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Short communication

Validation and application of a liquid chromatographic–mass spectrometric method for determination of artesunate in pharmaceutical samples

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Abstract

A simple and rapid liquid chromatographic–mass spectrometric assay for the evaluation of artesunate in vials for injection has been developed and validated.

The content of each vial was dissolved in 3.0 mL of methanol using a SGE analytical syringe (1.0 mL). Each sample was diluted to a theoretical concentration of 1000 ng/mL and analysed in triplicate. Three replicates of calibration standards at concentrations 500, 1000 and 1500 ng/mL were used to construct a calibration curve. Artesunate was analysed by liquid chromatography with atmospheric pressure chemical ionisation (APCI) mass spectrometric (MS) detection on a Hypersil Gold column (100 mm × 4.6 mm) using a mobile phase containing methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) at a flow rate of 1 mL/min. The assay was implemented for the analysis of artesunate for injection purchased from Guilin Pharmaceutical Company in China.
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1. Introduction

Malaria, caused by the mosquito-borne protozoan parasite *Plasmodium*, is the most important parasitic disease in the world. The World Health Organisation estimates that annually between 300 and 400 million people are infected causing between 1 and 3 million deaths. Children below the age of 5 in Africa constitute the majority of these.

Drug resistance is emerging to almost all antimalarial drugs except for the artemisinin derivatives. The increasing drug resistance and widening geographic distribution of *P. falciparum* emphasise the need for new and effective antimalarial drug combinations. To delay and prevent arising resistance the use of drug combinations and especially artemisinin based combination therapy is now generally accepted and encouraged [1]. In the treatment of severe malaria, parenteral artesunate has been shown to reduce the mortality in South East Asian patients by 35% compared to quinine [2]. Parenteral artesunate is now the recommended treatment for severe malaria in South East Asia.

Artesunate is a hemisuccinate ester, which is rapidly and extensively hydrolysed *in vivo* to the active metabolite dihydroartemisinin (DHA), which has a relative antimalarial activity of approximately 1.4 compared to the mother compound [3].

Pharmaceutical counterfeiting is a well-recognised global health problem with a particular impact in developing countries where drug-regulatory systems are weak or ineffective [4]. There have been many alarming reports in recent years of counterfeit antimalarials, antibiotics, hormones and steroids, analgesics and antipyretics, anti-asthma and anti-allergy drugs [5–11]. Non-destructive analytical techniques such as desorption electrospray ionisation mass spectrometry (DESI MS) has recently been successfully implemented for rapid screening of counterfeit artesunate tablets [12,13]. To date there are no published methods for determination of artesunate in pharmaceutical samples and only a few for the determination of artesunate and DHA in plasma [14–16]. All previous methods employ extensive sample preparation (i.e. liquid-liquid extraction or solid-phase extraction) and long chromatographic run times in order to avoid interferences present in the plasma matrix.

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The aim of the work described in this paper was to develop a simple readily applicable rapid liquid chromatographic–mass spectrometric assay for quality control and authentication of artesunate for injection. The assay was implemented for the analysis of artesunate for injection in vials purchased from Guilin Pharmaceutical Co., China, for use in a clinical trial.

2. Materials and methods

2.1. Chemicals

Artesunate and DHA were obtained from Holley-Cotec (China). The structure is shown in Fig. 1. Methanol (pro analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Ammonium acetate (LC–MS grade) was from FLUKA (Sigma–Aldrich, St. Louis, USA). Ammonium acetate buffer solutions were prepared by dissolving appropriate amounts of ammonium acetate in HPLC-water and adjusting pH with acetic acid (Merck Darmstadt, Germany).

2.2. Liquid chromatography–mass spectrometry

The LC system was a LaChrom Elite system consisting of two L2130 LC pumps, a L2200 injector set at 4 °C and a L2300 column oven set at 25 °C (Hitachi, Tokyo, Japan). Data acquisition and quantification were performed using Hystar™ and QuantAnalysis™ (Bruker, Bremen, Germany). The compounds were analysed on a Hypersil Gold (100 mm × 4.6 mm) column (Thermo, Waltham, USA) using a mobile phase containing methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) at a flow rate of 1 mL/min. Artesunate was detected using an Esquire 4000 iontrap mass spectrometer equipped with an APCI interface (Bruker Daltonics, Bremen, Germany). MS parameters were as follows: scan range m/z = 200–300, scan speed 13,000 $m/z\,s^{-1}$ with unit resolution, nebulizer flow 65 psi, dry gas flow 4.0 L/min, dry temperature 350 °C, vaporizing temperature 450 °C, capillary −1.6 kV, corona current 2200 nA, ion charge control (ICC) target 10,000, maximum accumulation time 100 ms, spectra averages 5, rolling averages 2. Quantification was performed using extracted ion chromatogram (EIC) for m/z 221. Data were processed using QuantAnalysis™ (Bruker Daltonics, Bremen, Germany).

