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Simple high-performance liquid chromatographic method with electrochemical detection for the simultaneous determination of artesunate and dihydroartemisinin in biological fluids

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Abstract

A simple, rapid, sensitive, selective and reproducible high-performance liquid chromatographic method with reductive electrochemical detection is described for the simultaneous quantification of artesunate (ARS) and dihydroartemisinin (DHA) in plasma. The procedure involved the extraction of ARS, DHA and the internal standard (artemisinin, ARN) with a mixture of dichloromethane and *tert*.-methyl butyl ether (8:2, v/v). Chromatographic separation consisted of the mobile phase (acetonitrile–water containing 0.1 *M* acetic acid, pH 4.8; 45:55, v/v) running through the column (Nova-Pak C_{18}, 150 cm×3.9 mm I.D., 5 μm particle size). The retention times of α-DHA, β-DHA, ARS and ARN were 2.9, 4.2, 4.5 and 6.0 min, respectively. The average recoveries of ARS, α-DHA and ARN in the concentration range of 10–800 ng/ml were 81.9, 88.2, 101.1 and 84.3%, respectively. The coefficients of variation (precision and repeatability) were below 10% for all three compounds at concentrations of 50, 200, 400 and 800 ng/ml, and below 20% at a concentration of 10 ng/ml. The limits of quantification for both ARS and α-DHA in spiked plasma samples were 5 and 3 ng/ml, respectively. The method was found to be suitable for application to pharmacokinetic studies of both ARS and DHA. © 1998 Elsevier Science B.V.

Keywords: Artesunate; Dihydroartemisinin

1. Introduction

Resistance of *Plasmodium falciparum* to the drugs that are currently used is rapidly increasing [1]. This has drawn attention to the use of artemisinin and derivatives (artemether, artesunate, arteether, dihydroartemisinin) as possible alternative antimalarials [2]. This group of compounds has been used successfully in the treatment of patients with multidrug resistant *P. falciparum* malaria, both in severe and uncomplicated cases [2–4]. Artesunate (ARS; Fig. 1), the hemisuccinate ester of dihydroartemisinin (DHA; Fig. 1) has been reported to be highly potent [5] and, due to its high water solubility, it is suitable for intravenous administration in which a rapid onset of action is desirable, such as in cases of cerebral malaria. In humans, ARS is rapidly and extensively biotransformed in liver to DHA, which is three–four times more potent as an antimalarial than ARS itself [6] and, therefore, ARS is regarded as a prodrug of DHA. Apart from its potential role as an antimalarial, from a cost-effective point of view,

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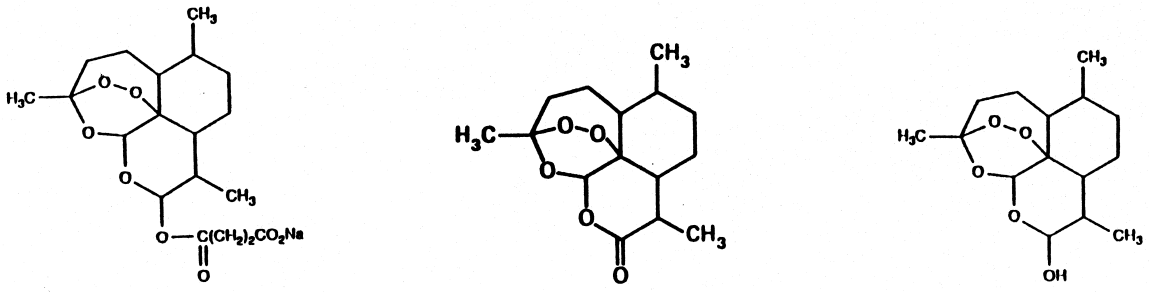

Fig. 1. Chemical structures of artesunate (ARS), artemisinin (ARN) and dihydroartemisinin (DHA) (from left to right).

DHA may also be a valuable, alternative candidate as an artemisinin derivative in the treatment of uncomplicated falciparum malaria when the compound is used as an oral drug formulation [7]. Detailed pharmacokinetic studies of ARS and DHA, when administered directly as the formulated drugs, as well as the kinetics of DHA as the principal plasma metabolite following the administration of ARS, are required for optimization of therapy with these drugs.

