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RESEARCH ARTICLE

Assessment of Thermal and Hydrolytic Stabilities and Aqueous Solubility of Artesunate for Formulation Studies

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Abstract

For the purpose of establishing the optimum processing parameters and storage conditions associated with nanolipid formulations of the artemisinin derivative artesunate, it was necessary to evaluate the thermal stability and solubility profiles of artesunate in aqueous solutions at various temperatures and pH. The effect of increased temperature and humidity on artesunate was determined by storing samples of the raw material in a climate chamber for 3 months and analyzing these by an established HPLC method. Artesunate remained relatively stable during storage up to 40°C ± 0.5°C and 75% relative humidity for 3 months, wherein it undergoes approximately 9% decomposition. At higher temperatures, substantially greater decomposition supervenes, with formation of dihydroartemisinin (DHA) and other products. In solution, artesunate is relatively stable at 15°C with less than 10% degradation over 24 h. The aqueous solubility of artesunate at different pH values after 60 min are pH 1.2 (0.1 M HCl) 0.26 mg/mL, pH 4.5 (acetate buffer) 0.92 mg/mL, distilled water 1.40 mg/mL, and pH 6.8 (phosphate buffer) 6.59 mg/mL, thus relating to the amount of ionized drug present. Overall, for optimal preparation and storage of the designated formulations of artesunate, relatively low temperatures will have to be maintained throughout.

Keywords artesunate · decomposition · dihydroartemisinin · solubility · stability

Introduction

The antimalarial drug artemisinin, the active principle of the blue-green herb qīng hāo 青蒿 (*Artemisia annua*) that has long been used in traditional Chinese medicine, was isolated from *A. annua* in the early 1970s by Chinese scientists working under the remarkable Project 523 [1–3]. Artemisinin is a sesquiterpene lactone containing a peroxide bridge that bestows its antimalarial properties but at the same time confers an instability that necessitates relatively careful handling, storage, and formulation with respect to other auspiciously robust antimalarial drugs such as chloroquine or mefloquine. It is converted by reduction of the lactone carbonyl into the lactol dihydroartemisinin (DHA) that is a mixture of stereoisomers epimeric at C-10. DHA in turn is converted into the hemi-succinate α-ester artesunate and the β-methyl acetal artemether respectively (Fig. 1); these three derivatives are used in front-line treatment of malaria. Currently, the combination of artesunate with pyronaridine, as an oral tablet formulation, is included in the WHO Model List of Essential Medicines for the acute treatment of both *Plasmodium falciparum* and *P. vivax* malaria, highlighting the significant impact of artesunate for the effective treatment of this disease [4]. Our particular interest in artesunate stems from the need to use this as one of several comparator drugs in the preparation of various nano-vesicular and other formulations associated with applications of artemisinins for potential treatment of cancer [5, 6] and infectious diseases [7, 8]. In this respect, we have conducted a preliminary evaluation of nano-vesicular formulations of the amino-artemisinin artemisone for potential treatment of melanoma [9] and are proceeding with development of other formulations for treatment of cutaneous tuberculosis (TB) [10]. Earlier, formulation studies involving the preparation of artesunate-loaded chitosan/lecithin nanoparticles indicated enhanced *in vivo* antimalarial activity, further highlighting the potential of this drug in tailored and novel drug delivery systems [11]. Overall, artesunate attracts as a comparator

