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Selective high-performance liquid chromatographic determination of artesunate and α- and β-dihydroartemisinin in patients with falciparum malaria

Kevin T. Batty[a,b,*], Timothy M.E. Davis[b], Le Thi Anh Thu[c], Tran Quang Binh[c], Trinh Kim Anh[c], Kenneth F. Ilett[a,d]

[a]*Department of Pharmacology, University of Western Australia, Nedlands, Western Australia 6907, Australia*
[b]*Department of Medicine, University of Western Australia, Fremantle Hospital, Fremantle, Western Australia 6160, Australia*
[c]*Tropical Diseases Research Centre, Cho Ray Hospital, Ho Chi Minh City, Viet Nam*
[d]*Clinical Pharmacology and Toxicology Laboratory, The Western Australian Centre for Pathology and Medical Research, Nedlands, Western Australia 6009, Australia*

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Abstract

A novel solid-phase extraction and a robust high-performance liquid chromatographic (HPLC) separation procedure for artesunate and α- and β-dihydroartemisinin, using post-column alkali decomposition and UV detection, is described. Extraction was performed with Bond-Elut Phenyl solid-phase extraction cartridges and analysis by HPLC was carried out using a Waters Symmetry C_8 5-μm 150 × 3.9 mm I.D. column. The mobile phase was 50% acetonitrile in 0.1 *M* acetate buffer (pH 4.8) delivered at a flow-rate of 0.7 ml/min. The column eluate was mixed with 1.2 *M* potassium hydroxide in 90% methanol delivered at 0.3 ml/min, in a 1-ml reaction coil at 69°C, to form UV-absorbing chromophores which were detected at 290 nm. The recovery of all analytes was greater than 80%. There was no significant difference in the peak-area ratio of α- and β-dihydroartemisinin in plasma. Preliminary pharmacokinetic data from six adult Vietnamese patients who received 120 mg of artesunate by intravenous injection for the treatment of acute falciparum malaria are presented. Despite limited data, the mean half-life of artesunate was approximately 3.5 min while that for dihydroartemisinin was 34 min. These data confirm the relatively rapid clearance of both artesunate and its principal active metabolite, dihydroartemisinin.

Keywords: Artesunate; Dihydroartemisinin

1. Introduction

Artesunic acid (artesunate, ARTS, Fig. 1A) is one of the semi-synthetic antimalarial analogues of artemisinin (qinghaosu, QHS, Fig. 1C). ARTS is the only one of the analogues currently used in clinical practice in South-East Asia that can be administered by intravenous (i.v.) injection [1,2]. As with the other analogues (artemether and arteether), ARTS is converted in vivo to the active metabolite dihydroartemisinin (DQHS, Fig. 1B). However, in the case of ARTS, the rate of metabolism is extremely rapid [3–6].

*Corresponding author. Address for correspondence: Department of Pharmacology, University of Western Australia, Nedlands, Western Australia 6907, Australia.

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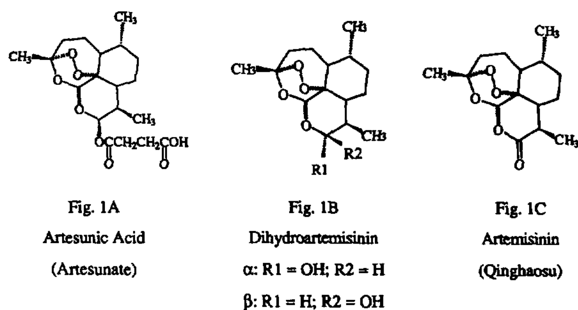

Fig. 1. Chemical structures of artesunate, dihydroartemisinin and artemisinin.