A few high-performance liquid chromatographic (HPLC) techniques with ultraviolet or electrochemical detection for the quantification of ARS and DHA have been published [8–17]. However, some of these methods do not retain high sensitivity and are not reliable enough for pharmacokinetic studies, and some require sophisticated procedures. The aim of this report was to search for a simple, rapid, specific, sensitive and accurate HPLC method with electrochemical detection (HPLC–ED) that was reliable enough to enable pharmacokinetic assessment of ARS and DHA in biological fluids. Simple deoxygenation prior to injection using a modified hypodermic syringe and a Rheodyne injector was used for the system [17].

2. Experimental

2.1. Chemicals

Standard powders of ARS and DHA were obtained from Walter Reed Army Research Institute, and artemisinin (ARN: Fig. 1) powder was a gift from Arenco Pharmaceutica, Belgium. All were prepared as 0.5 μg/μl stock solutions in 50% ethanol. Working solutions were prepared by diluting the stock standard solution with 50% ethanol to a concentration of 10 ng/μl, and these were stored as aliquots in 1 ml glass vials. Standard solutions were stored at −70°C until use.

All of the organic solvents used were of HPLC-grade. Absolute ethanol and *tert*.-methyl butyl ether were obtained from BDH (Poole, UK), and dichloromethane and acetonitrile were obtained from Fisons (Loughborough, UK). Other reagents and solvents (sodium chloride, acetic acid, sodium hydroxide and dimethyldichlorosilane) were of analytical grade quality and were supplied by BDH. High purity distilled deionized water was used to prepare all buffers and solutions.

2.2. Chromatography

The method was developed on a model BAS 200B liquid chromatography system coupled to an electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA), a Rheodyne 7125 injector (Rheodyne, Berkeley, CA, USA) with a 20-μl sample loop. The system consisted of mobile phase reservoirs (three bottles), solenoid proportioning valves, a dual piston pump, a pulse dampener, a column and a detector oven, dual thin-layer electrodes with a Ag/AgCl reference electrode. Stainless steel connectors and tubing were used throughout the system.

A number of HPLC chromatographic systems and extraction procedures were investigated to optimize the simultaneous, isocratic separation of ARS, DHA and ARN. A Nova-Pak C_{18} column (Waters, USA), together with the mobile phase consisting of 45% (v/v) acetonitrile in 0.1 *M* acetic acid (pH 4.8) gave

a satisfactory separation with a short run time (10 min). One major criterion for column selection was that sufficient DHA was retained on the column to avoid interference from the oxygen peak. Freshly prepared mobile phase was rigorously deoxygenated with helium (ultra high pure, 99.99%) at a flow-rate of 1 ml/min for 2 h, to remove dissolved oxygen. The system was operated at the following temperatures: mobile phase (35°C), column and detector oven (20°C). The mobile phase was delivered at a flow-rate of 1.5 ml/min (back pressure, 1500–2000 p.s.i.; 1 p.s.i.=6894.76 Pa). The system was run continuously with the mobile phase being recycled back into the reservoir and used for a period of two weeks. Rigorous sample deoxygenation was done prior to injection according to a method described previously [17]. The electrochemical detector was operated in the reductive mode at an applied potential of −1.0 V, using a thin-layer dual glassy-carbon electrode (Model MF-1000, BAS) and a Ag/AgCl reference electrode (Model MW-2021, BAS) at a sensitivity of 50 nA. Our study showed that glassy carbon at −1.0 V dramatically increased the detector response to the compounds of interest with only a modest increase in background current. The sensitivity of the detector was maintained by a combination of manual (abrasion-based) and electropolishing (−2.0 V versus AgCl for 2 h) techniques. The chromatograms were analyzed with software provided with the instrument (ChromGraph Report Software).