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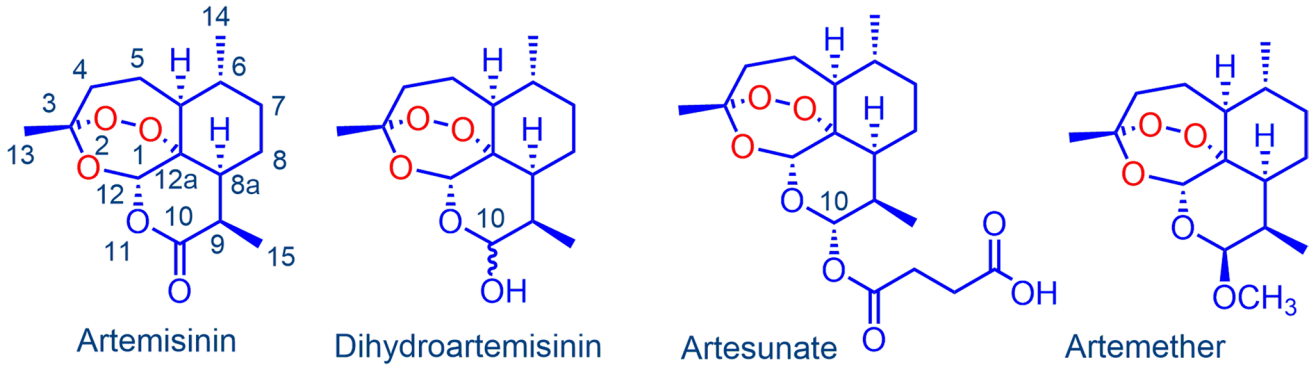

Fig. 1 The lactone artemisinin, its reduction product, the hemiacetal dihydroartemisinin (DHA), the α-hemi-ester artesunate, and the β-methyl acetal artemether

drug because it has been thoroughly characterized from a clinical viewpoint.

Artesunate is currently widely used in fixed combination therapies with other malarial drugs for oral treatment of non-severe malaria and alone as a dual pack formulation for treatment of severe/cerebral malaria via intravenous or intramuscular injection [3, 4]. In the latter case, solid artesunate is mixed with 5% aqueous sodium bicarbonate and diluted with aqueous saline or dextrose immediately prior to injection, which must be carried out rapidly to avoid hydrolysis to DHA [12–14]. Artesunate administered intravenously has a half-life of less than 15 min; in comparison, DHA generated from artesunate *in situ* has a half-life of 30–60 min [15, 16]. As artesunate is a hemi-ester of DHA, it is intrinsically unstable to hydrolysis via alkyl-oxygen cleavage under acidic conditions, or via acyl-oxygen cleavage under basic conditions [17]. It is most unlikely that formal metabolism *in vivo* via intercession of esterases is involved; that is, DHA arises via hydrolytic decomposition of artesunate *in vivo*. In this respect, it is noteworthy that oral administration of artesunate leads to a substantially higher relative bioavailability for DHA than does administration of DHA itself. That is, artesunate is more resistant to first pass metabolism than is DHA and is hydrolyzed in the systemic circulation to DHA [18].

With respect to thermal stability, although solid crystalline artesunate is stable to storage at temperatures of 2–8°C, storage at temperatures of 25°C or greater is reported to result in 8–10% decomposition after 1 week [19]. However, according to thermogravimetric analyses, crystalline artesunate is thermally relatively robust, with a decomposition threshold of 152°C; in contrast, DHA has a decomposition threshold of 110°C [20]. The facile thermal decomposition of DHA in particular is associated with a distinct decomposition pathway involving a direct "unzipping" process characteristic of hemiacetals to provide a variety of products, including a rearranged peroxy-hemiacetal which although possessing antimalarial activity also decomposes rapidly to inert end products [20]. Overall, although it is noteworthy that artesunate is so widely used given its extremely short half-life with respect to hydrolysis to DHA, it is the preferred drug—it is more stable under storage conditions than is DHA and, as noted above, serves as a better source of DHA *in vivo* than does DHA itself. In any event, products obtained from the thermal decomposition of artesunate have been thoroughly characterized; the products relevant to the current work are DHA and 9,10-anhydrodihydroartemisinin (glycal, Fig. 2) [20, 21].

