}{n+} - \frac{\sum Y(-)}{n-}$$

where $\sum Y(+)$ and $\sum Y(-)$ and $n+$ and $n-$ represent the sum of the responses and the number of design experiments,

Table 1
2^2 experimental design with three replicates at medium level

Factor			Response		
Experiment no.	pH	CH_3CN%	Rs α-DHA/β-DHA	Rs β-DHA/ART	ART area[a]
1	−1	38	5.14	3.61	336.5
2	−1	42	4.23	2.20	328.7
3	1	38	5.46	3.64	334.3
4	1	42	4.26	2.03	335.1
5	0	40	4.76	2.7	332.5
6	0	40	4.77	2.69	332.0
7	0	40	4.63	2.59	335.2
High level (+1)	2.6	38			
Medium level (0)	2.9	40			
Low level (−1)	3.2	42			

[a] Solutions at 0.35 mg mL^{-1}.

Table 2
Preparation of the ART solutions for the validation study

	Nominal concentration (%)				
	50	70	90	100	110
Stock solution					
ART (g) in 9 g of ethanol	0.15	0.21	0.27 9	0.30	0.33
Analytical solution[a]					
Final concentration (mg mL^{-1})	0.33	0.47	0.6	0.67	0.73

[a] Each solution correspond to 0.5 g of stock solution in 25 mL of CH3CN:water (40:60).

respectively, when factor X is at a low (−) or a high (+) level.

The standard error effect $(S.E.)_e$, which represents the experimental variability, was estimated with the standard error of three replicates of a design experiment were X is at the medium level ($p = 0.05$).

An effect of a factor is statistically significant when $|Ex \geq 2(S.E.)_e|$ [13].

In Table 1, each value is the mean of injections made in triplicate.

2.3.2. Linearity and accuracy

The range and the preparation of the five ART solutions for the linearity and accuracy studies are detailed in Table 2. All samples were prepared by weighing. After 1 min under a gentle agitation, the stock solutions were ready to be solubilized in the CH_3CN:water (40:60). The aqueous solutions must be prepared just before injection because ART is not very stable in aqueous media. They were injected by duplicate into the chromatograph.

2.3.3. Precision

Precision of the method was tested with 18 different samples corresponding to 100%. The Intra-day precision was assessed with six samples analysed the same day. The same protocol was repeated two other days for constituting the Day-to-day precision.

2.4. Stability study of the ART solutions

Solutions were prepared at 41.8 mg mL^{-1} in ethanol, at 31.7 mg mL^{-1} in PG, at 34.5 mg mL^{-1} in PG:ethanol (2:1) and at 44.4 mg mL^{-1} in PEG 400. Due to the viscosity of these solvents, the required amount was taken by weighting. For chromatographic analysis, 0.5 g of solutions was weighted in a 25 mL volumetric flask, and filled with CH_3CN:water (40:60), except for solutions in PEG 400 for which a 50 mL volumetric flask was used.

2.5. Acid reaction condition

Acidified ethanol was prepared by adding 100 μL of HCl in 100 mL of absolute ethanol. Absolute ethanol and acidified ethanol were dried with molecular sieve during 72 h before use. 100.1 mg of ART and 61.8 mg of DHA were weighted and solubilized in 1.0393 and 0.6227 g of ethanol, respectively. 3.019 and 1.8182 g of acidified ethanol were then added in the ethanolic solution of ART and DHA, respectively. These reactions were carried out under stirring at room temperature.

3. Results and discussion

The stability of ART was studied in ethanol, PG and PEG 400. An analytical method enabled to monitor ART degradation was thus developed.

3.1. Method development

ART absorbs light only at low wavelengths and has a relatively low molar extinction coefficient. Similar spectrometric properties should be expected from its degradation products. Consequently a simple UV detection without derivatisation was selected. The main methods for analysing ART in HPLC were performed by RP-HPLC using mixture of CH_3CN and buffer at pH around 5 as mobile phase [5,6,8,12]. However ART is a weak acid with pK_a value equal to 4.6 [14]. Therefore a mobile phase using pH value largely smaller than 4.6 should be more appropriate in RP-HPLC. Such pH condition was applied with octadecyl grafted silica in the method of the International Pharmacopoeia where the mobile phase was 50% of potassium phosphate buffer at pH 3.0 and 50% of CH_3CN [15]. The same pH value was also carried out using 31% of aqueous trifluoroacetic acid with 69% of CH_3CN. These conditions had permitted hyphenated-liquid chromatography with ELSD [10]. This pH value increased ART retention in RP-HPLC, so C8 column was first envisaged. Shorter graftings were also mainly used for ART analysis in previous studied [5,6,8].