2.3. Preparation of standards

Stock solutions of artesunate 2 mg/mL were prepared in methanol. Calibration standards at 500, 1000 and 1500 ng/mL were prepared by dilution of the stock solution with methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v). A calibration curve was constructed from triplicates at each calibration level using peak-area against concentration and $1/x$ weighted linear regression for quantification. The calibration standards were prepared freshly on each day of analysis.

2.4. Analytical procedure

Exactly 3.0 mL methanol was transferred to each vial using a SGE analytical syringe (1.0 mL) to produce a theoretical concen-

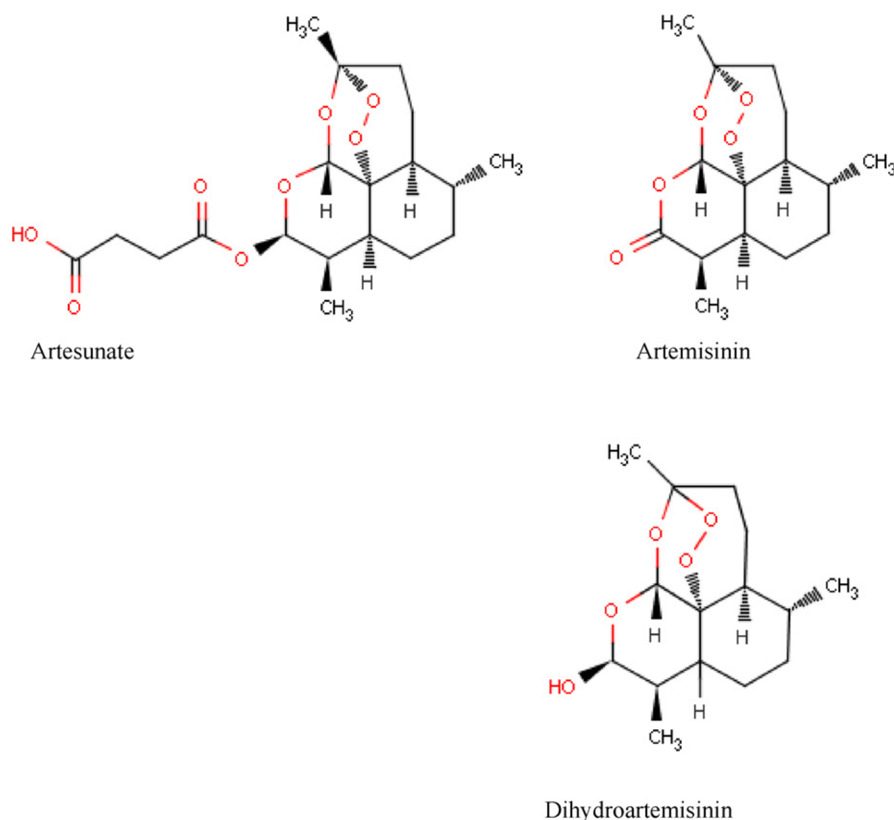

Fig. 1. Structure of artesunate, dihydroartemisinin and artemisinin.

tration of 20.0 mg/mL. Portions from each vial were then diluted with methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) in serial dilution steps to a final theoretical concentration of 1000 ng/mL. One milliliter of sample was transferred to an amber autosampler vial and three replicates of 10 μL were injected into the LC–MS system.

2.5. Validation

The accuracy and precision of the assay was evaluated over 7 days using 6 replicates of quality control samples at 500, 1000 and 1500 ng/mL each day. The accuracy and precision for a real sample was further evaluated using six replicates of dissolved artesunate from a vial (theoretical concentration 1000 ng/mL) each day. Linearity and regression model was evaluated using back-calculated values for the calibration curves. Stability of artesunate in methanol and methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) was evaluated at room temperature and at 4 °C for 24 h.

Selectivity was evaluated by analysis of blank methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) solution, artemisin and dihydroartemisinin. The procedure described under Section 2.4 was applied for the analysis of artesunate for injection from different batches purchased from Guilin Pharmaceutical Company.