In focusing on solubility, for a drug to exert a systemic effect, it must be dissolved. Solubility is also important for enabling preparation of various formulations of a given drug [22]. In the case of artesunate, the free carboxyl group through ionization at appropriate pH values in aqueous solution will enhance aqueous solubility, which contrasts with the generally very low aqueous solubilities of artemisinin and artemether [19]. DHA itself is evidently insoluble [23], but it turns out not to be possible to measure aqueous solubility because of decomposition [24, 25]. Thus, among the clinically proven artemisinins, artesunate attracts because of its higher solubility. Reported solubilities in water at 25°C vary from 0.06 to 0.08 mg/mL [19, 26, 27]. In aqueous 0.1 M HCl solution at pH 1.2, solubility is 0.30 mg/mL and in phosphate buffer at pH 7.0, solubility is 0.59 mg/mL. It has also been demonstrated that addition of small amounts of protic organic solvents such as ethanol to the water increases solubility of the drug [19, 28]. The overall problem in establishing solubility in aqueous solution at different pH values is the competing hydrolysis. Thus, at pH 1.2, the conversion into DHA is rapid, with a half-life ($t_{1/2}$) of 26 min [12]. At pH 7.4, the $t_{1/2}$ is about 10 h at 23°C. Thus, any measurement of solubility must monitor the extent of decomposition as well.

In order to assay for formation of DHA from artesunate under thermal or aqueous conditions, it must be noted that

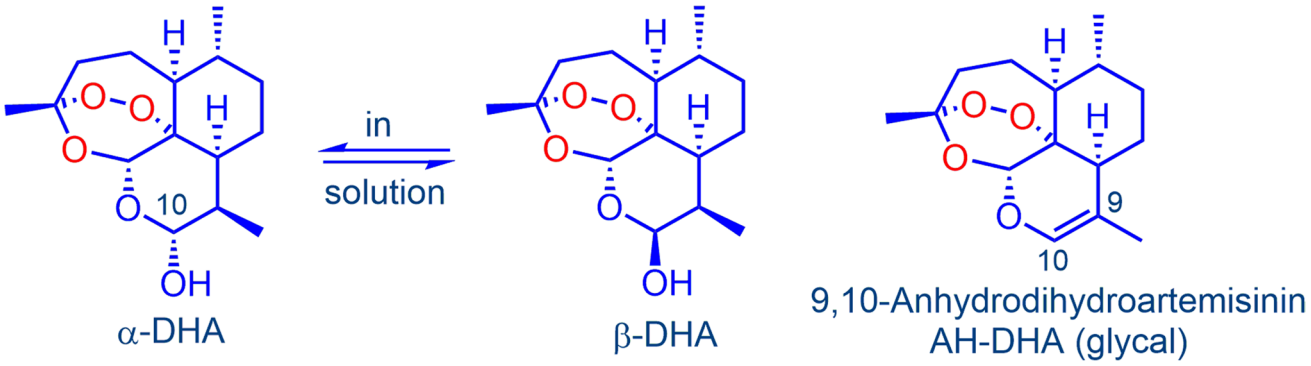

Fig. 2 Chemical structures of crystalline DHA which is the β-epimer (β-DHA), the α-epimer (α-DHA), and 9,10-anhydrodihydroartemisinin (AH-DHA)

DHA as a hemiacetal exists as a mixture of stereo-isomeric epimers incorporating either an axial "alpha" hydroxyl group or equatorial "beta" hydroxyl group (Fig. 2) [20, 24]. Pure crystalline DHA, m.p. 153–155°C [24], is the β-epimer as determined by an X-ray crystallographic determination [29]. Dissolution of solid DHA in organic solvents results in equilibration to provide mixtures of the alpha and beta epimers, whose final composition depends upon the nature of the solvent [20]. In any event, it is important to note that HPLC analyses of DHA always presents two peaks due to each of the alpha and beta epimers. Under reverse phase conditions, that is, where relatively polar eluting solvents are used in association with a non-polar stationary phase, the alpha epimer is usually the major component and has the lower elution time [30–33].