The composition of the mobile phase was tuned using a solution of ART, in ethanol, degraded by heating (50 °C). Forty-five percent of CH_3CN led to the separation of several degradation products (Fig. 2A). α-DHA and β-DHA were identified (i.e. peaks 1 and 2) and eluted before ART. However some more hydrophobic compounds were also observed which induced an analysis time equal to 30 min. In order to shorten the analysis, a shorter column with smaller particle size was tested. Although the stationary phase of this shorter column was an octadecyl grafted silica, the similar separation was achieved in 11 min (Fig. 2B) with only 40% of CH_3CN.

For the stability study, the chromatographic system was thus CH_3CN:potassium phosphate buffer 10 mM (40:60; v:v; pH 2.9) at 0.7 mL min^{-1} with UV detection at 220 nm using C18 stationary phase. Moreover phosphate buffer instead of acetate buffer

Table 3
Interpretation of effect significance on the responses

	R_S α-DHA/β-DHA			R_S β-DHA/ART			ART area		
	Effect	$2(S.E.)_e$	Significant	Effect	$2(S.E.)_e$	Significant	Effect	$2(S.E.)_e$	Significant
pH	0.09	0.82	No	−0.04	1.17	No	1.04	4.89	No
CH_3CN%	−0.53	0.82	No	−0.76	1.17	No	−1.76	4.89	No

divided by five-fold the background noise at this wavelength of detection.

The method was validated with reference to ICH guidelines Q2A and Q2B monitoring the validation characteristics, which need to be evaluated [16]. Small analysis time was obtained (i.e. 3 min) and good separation of ART and its main degradation product (i.e. DHA) was achieved (i.e. $R_s = 2.66$ between ART and β-DHA which is the closest DHA isomer to ART).

3.2. Method validation

The developed method was validated with respect to robustness, linearity, accuracy, precision, limit of detection (LOD) and quantification (LOQ).

3.2.1. Robustness

Among the parameters, which can be tested for the robustness, pH and CH_3CN % were the more decisive because they were not controlled by the apparatus. These parameters depend on the users, which prepared the mobile phase. The pH may mainly affect the separation between ART and DHA since only ART retention depends on this factor. DHA retention is not affected by pH variation. These parameters were then studied with regard to separation and quantification properties of this method. The effect of these two factors on different responses (ART area, resolution factor between α-DHA and β-DHA and between β-DHA and ART) is evaluated with a 2^2 experimental design (Table 1). Furthermore three experiments at the nominal levels (i.e. the conditions tested in the assay procedure) were performed to establish standard error effect $(S.E.)_e$.

The robustness evaluation indicated that neither pH nor CH_3CN percentage have significant effect on the different responses (Table 3).

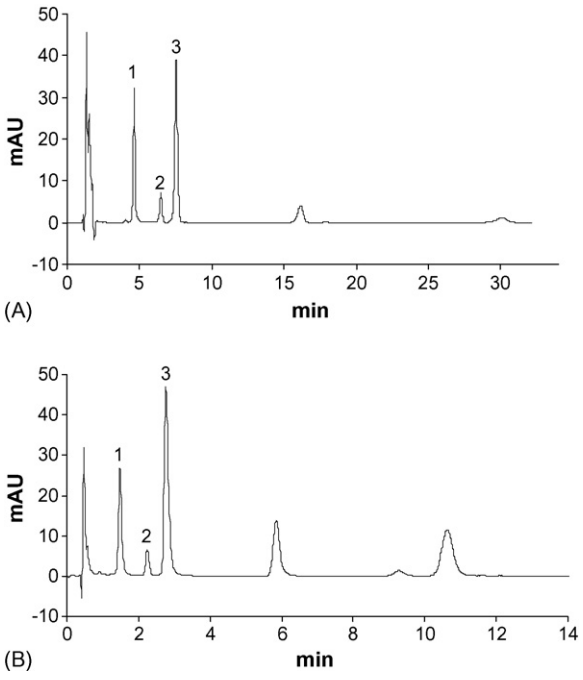

Fig. 2. Degraded solution of ART in ethanol, α-DHA (1); β-DHA (2); ART (3). (A) C8, CH_3CN:potassium phosphate buffer 10 mM (45:55, v:v; pH 2.9) at 1 mL min^{-1}; (B) C18, CH_3CN:potassium phosphate buffer 10 mM (40:60, v:v; pH 2.9) at 0.7 mL min^{-1}.