3. Results and discussion

Artesunate is freely soluble in methanol and has one acidic pK_a estimated at 4.3 [17]. Artesunate is rapidly metabolised *in vivo* to DHA by non-specific esterases but will also undergo *ex vivo* hydrolysis to DHA particularly in acid conditions. The stability of the artemisinin derivatives in solution is in general quite poor. The stability of artesunate has shown to be enhanced if dissolved in solvents (e.g. ethanol) instead of water [18]. Dissolving it in methanol instead of water or physiological saline solution therefore enhanced the stability of artesunate in this study. During development of the method several different approaches were evaluated. The initial approach was to take a small aliquot of the powder and dissolve it for further quantification. It appeared, however, very difficult to get an accurate estimate of the total amount present in the vial. The powder was particularly retained by the rubber vial cap making it difficult to extract the whole amount for weighing, and the glass vial was significantly heavier than the powder making it difficult to get adequate precision and accuracy if differential weighting was used.

The next approach was to mimic the instructions for use of the drug in patients, that is dissolution in 1 mL sodium bicarbonate (in the commercial packaging a glass ampoule containing 5% sodium bicarbonate is included) and further dilution with 5 mL physiological saline. It was however not practical to add the two different solutions with the analytical syringe and, moreover, the stability of the artesunate was relatively poor in the final solution. Another disadvantage was that the volume of sodium bicarbonate supplied with the artesunate vials varied considerably, with a range from 0.9 to 1.2 mL. The best approach appeared to be dissolving the artesunate in the vials by adding 3.0 mL methanol with a SGE analytical syringe as it enabled rapid dissolution and enhanced stability. A small aliquot from each vial was then serially diluted with methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) to a theoretical concentration of 1000 ng/mL.

Initially LC–MS/MS was investigated as an alternative to LC–MS. However since an iontrap was used the added fragmentation step also adds a time constant which leads to band broadening and loss of resolution. MS/MS did not offer any

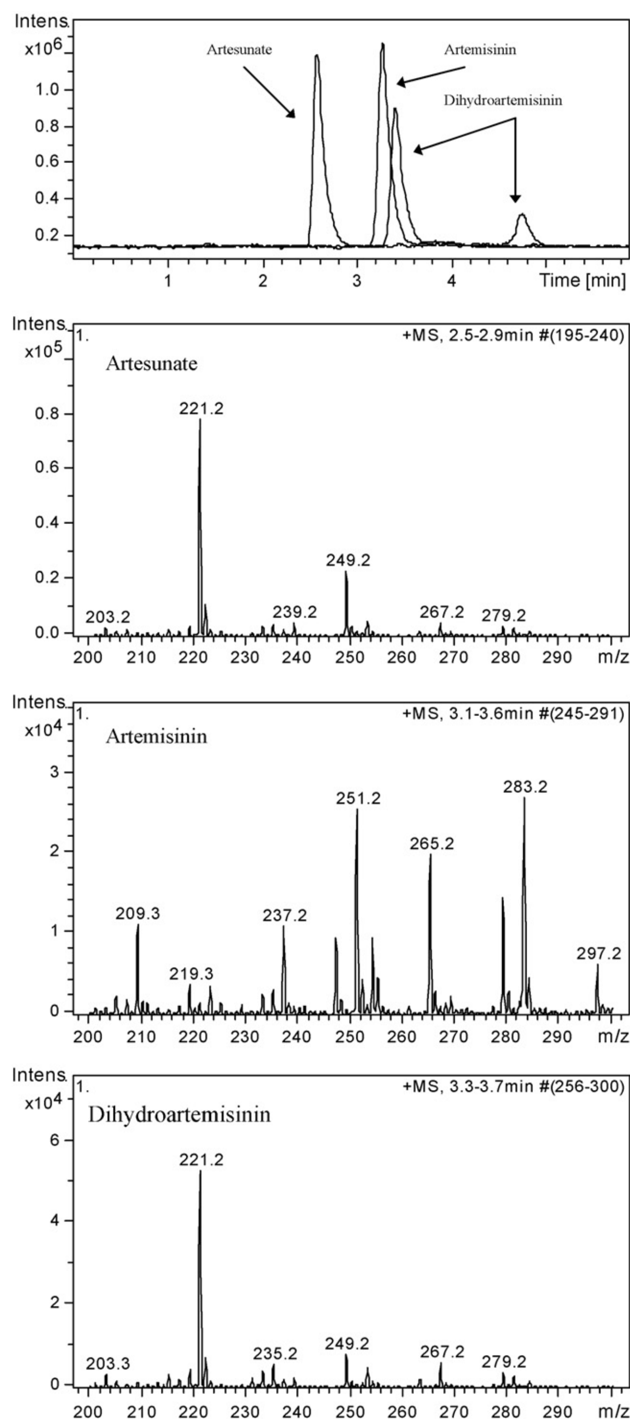

Fig. 2. LC chromatogram and MS spectra of artesunate, dihydroartemisinin and artemisinin.