Given the lack of a systematic study on the thermal and aqueous stabilities of artesunate, we have now evaluated its thermal stability and solubility profiles in aqueous solutions at various temperatures and pH ranges. The data is important for enabling selection of the conditions required for preparation of formulations of artesunate without incurring untoward decomposition to DHA, and the various decomposition formed from the latter compound.

Materials and Methods

Materials

Each of artesunate and dihydroartemisinin (DHA) was purchased from DB Fine Chemicals (Johannesburg, South Africa) and purity assessed to be greater than ≥ 98% by HPLC analyses. Chromatography grade acetonitrile was purchased from ACE Chemicals (Pty) Ltd. (Johannesburg, South Africa). Potassium hydrogen orthophosphate, ammonium acetate, and 32% v/v analytical grade hydrochloric acid were purchased from Merck, Johannesburg, South Africa. Distilled and ultrapure water (resistivity of > 18.2 MΩ.cm^{-1}) were obtained from a Rephile Direct Pure water purification system (LabDynamic, Johannesburg, South Africa).

High Performance Liquid Chromatography

For the HPLC analyses of artesunate, the protocols of the International Pharmacopoeia and ICH Guidelines were followed [33, 34]. A Shimadzu chromatographic system (Shimadzu, Japan) and Phenomenex® Luna C_{18} (5 µm) 150 mm × 4.6 mm reverse phase column were used. The mobile phase consisting of 44 parts acetonitrile and 56 parts of 0.01 M phosphate buffer at pH 3.0 (pH adjusted with phosphoric acid) was used at a flow rate of 1.0 mL/min. All samples were analyzed at a wavelength of 216 nm. The column temperature was maintained at 30°C. The injection volume for each sample was 20 µL. This is a verified method with a linear regression of (r^2) = 0.9993 and a limit of detection (LOD) of 4.98 µg/mL and limit of quantification (LOQ) of 15.08 µg/mL. The previously recorded relative retention times for artesunate and the degradation products were used in this study: α-DHA 0.6 min, β-DHA 0.9 min, artesunate 1.0 min, and AH-DHA 2.7 min [33].

Accelerated Stability Studies

The effect of increased temperature and humidity was determined by storing approximately 500 mg of drug in a Petri dish. The sample was distributed evenly on the surface of the Petri dish, and three samples were prepared for every storage condition. The samples were stored in a climate chamber (Binder, Tuttlingen, Germany) at 25°C/60% relative humidity (RH), 30°C/65% RH, and 40°C/75% RH for a period of 3 months. Sufficient sample was removed at monthly intervals (0, 1, 2, and 3 months). Samples were also stored at elevated temperatures (60, 65, and 70°C) without the addition of relative humidity, for a period of 2 weeks to

ascertain the effect that typical processing could have on artesunate stability. The samples were assayed in triplicate to determine purity and possible degradation, by means of HPLC analysis [33], and data was interpreted according to the International Conference on Harmonisation (ICH) Q1A (R2) Guidelines [34].

Solubility Studies

To determine equilibrium solubility, an excess of API powder was weighed and placed in amber test tubes with screw cap closures. Water (120 mg), 0.1 N HCl (140 mg, pH 1.2), phosphate buffer (140 mg, pH 6.8), and acetate buffer (225 mg, pH 4.6) were added to nine test tubes per medium and placed in a water bath at 37 ± 2°C for the media other than water. The water solubility tests were performed at the designated temperatures of 5, 15, 25, 37, and 45 ± 2°C. The test tubes were fixed to a rotating axis (54 rpm) for the duration of the experiment. The buffered aqueous media were prepared according to the pharmacopeia specifications [35]. The solutions subsequently obtained were filtered through 0.45 µm PVDF (polyvinylidene difluoride) filters, after which HPLC analyses were performed on the filtrates according to the specifications of the HPLC method.