3.2.2. Linearity

Linearity was established with 15 data from 5 solutions at concentration levels ranging from 50% to 110% prepared 3 times and injected at 3 different days. Such range of concentrations was selected in order to be able to quantify ART in case of degradation. The relationship was linear and the calculated curve went through the origin (Table 4).

3.2.3. Accuracy

Accuracy was tested with the same set of data used for the linearity study. Table 4 shows percentage recoveries, which indicate that the method is accurate. Recovery data were obtained within the range of 99.57–100.42%. The mean '*t*' value versus the true value with 95% confidence shows that the experimental mean is not significantly different from true value $t_{(0.05;N-1)} = 2.145$.

3.2.4. Precision

As the Intra-day and the Day-to-day precision are within the acceptance criteria of 5%, the method is precise (Table 4).

Table 4
Validation results of the chromatographic method for ART analysis

Concentration range (mg mL^{-1})	0.33–0.73
Linearity	
Determination coefficient r^2	0.9995
Slope	866.41
Intercept	9.58
Accuracy	
% Recovery	99.99
% R.S.D.	0.20
Precision	
Intra-day precision (% R.S.D.)	0.70
Day-to-day precision (% R.S.D)	0.74

3.2.5. Limit of detection and quantification

The LOD and LOQ determination can be based on standard deviation of the intercept (σ) and slope (S) evaluated from the calibration curve.

$$\mathrm{LOD} = \frac{3\sigma}{S}, \qquad \mathrm{LOQ} = \frac{10\sigma}{S}$$

By this approach, LOD and LOQ were estimated at 14 and 42 $\mu g\,mL^{-1}$, respectively. Experimentally the LOD was observed at 10 $\mu g\,mL^{-1}$.

The results of this validation study led to the conclusion that the use of a single ART calibration sample was enough for quantitative determination.

3.3. Stability study of ART in pharmaceutical solvents

The study of the chemical stability of ART was performed in four different solvent solutions: absolute ethanol, PG, PG:ethanol (2:1) and PEG 400. All these solvents are substances for pharmaceutical use described in European Pharmacopoeia [17].

Preliminary results with 1-month-old solutions showed that ART degradation occurs in PG. Moreover the degradation products were almost co-eluted with ART preventing its quantification. Therefore the quantitative stability study was then carried out in PEG 400 and ethanol (Fig. 3). The degradation occurs more rapidly in PEG 400. 45 days at room temperature in dark environment led to 20% of ART degradation in PEG 400, whereas in ethanol 4 months were required to reach the same percentage of degradation. However none of these solvents prevented ART degradation.

To discuss about ART degradation, 1-year-old solutions in the four different solvent conditions were analysed (Fig. 4). Chromatographic profiles were qualitatively and quantitatively different in the four conditions. However three peaks were present in all chromatograms. Two of them correspond to α and β DHA (i.e. peaks 1 and 2) and the third is more hydrophobic than ART since it elutes at 5.7 min (i.e. peak c). Ethanol and PG led to the highest number of degradation products. Five additional peaks than ART were observed (Fig. 4A and B). The solvent effect appeared

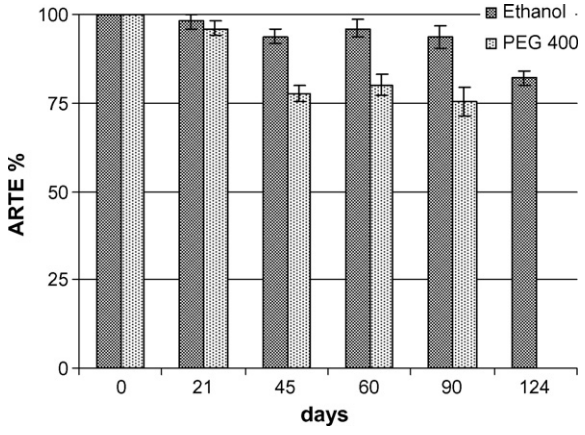

Fig. 3. Percentages of ART in ethanol and PEG 400 at 41.8 and 44.4 mg mL^{-1}, respectively.