2.3. Sample extraction procedure

In order to minimize adsorption of drugs to glass, extraction was carried out in 15-ml screw-capped glass test tubes that had been precoated with dimethyldichlorosilane in toluene (5%, v/v). To a 1-ml plasma sample, the internal standard, ARN (300 ng), was added, followed by vortex-mixing for 30 s. The resultant mixture was extracted twice with 5 ml of the mixture of dichloromethane–*tert*.-methyl butyl ether (8:2, v/v) by mechanical tumbling for 20 min. This organic solvent mixture was chosen because it resulted in a clean and high recovery of all analytes. After centrifugation at 1200 *g* for 15 min (4°C), the clear organic layer was transferred to a clean tube using a pasteur pipette. Evaporation to dryness was by a stream of nitrogen at room temperature. The residue was dissolved in 60 μl of 50% ethanol and left for at least 16 h at 4°C, in order to allow stabilization of the ratio of α- and β-anomers of DHA. After rigorous deoxygenation, 20 μl were injected onto the column.

2.4. Calibration curves

Solutions of ARS and DHA in 50% ethanol, ranging from 1 to 800 ng/ml, were injected into the HPLC system to assess detector linearity. Peak heights were plotted against the quantities of ARS and DHA injected. ARS and DHA were linear ($r>$ 0.9999) over the concentration range used.

Calibration curves were prepared by triplicate analysis of 1-ml plasma samples spiked with concentrations of ARS and DHA in the ranges of 10–800 ng/ml, with a fixed concentration of internal standard (300 ng). Samples were analyzed as described in Section 2.3, and the peak-height ratios of ARS and DHA to internal standard were plotted against the corresponding drug concentrations. Peak-height ratios of the samples were determined and the concentrations calculated from the standard curves. The ratio of α- and β-DHA in spiked samples was stabilised when injection was postponed for 16–18 h after reconstitution; quantification of DHA was therefore only determined from the predominant anomer (α-DHA).

2.5. Method recovery, precision, accuracy, stability and selectivity

The analytical recoveries of the extraction procedure for ARS, α-DHA and ARN were estimated by comparing the peak heights obtained from an extracted sample with those measured with equivalent amounts of each analyte in 50% ethanol. The concentrations used were 10, 50, 200, 400 and 800 ng/ml for ARS, DHA and ARN.

The precision of the method, based on within-day repeatability, was determined by replicate analysis of five samples spiked with five different concentrations of ARS, DHA and ARN (10, 50, 200, 400 and 800 ng/ml). The reproducibility (day-to-day variation) of the method was established using the same concentration range as above, but only a single de-

termination of each concentration was made on five different days. Coefficients of variation (C.V.) were calculated from the ratios of standard deviation (S.D.) to the mean.

The stability of each compound was determined by storing plasma spiked with drugs and working standard solutions for six months at −70°C. Concentrations were measured periodically (after two weeks, one, two, four and six months) using the described HPLC method.

The selectivity of the method was verified by checking for interference by commonly used antimalarials (mefloquine, quinine, chloroquine, pyrimethamine, primaquine, artemether, arteether) after subjecting them to the extraction procedure.

2.6. Application of the method to biological samples

Since there was no significant difference in the peak-height ratio of α- to β-DHA in spiked plasma samples compared to plasma samples from patients and because α-DHA is the predominant anomer, the method was used for investigation of the pharmacokinetics of ARS and α-DHA in two male Thai volunteers (aged 23 and 25 years, weighing 55 and 53 kg, respectively), following the administration of a single oral dose containing 300 mg of ARS (Arenco Pharmaceutica; 50 mg per tablet), or 300 mg of DHA (Arenco Pharmaceutica; 100 mg per capsule). The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. Prior to the study, on both occasions, the subjects fasted overnight. Venous blood samples (3 ml each) were collected in heparinized plastic tubes at 0 h (pre-dose), and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 18, 24, 36, 48 and 72 h after dosage. Plasma was separated by centrifugation at 2000 *g* for 10 min, immediately after collection and frozen at −70°C until analysis.