Results and Discussion

Accelerated Thermal Stability Studies

During accelerated stability testing, the percentage of the amounts of artesunate remaining with acceptable deviation (less than 2.0%) [34] between testing intervals is presented in Table I.

No discoloration of the raw material was observed and degradation products were not detected for the 25°C/60% RH and 30°C/65% RH test conditions. At 40°C/75% RH, although slight discoloration of the raw material and a decrease in drug quantity were observed, no degradation products were detected. This may be attributed to either the sensitivity of the HPLC method used or that a higher injection volume should have been used in order to detect artesunate degradation product peaks.

Thermal Stress Testing

In order to inform artesunate stability during formulation studies, where possible temperature excursions substantially greater than ambient may exist, we conducted a brief survey of the effects of submitting neat, solid artesunate to storage in a laboratory oven at 60, 65, and 70°C for a period of 2 weeks, with samples being analyzed at the end of weeks 1 and 2. The percentage amounts of artesunate remaining at the end of the testing intervals are presented in Table II. For the 60°C tests, no apparent degradation was detected.

For the results obtained from stress testing at 65°C, the percentage artesunate was detected with acceptable deviation between the initial sample and week 1 (<2.0%) [33, 34]. For the week 2 sample, trace amounts of DHA of DHA were detected. At 70°C, artesunate underwent substantial decomposition. The HPLC chromatograms revealed the presence of α-DHA, β-DHA, and the glycal (*cf.* Figure 2) in the week 1 and week 2 samples, with the latter containing higher concentrations of the decomposition products. Thus, reassuringly, artesunate is a relatively robust artemisinin derivative from a purely thermal standpoint. This also confirms the solid-state stability of artesunate demonstrated by Agnihotri et al. [19], and it is clear that preparation of any formulation that may require heating of neat artesunate for short periods of time (*ca* 1–2 days) can comfortably be conducted at temperatures of up to 60°C.

Solubility of Artesunate in Aqueous Solutions at Various pH Values

Here, we constructed a solubility profile of artesunate in distilled water (pH 7.0) over a temperature range from 5 to

Table II Summary of Quantified Purity of Artesunate (Mean ± SD) at Varying Storing Temperatures Over a 2-Week Period[a]

Temperature	60 ± 2°C[b]	65 ± 2°C[b]	70 ± 2°C[b]
Initial	97.9 ± 0.58%	97.9 ± 0.58%	97.9 ± 0.58%
7 days	95.3 ± 0.33%	97.9 ± 0.25%	93.9 ± 0.81%
14 days	97.3 ± 0.02%	93.4 ± 0.14%	85.9 ± 2.08%

[a]Average % artesunate remaining as established by HPLC analysis ($n = 3$): [b]for method see experimental section and [28]

Table I Accelerated Thermal Stability Assessment of Artesunate[a]

Temperature/RH[b]	25°C/60% RH	30°C/65% RH	40°C/75% RH
Initial	97.9 (±0.58%)	97.9 (±0.58%)	97.9 (±0.58%)
Month 1	98.2 ± 0.15 (±0.6%)	96.1 ± 1.30 (±0.4%)	96.4 ± 0.15 (±1.0%)
Month 2	99.9 ± 1.65 (±0.6%)	98.9 ± 0.05 (±0.4%)	93.2 ± 0.05 (±1.0%)
Month 3	97.3 ± 1.95 (±0.6%)	99.2 ± 2.80 (±0.4%)	91.9 ± 1.95 (±1.0%)

[a]Mean % artesunate (±SD) remaining as established by HPLC analysis at the end of each month ($n = 3$): for HPLC method see experimental section and [28]; [b]*RH*, relative humidity.