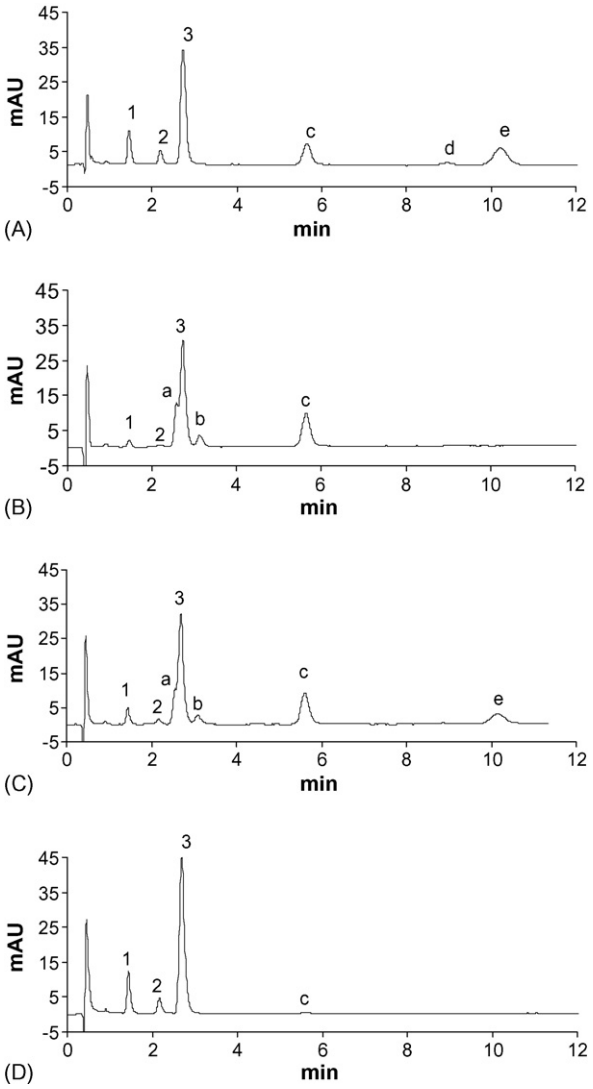

Fig. 4. One-year-old solutions of ART in different solvent mixtures at room temperature. (A) In ethanol at 41.8 mg mL^{-1}; (B) in PG at 31.7 mg mL^{-1}; (C) in PG:ethanol (2:1) at 34.5 mg mL^{-1}; (D) in PEG 400 at 44.4 mg mL^{-1} analysed with X-Terra RP C18, CH_3CN:potassium phosphate buffer 10 mM (40:60, v:v; pH 2.9) at 0.7 mL min^{-1}. Peaks (1), (2) and (3) see Fig. 1; peaks (a)–(e) are unknown degradation products different from DHA.

cumulative to degrade ART in solvent mixture PG:ethanol (2:1) (Fig. 4C). Peaks a and b were induced by PG whereas peaks d and e were observed only in presence of ethanol. In PEG 400, only DHA and peak c appeared. However c was present in trace amount (Fig. 4D).

As compounds c–e were more hydrophobic than ART, they may be provided by condensation between solvent and ART or DHA. In order to provide assumption about identity of these degradation products, the following experiments were carried out. DHA and ART were respectively stirred in acidified ethanol in free water environment (Fig. 5). DHA can be converted to arteether in acid condition with ethanol [18] whereas ART may be esterified by ethanol. Fig. 5A shows two further peaks labelled I and III which are thought to be α and β arteether, respectively, since these more hydrophobic compounds can be provided only by condensation between DHA and ethanol. Although the DHA

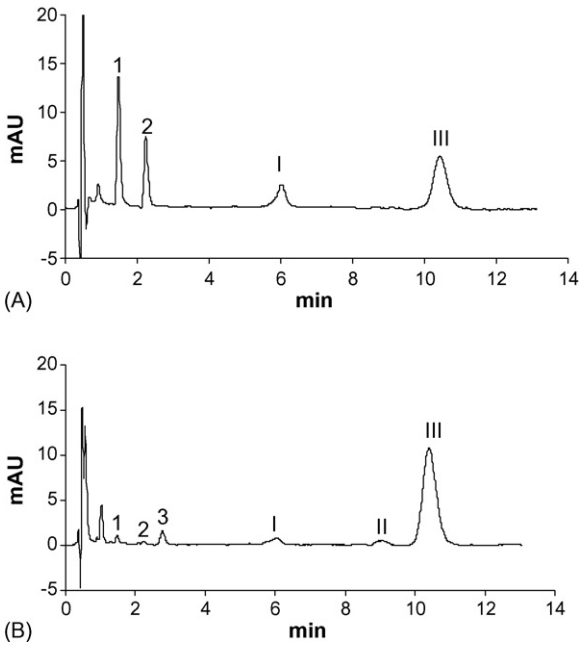

Fig. 5. DHA at 32.0 mg mL^{-1} (A) and ART at 31.3 mg g^{-1} (B) in acidic ethanol (0.0275% HCl) during 7 h analysed with X-Terra RP C18, CH_3CN:potassium phosphate buffer 10 mM (40:60, v:v; pH 2.9), at 0.7 mL min^{-1}.