Table 1
Accuracy and precision

	Mean found	% Deviation (found vs. added)	Intra-assay precision (%)	Inter-assay precision (%)	Total-assay precision (%)
500 ng/mL ($n = 42$)	496 ng/mL	−0.9	2.9	5.7	3.5
1000 ng/mL ($n = 42$)	978 ng/mL	−2.2	2.4	7.6	3.7
1500 ng/mL ($n = 42$)	1503 ng/mL	0.2	2.7	5.3	3.2
Vial 60 mg[a] ($n = 36$)	61.96 mg	3.3[b]	2.1	7.7	3.5

[a] Diluted to a theoretical concentration of 1000 ng/mL.
[b] Assuming a content of exactly 60.0 mg in the vial.

Table 2
Amount of artesunate in vials from different batches

	Found (ng/mL)	Artesunate/vial (mg)[a]	RSD (%)	% Deviation (found vs. stated)
Vial 1 batch 041101 ($n = 3$)	1119	67.1	1.5	11.9
Vial 2 batch 041101 ($n = 3$)	1032	61.9	0.5	3.2
Vial 1 batch 060107 ($n = 3$)	1062	63.7	1.4	6.2
Vial 2 batch 060107 ($n = 3$)	1044	62.6	1.7	4.4
Vial 1 batch 050401 ($n = 3$)	998	59.9	2.1	−0.2
Vial 2 batch 050401 ($n = 3$)	1095	65.7	3.6	9.5

[a] Artesunate/vial (mg) = x ng/mL (found)/1000 ng/mL (theoretical after dilution) × 60 mg (theoretical before dilution).

advantage since the background signal from the pharmaceutical sample was very low. Thus there was no gain in selectivity or increase in signal-to-noise ratio of MS/MS compared to MS. It is very important to have adequate LC separation between the degradation product DHA and artesunate since these two compounds produce the exact same precursor- and daughter ions (Fig. 2). Mobile phases containing a high content of acetonitrile provided good separation but unfortunately proved unsuitable as they caused carbonisation of the corona needle and loss of sensitivity up to 25% within a couple of hours. The mobile phase was changed to methanol–ammonium acetate, which provided good separation and a robust signal.

3.1. Validation

Back calculated values for the calibration standards validated the regression model using linear regression with 1/x weighting for quantification. The assay was linear in the tested calibration range (500–1500 ng/mL) and the accuracy and precision (RSD) for the back-calculated calibration standards were less than 3% for all series. The accuracy and precision of the assay for quality control samples and a real sample of dissolved artesunate from a vial (theoretical concentration 1000 ng/mL) are summarised in Table 1.

Artesunate was stable in methanol and methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) at room temperature and at 4 °C for at least 24 h.

Neither blank methanol–ammonium acetate 10 mM pH 5.3 (70:30, v/v) solution, artemisinin or dihydroartemisinin produced any signal at the retention time of artesunate. The procedure described under Section 2.4 was applied for the analysis of artesunate in vials for injection purchased from Guilin Pharmaceutical Company (Table 2).

The content of artesunate in all the vials except one were within the ±10% tolerance limits specified by the company for release of finished product (Guilin, personal communication). No vial contained less than the specified amount and in average they contained 5.8% more than specified. The reference standard used to prepare calibration standards was obtained from another source (i.e. Holley-Cotec) than the artesunate vials (i.e. Guilin Pharmaceutical Company) and it is possible that this could have contributed to a systematic difference between calibration standards and vials for injection.

4. Conclusion

A simple and rapid LC–MS assay for quality control and authentication of artesunate in pharmaceutical samples has been developed and validated. The assay is accurate and reproducible and can be used for quality control of artesunate in pharmaceutical samples. The assay was used for analysis of artesunate for injection from Guilin Pharmaceutical Co. All samples with one exception contained amounts of artesunate within specifications.

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