45°C. In distilled water, maximum solubility was attained relatively rapidly, with relatively little decomposition occurring during the 24-h time frame at solution temperatures of 5 or 15°C (Fig. 3). However, at temperatures of 25°C or higher, an overall decrease in artesunate concentration was observed over 24 h (Fig. 3). This decrease was the result of the hydrolysis of artesunate in solution.

At 5 and 15°C, a maximum apparent solubility was reached relatively rapidly, with a slow and relatively minor amount of hydrolytic degradation (< 10%) occurring over the 24-h time frame (Fig. 3). Interestingly, the dissolved artesunate concentration appears to oscillate for the first 8 h with a marginal decline then being observed up to 24 h (Fig. 3). This phenomenon is attributed to the increase in temperature of the solutions occurring upon withdrawal from the test solution and subsequent analysis of the filtrate. The HPLC sampling tray was not temperature controlled, and thus an increase in the temperature of the sample was unavoidable. The lowest percentage of artesunate detected relative to all detected peaks was 97.2% and 89.6% at 5 and 15°C respectively. At 25°C, after an initial period of 3 h, hydrolytic degradation of artesunate becomes substantial (Fig. 3) which according to HPLC analysis corresponds to formation of α- and β-DHA. At both 37 and 45 °C, the artesunate concentration decreases rapidly, tending to a maximum at 24 h (Fig. 3). Lower amounts of degradation at 37°C were observed over the period of 1–8 h but reached the same levels after 24 h (Fig. 4). At a solution temperature of 45°C, the amount of artesunate dissolving was 1.15 mg/mL with substantial degradation occurring after 2 h, wherein the concentration of dissolved artesunate was 0.52 mg/mL; after 24 h, this had reduced to 0.49 mg/mL (Fig. 3). Degradation products detected by means of HPLC analysis were α-DHA, β-DHA, and 9,10-anhydrodihydroartemisinin (AH-DHA, *cf.* Figure 2). At 45°C after 1 h, 18.4% of α-DHA and 3.1% of ß-DHA were detected. AH-DHA was detected after 4 h (24.5% α-DHA; 3.7% ß-DHA; 9.6% AH-DHA). After

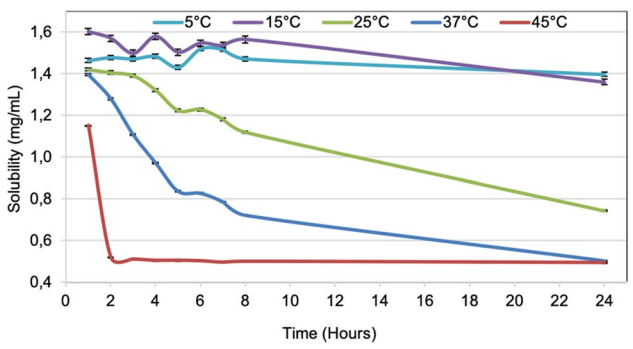

Fig. 3 Amount of dissolved artesunate in water at different temperatures of the solution (5, 15, 25, 37, and 45°C (±2°C) over a 24-h period

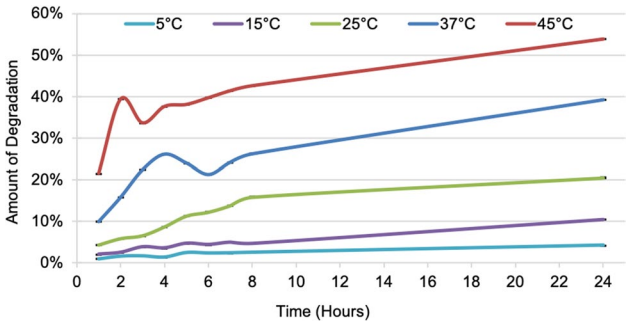

Fig. 4 Total amounts of degradation products comprising α-DHA, ß-DHA, and 9,10-anhydrodihydroartemisinin (AH-DHA) detected over a 24-h period at 5, 15, 25, 37, and 45°C (±2°C), in distilled water

24 h, the total degradation was 53.8% (18.8% α-DHA; 2.5% ß-DHA; 32.5% AH-DHA) (Table III). The graphical summation of all degradation products formed at the various temperatures from artesunate is presented in Fig. 4. It is clear that degradation is minimized at 5 and 15°C.