Since QHS and its analogues do not have ultraviolet (UV) or fluorescent chromophores, chromatographic analysis of these drugs in biological fluids has proved difficult [7]. Nevertheless, several high-performance liquid chromatography (HPLC) analytical methods have been developed, including post-column alkali [8,9] and pre-column acid [10–12] decomposition with UV detection, as well as standard isocratic separation with reductive electrochemical (EC) detection [13,14]. Recently, HPLC with chemiluminescent detection [15] and gas chromatography–mass spectrometry [16] methods that are suitable for analysis of artemisinin have been published. Whilst HPLC–EC is currently the most sensitive method for determination of artemisinin and its derivatives in biological fluids, with a limit of detection in the order of 5–10 μg/l, the method is labour-intensive and depends on complex, expensive equipment that can provide a constantly oxygen-free environment [7]. In view of these limitations, we sought to develop a more robust and practical method for clinical pharmacokinetic studies in patients with falciparum malaria who are treated with ARTS or other artemisinin analogues in currently recommended doses.

This report describes a novel solid-phase extraction and isocratic HPLC separation procedure for ARTS and DQHS, with an adapted post-column alkali decomposition and UV detection method [8]. We present preliminary pharmacokinetic data from six adult Vietnamese patients who received a conventional initial intravenous dose of ARTS for the treatment of acute falciparum malaria. The drug and metabolite plasma concentration profiles confirm the rapid metabolism of ARTS and show that both the α- and β-anomers of DQHS are present.

2. Experimental

2.1. Materials

ARTS, DQHS and QHS reference standards were gifts from Colonel Brian Schuster (Walter Reed Army Institute of Research, Washington DC, USA). Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt (Paris, KY, USA). Analytical grade glacial acetic acid and sodium acetate (anhydrous) were obtained from Ajax (Auburn, NSW, Australia). Analytical grade potassium hydroxide, ethyl acetate and butyl chloride were obtained from BDH (Poole, UK). De-ionised water was used for preparation of all buffers and aqueous solutions. All glassware was silanised with Repel-Silane (LKB, Bromma, Sweden).

2.2. Extraction procedure

Plasma (1 ml aliquots) was spiked with 250 ng of QHS as internal standard (20 μl of 12.6 mg/l solution in methanol). Extraction was performed with Bond-Elut Phenyl (100 mg, 1 ml) solid-phase extraction cartridges (Varian, Harbor City, CA, USA). The cartridges were primed with 1 ml of methanol and 1 ml of 1 *M* acetic acid prior to applying the plasma sample. The cartridge was washed with 2 × 1 ml of 1 *M* acetic acid, followed by 1 ml of 20% methanol in 1 *M* acetic acid before eluting the analytes with 2 × 1 ml of 20% ethyl acetate in butyl chloride. A small quantity of aqueous solution was aspirated and the organic phase was evaporated under N_2 at 40°C. The residue was reconstituted with 200 μl of HPLC mobile phase and aliquots (50–75 μl) were injected onto the column.

2.3. Chromatography

Analysis by HPLC (Fig. 2) was carried out using a 590 programmable solvent delivery pump and a Symmetry C_8 5 μm, 150 × 3.9 mm HPLC column

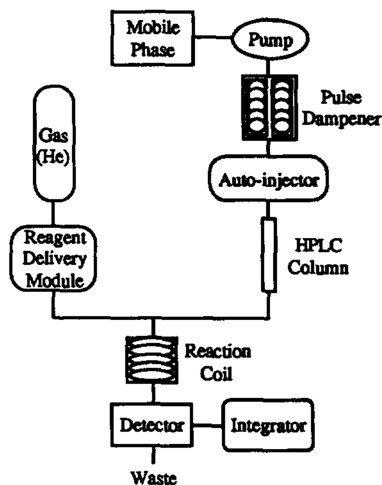

Fig. 2. Schema of HPLC apparatus.

with integrated guard column (Waters, Milford, MA, USA). Injections were made via a K65B automated sample injector (ETP Kortec, Ermington, NSW, Australia). The post-column reagent was 1.2 *M* potassium hydroxide in 90% methanol, delivered by a waters reagent delivery module, at a flow-rate of 0.3 ml/min using high-purity helium at 42 p.s.i.