used for the reaction was only β-DHA, the transformation into α-DHA readily occurs as the reaction is performed in ethanol. With ART placed in the same acid condition (Fig. 5B), five degradation products are obtained (i.e. peaks 1, 2, I, II and III). However the main obtained product was peak III, which may be β arteether since ART is also in β configuration. This latter element suggested that the assignment of peak III may be β arteether. However if DHA is the intermediate product of this reaction, trace of α-DHA should also be observed in this chromatographic profile (Fig. 5B). Due to the competition between the rapid interconversion between α and β-DHA [19] and etherification reaction, α arteether is also obtained (peak I). However peak c in Fig. 4 cannot be α arteether since it appears in conditions without ethanol. Moreover its presence in all conditions suggests that it may not be the result of a condensation between ART and the solvent.

Peak d may be the product resulting from the esterification of ART with ethanol. Since it appeared only in this condition, it suggested that the solvent was involved in the product structure. However such reaction is less favourable, therefore this product is always recovered in trace amount (Figs. 4A and 5B).

4. Conclusion

The chromatographic method optimised herein provides appropriate performance for quality control applications. It is simple, robust and rapid.

Although the degradation of ART in pharmaceutical solvents is slowed down compared to water, none of the tested solvents have prevented the degradation of ART.

Ethanol was the solvent, which most impeded ART degradation. However its degradation in this condition led to the highest number of degradation products. In PEG 400, degradation started after 1 month, however DHA was the unique degradation product. For pharmaceutical formulations, ethanol could be a solvent of choice for use within a short period of time (less than 3 months). However PEG 400 remains an interesting excipient for ART formulations due to DHA formation only. Nevertheless, our results indicate that ART formulations should be limited to dry forms or extemporaneous solutions.

Acknowledgement

We thank Cyril Rubio (COMIPSO, Bordeaux, France) for the synthesis of β-DHA used in this study financially supported by DNDi, which is used in this study.

References

[1] J. Karbwang, K. Na-Bangchang, A. Thanavibul, D. Bunnag, T. Chongsuphajaisiddhi, T. Harinasuta, Lancet 340 (1992) 1245–1248.
[2] R.K. Haynes, S. Krishna, Microb. Infect. 6 (2004) 1339–1346.
[3] K.T. Batty, K.F. Ilett, T. Davis, M.E. Davis, J. Pharm. Pharmacol. 48 (1996) 22–26.
[4] Z.M. Zhou, J.C. Anders, H. Chung, A.D. Theoharides, J. Chromatogr. 414 (1987) 77–90.
[5] K. Na-Bangchang, K. Congpuong, L.N. Hung, P. Molunto, J. Karbwang, J. Chromatogr. B 708 (1998) 201–207.
[6] V. Navaratnam, M.N. Mordi, S.M. Mansor, J. Chromatogr. B 692 (1997) 157–162.
[7] A.-Q. Zou, M. Xie, X.-D. Luo, J. Chromatogr. 410 (1987) 217–221.
[8] K.T. Batty, T.M.E. Davis, L.T. Thu, T.Q. Binh, T.K. Anh, K.F. Ilett, J. Chromatogr.B 677 (1996) 345–350.
[9] R.B. Taylor, M.I. Awad, R.G. Reid, R.R. Moody, J. Chromatogr. B 744 (2000) 415–421.
[10] P. Christen, J.-L. Veuthey, Curr. Med. Chem. 8 (2001) 1827–1839.
[11] D. Ortelli, S. Rudaz, E. Cognard, J.-L. Veuthey, Chromatogr. 52 (2000) 445–450.
[12] H. Naik, D.J. Murry, L.E. Kirsch, L. Fleckenstein, J. Chromatogr. B 816 (2005) 233–242.
[13] M. Feinberg, Validation des Méthodes D'analyse, Dunod, Paris, 2004, pp. 229–234.
[14] P. Augustijns, A. D'Hulst, J. Van Daele, R. Kinget, J. Pharm. Sci. 85 (1996) 577–579.
[15] The International Pharmacopoeia, Monographs for Antimalarial Drugs, Artesunate, vol. 5, pp. 222–227, 252.
[16] ICH guidelines: http://www.emea.eu.int/htms/human/ich/quality/ichfin.htm.
[17] European Pharmacopoeia, 5th ed., Council of Europe, Strasbourg, France, 2005.
[18] C. Singh, P. Tiwari, Tetrahedron Lett. 43 (2002) 7235–7237.
[19] X.-D. Luo, H.J.C. Yeh, A. Brossi, J.L. Flippen-Anderson, R. Gilardi, Helv. Chim. Acta 67 (1984) 1515–1522.