According to previous work, the activation energy (E_a) necessary to trigger the degradation of artesunate when present in water was calculated using the calculated degradation rate constants (k) and applying the Arrhenius equation:

$$lnK = -\frac{Ea}{RT} + lnA$$

A linear plot of the inverse log calculated for each reaction rate *versus* 1/T (K) provided a slope of − 2880.29 from which an E_a = 23.95 kJ/mol was quantified. Unlike most APIs, of which the solubility in water increases with an increase in temperature, the accelerating influence of additional energy on hydrolysis far outweighs any benefit on solubilization. Therefore, the highest apparent aqueous solubility values for artesunate were observed at low temperatures. Equilibrium solubility tests were also performed in 0.1 M HCl (pH 1.2), acetate buffer (0.1 M, pH 4.5), and phosphate buffer (0.2 M, pH 6.8) at 37 ± 2°C. As noted above, artesunate undergoes hydrolysis via alkyl-oxygen cleavage under acidic conditions, or acyl-oxygen cleavage under basic conditions according to classical ester hydrolysis mechanisms [17, 20]. Thus, we need to take account of competing hydrolysis in relation

Table III Degradation Products (%) Measured Over 24 h at 45°C in Distilled Water

Degradation products	1 h	4 h	24 h
α-DHA	18.4	24.5	18.8
ß-DHA	3.1	3.7	2.5
AH-DHA	-	9.6	32.5

to the pH of the medium in addition to its temperature. In terms of solubility in pH-buffered media, phosphate buffer showed the highest solubility concentration solubility medium for artesunate. The solubility of artesunate in different media at 37 ± 2°C after 60 min was as follows: 0.1 M HCl: 0.26 mg/mL; acetate buffer: 0.92 mg/mL; water: 1.40 mg/mL; and phosphate buffer: 6.59 mg/mL. After 60 min in 0.1 M HCl 20.26% ß-DHA, in pH 4.5 buffer 10.18% α-DHA and 2.04% ß-DHA, in water 8.36% α-DHA and 1.53% ß-DHA, and in phosphate buffer 4.12% α-DHA, 0.60% ß-DHA and 0.76% AH-DHA were detected. The degradation was greater than that reported previously; however, it must be noted that those studies were conducted at 2–8°C and 25°C [19]. Therefore, it may be concluded that in terms of pharmaceutical processing of artesunate in aqueous buffers, degradation can be minimized by maintaining the temperature at 25°C.

Conclusion

To conclude, it is clear that artesunate is an intrinsically unstable drug, in line with previous work. With respect to aqueous solubility, determination of the solubility of artesunate must be carried out at temperatures of 15°C or lower so as to minimize hydrolytic degradation of artesunate. The instability of artesunate in aqueous solutions is particularly manifesting here, and it is clear that any manipulation involving artesunate in aqueous solutions has to be conducted with care. This already is amply reflected in the need for rapid handling in the preparation of aqueous solutions of artesunate for injectable formulations. Overall, it is clear that the use of protic nucleophilic solvents such as water or alcohols for preparing stable formulations of artesunate will have to be avoided.

Author Contribution Post-graduate who was responsible for a substantial contribution to most of the analytical work: Bezuidenhout JW.

Conceptualization of the project: Liebenberg W, Aucamp M, and Stieger N.

Drafting and revising it critically for important intellectual content: Haynes RK and Aucamp M.

Final approval of the version to be published: Haynes RK, Liebenberg W, and Aucamp M.

All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data Availability All data generated during this study is available upon request to the authors.

Declarations

Conflict of Interest The authors declare no competing interests.

Disclaimer Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

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