The mobile phase was 50% acetonitrile in 0.1 *M* acetate buffer (40 ml 1 *M* acetic acid + 60 ml 1 *M* sodium acetate per litre, pH 4.8) delivered at a flow-rate of 0.7 ml/min. The mobile phase and post-column reagent were mixed and passed through a 1-ml reaction coil (Waters) housed in a Waters HPLC column heater at 69°C. Out-gassing was minimised by preparation of fresh mobile phase on each day of operation and sonication (10 min) and filtration of both the mobile phase and post-column reagent.

Detection was at 290 nm, using a Waters Lambda-Max 481 LC spectrophotometer, linked to a 3380A integrator (Hewlett-Packard, Avondale, PA, USA). Retention times for ARTS, α-DQHS, β-DQHS and QHS were approximately 6.3, 7.5, 10 and 13 min, respectively (Fig. 3).

2.4. Recovery

Preliminary evaluation of a range of solid-phase extraction cartridges showed that recovery from

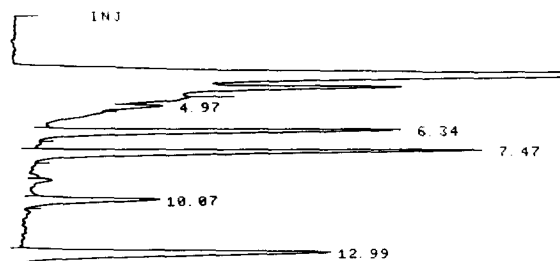

Fig. 3. Chromatogram of an extract of plasma from a patient, 10 min after intravenous administration of ARTS (2.4 mg/kg). Peaks: 6.34 min = ARTS; 7.47 = α-DQHS; 10.07 = β-DQHS; 12.99 = QHS (internal standard; 250 μg/l). Concentrations of ARTS and DQHS (racemate) were 1770 and 2540 μg/l, respectively.

Varian Bond-Elut Phenyl, C_8, and C_{18} and Waters trifunctional C_{18} was 85%, 95%, 70% and 75%, respectively, for ARTS, and 90%, 95%, 75%, and 75%, respectively, for DQHS. Optimal extraction results were achieved with the Bond Elut Phenyl cartridge, and therefore this was chosen for all subsequent procedures.

The recovery of ARTS, DQHS and QHS was evaluated by spiking drug-free plasma with 2300, 690 and 235 μg/l of ARTS, 1750, 520 and 175 μg/l of DQHS (racemate) and 250 μg/l of QHS as the internal standard. Extraction and chromatography procedures were as described previously. Recovery was assessed by comparing peak-area measurements for each analyte from the extracted plasma with measurements from direct injection on-column of solutions of the same concentration in mobile phase.

2.5. Calibration and reproducibility

ARTS and DQHS were quantified using the peak-area ratio of analyte to internal standard. A five-point standard curve in the range of 230–2300 μg/l for ARTS and 175–1750 μg/l for DQHS was constructed on each day of analysis. The within-day coefficient of variation was determined by duplicate analysis of at least two samples from the standard curve. The between-day coefficient of variation was assessed from aliquots of plasma containing 1450 μg/l ARTS and 1100 μg/l DQHS which were stored at −70°C and analysed with each batch of patient samples.

2.6. Patients and clinical methods

Six Vietnamese patients (three males, three females) aged 24 to 65 years and with body weights ranging from 41.5 to 50.0 kg were studied. Four had presented to the General Hospital, Dalat, Lam Dong Province and two to Cho Ray Hospital, Ho Chi Minh City with slide-positive, uncomplicated falciparum malaria. Three had received no antimalarial drugs before presentation. The other three had been treated with oral artesunate or unknown medication at least 12 h prior to recruitment, but had an unsatisfactory initial clinical response. None of the patients were jaundiced and serum creatinine concentrations were all less than 120 μmol/l. Venous haematocrits at the time of study were 19 to 40%. All patients gave informed consent to the study procedures which were approved by the University of Western Australia Human Rights Committee, the Ethics Committee, Cho Ray Hospital and the Ministry of Health, Vietnam.

Venous blood samples (5 ml) were obtained immediately before (0 min) and then at 10, 20, 30, 45, 60, 90 min and 2, 4 and 6 h after i.v. administration of 120 mg of ARTS (Guilin No. 2 Pharmaceutical Factory, Guangxi, China). The blood was drawn directly into sodium fluoride/potassium oxalate blood collection tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) to prevent hydrolysis of ARTS by plasma esterases. Sample tubes were placed in ice and centrifuged within 30 min of sampling. Separated plasma was stored below −20°C until analysed. Preliminary studies showed that both ARTS and DQHS can be stored at −20°C or lower for up to 6 months with no significant degradation (data not shown). On the day of analysis the plasma samples were thawed to room temperature, centrifuged at 1000 *g* for 5 min, and 1-ml aliquots taken for analysis.

2.7. Data analysis

Statistical analyses were performed with SigmaStat for Windows (Jandel Scientific, San Rafael, CA, USA). Pharmacokinetic parameters were determined from the plasma concentration–time data using a non-compartmental approach [17]. Mean data were compared by *t*-test with a level of significance of 0.05.

3. Results and discussion

3.1. Extraction and assay procedure

The recovery of ARTS, α-DQHS, β-DQHS and QHS was 93% ± 7% (mean ± S.D.; $n = 12$), 88% ± 9.5% ($n = 12$), 80% ± 4% ($n = 8$) and 89% ± 4% ($n = 12$), respectively. There was no significant difference in the ratio between α-DQHS and β-DQHS for recovery efficiency or analyte to internal standard peak-area ratio.

The correlation coefficient for the standard curve was greater than 0.98 on all occasions. The limits of detection were 30 μg/l for ARTS and 20 μg/l for DQHS, respectively, which are directly comparable to those of previously published methods of analysis of artemisinin derivatives in biological fluids. Edlund et al. [8] reported limits of detection for ARTS and DQHS of 19 and 14 μg/l, respectively, whilst Thomas et al. [11] reported a limit of detection for DQHS of 25 μg/l. The HPLC–EC detection method can achieve a limit of detection for DQHS of 10–25 μg/l [4,14].

Since the range of concentrations appropriate for our clinical study was approximately 200 to 3000 μg/l for ARTS and 150 to 2000 μg/l for DQHS, the standard curve was consistent with these requirements. The limit of sensitivity for the standard curve (defined as the intercept of the lower 95% confidence limit with the *x*-axis) was approximately 50 μg/l in all cases.

The within-day coefficient of variation ranged from 2% to 10% for both ARTS and DQHS. The between-run coefficient of variation was 6% for ARTS and 8% for DQHS ($n = 14$).

There was no significant difference in the peak-area ratio (3.25 ± 0.44; $n = 24$) of α-DQHS to β-DQHS in spiked plasma samples compared to plasma samples from patients (3.30 ± 0.54; $n = 23$). Given the consistency of these data, and the predominance of the α-DQHS anomer, it was considered appropriate to quantify DQHS from the α-DQHS peak for all patient samples.

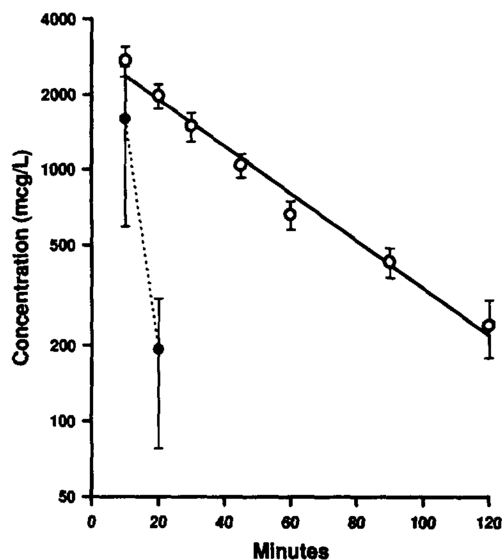

Fig. 4. Plasma concentration–time data for artesunate (●) and dihydroartemisinin (○). Data (mean ± S.D.) from six Vietnamese patients has been pooled. The solid line shows the line of best fit of dihydroartemisinin estimated by log-linear regression analysis.

3.2. Clinical study

The blood collection protocol did not provide sufficient data for accurate determination of pharmacokinetic parameters for ARTS. All patients had measurable levels of ARTS 10 and 20 min after administration and concentrations were below the limit of detection in all subsequent samples. The mean concentration–time data are shown in Fig. 4. Although it was not possible to accurately quantify the half-life of ARTS from the two data points, a mean value of approximately 3.5 min was estimated from direct log-linear interpolation. This result is comparable to the half life of 2.3 min reported in an earlier volunteer study [5], but shorter than the half life of 29 min reported after intramuscular administration of 2 mg/kg ARTS to six Vietnamese patients [4].

Preliminary pharmacokinetic data for DQHS were obtained from seven plasma samples for each patient. The concentration of DQHS was below the limit of sensitivity in the 4- and 6-h plasma samples. The mean half-life was 34 ± 8 min ($n = 6$; Fig. 4). By comparison, mean half-lives reported by Benakis et al. [4] and Yang et al. [5] were approximately 90 min and 48 min, respectively. Whilst the limit of sensitivity used in the present clinical study was approximately 50 μg/l, the high peak plasma concentrations of DQHS allowed determination of the pharmacokinetic parameters over a time period equivalent to four half-lives for the drug. The additional sensitivity offered by HPLC–EC detection (limit of detection for DQHS of 10–25 μg/l [4,14]) would not significantly improve the determination of pharmacokinetic descriptors. The data from this and previous studies [4,5] are consistent with a single exponential decay of DQHS over the concentration range of 2500 to 25 μg/l in plasma. In future studies, more frequent sampling should provide better data for the determination of pharmacokinetic parameters for ARTS and DQHS.

Our data show that the peak plasma concentration for ARTS is of the order of 2 mg/l, and that the drug is metabolised rapidly in vivo. Although considered a pro-drug, ARTS may make a significant initial contribution to parasite kill. The potent metabolite DQHS has a substantially longer elimination half-life than ARTS but, extrapolating from our data, the plasma concentration falls to less than 1 μg/l approximately 6.5 h after a 120-mg intravenous dose of ARTS. The minimum effective in vivo concentration is unknown, but conventional dosage regimens based on twelve-hourly administration of ARTS may result in substantial periods where concentrations of both ARTS and DQHS are sub-therapeutic. Whilst there is a paucity of pharmacodynamic information about the artemisinin derivatives, more frequent (e.g. six-hourly) ARTS injections or perhaps constant infusions may provide better therapeutic efficacy.

4. Conclusion

This solid-phase extraction procedure and HPLC assay provides an efficient, relatively inexpensive and robust method of analysing ARTS and the α- and β-anomers of DQHS in plasma samples. The ratio of the α- and β-anomers of DQHS in both clinical samples and standard solutions was similar and therefore quantification of DQHS can be reliably

assessed from the α anomer. Whilst the method is less sensitive than HPLC–EC, the precision and sensitivity are adequate for plasma concentrations achieved following conventional doses. Preliminary pharmacokinetic data from six Vietnamese patients with falciparum malaria show that ARTS and DQHS are cleared rapidly, with elimination half-lives of 3.5 and 34 min, respectively.

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