Pharmacokinetic analysis was performed using either the model-independent or model-dependent method [18]. The maximum plasma concentration (C_{max}) and the time to maximum concentration (t_{max}) were observed values. The terminal phase elimination rate constant (λ_z) was determined by least squares regression analysis of the terminal plasma concentration–time data, and the terminal phase elimination half-life ($t_{1/2z}$) was determined from the ratio $0.693/\lambda_z$. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule. Oral clearance (Cl/F) was calculated from dose/AUC. The apparent volume of distribution (V_z/F) was calculated from Cl/F divided by λ_z. To characterise the absorption phase better, a one- or two-compartment open model with first-order elimination was fitted to the data by an iterative weighted non-linear regression using the TopFit programme. The observed concentrations were weighted as the reciprocal of the analytical variance.

3. Results and discussion

Analytical techniques for artemisinin and derivatives available nowadays include chemical assay [19], thin-layer chromatography [20–25], gas-chromatography (GC) [26–28], GC combined with mass-spectrometry (GC–MS) [29–31], MS–MS [32], radioimmunoassay (RIA) [33], enzyme-linked immunosorbent assay (ELISA) [34], HPLC with ultraviolet or electrochemical detection (HPLC–UV, HPLC–ED) [10–17] and superficial fluid chromatography with electrochemical or chemiluminescent detection [35,36]. Some have been applied to pharmacokinetic studies [13–17]. A sensitive bioassay has been developed [37,38], however, the lack of specificity precluded its use as a reference assay for pharmacokinetic studies. Among these assays, reductive mode HPLC–ED provides a sensitive, selective, accurate and reproducible assay methodology and is regarded as the method of choice for pharmacokinetic studies of these compounds, despite its cost and level of sophistication. More specifically, quantification of ARS and DHA has been described previously using either HPLC–UV [15] or HPLC–ED method [16], which required a laborious sample preparation procedure using pre-column solid-phase extraction. The advantages of the method developed in the present study over that previously reported are basically the simplicity of sample preparation and deoxygenation procedures, whilst maintaining the high sensitivity and selectivity. The methods can separate ARS from DHA without a laborious pre-column separation step, such as that reported previously [31,32]. Furthermore, high sample throughput

makes the method attractive for application in pharmacokinetic studies. In a previous method with HPLC–ED [32], there was a requirement for automatic deoxygenation when the system was coupled to an autosampling injector, which increased the degree of sophistication and required that the system be operated by highly trained personnel.

Calibration curves for ARS and α-DHA were linear over the range of 10 to 800 ng/ml, with correlation coefficients of greater than 0.990. The coefficients of variation (precision and repeatability) were below 10% for all three compounds at concentrations of 50, 200, 400 and 800 ng/ml, and below 20% at a concentration of 10 ng/ml. The inter-assay (day-to-day) and intra-assay (within-day) precision for ARS, α-DHA and ARN at five different concentrations are given in Table 1. All analytes were stable in plasma when samples were stored at −70°C for six months. The limits of quantification, defined as peak heights at a signal-to-noise ratio of three at a sensitivity of −20 nA in 1-ml plasma samples, were 5 and 3 ng/ml, for ARS and α-DHA, respectively. There was no significant difference in the ratio between α- and β-DHA for efficiency of recovery of the analytes to internal standard peak-height ratio and, therefore, quantification of DHA was reliably assessed from the α-anomer. Average recoveries for ARS, α-DHA and ARN in plasma over the concentration range of 10–800 ng/ml were 81.9, 88.2, 101.1 and 84.3%, respectively.

The chromatographic separation of a standard solution containing DHA (α, β), ARS and ARN (in 50% ethanol) is shown in Fig. 2a. Fig. 2b–d illustrate typical chromatograms of blank plasma, spiked plasma with DHA, ARS and ARN, and plasma obtained from one healthy male Thai 1 h after being given a dose containing 300 mg of ARS. The two anomers of DHA (α, β) were eluted at 2.9 and 4.2 min, respectively, followed by ARS (4.5 min) and ARN (6.0 min). The method was free of chromatographic interference from endogenous compounds and the commonly used antimalarials, including other artemisinin derivatives, i.e., artemether and arteether.

To demonstrate the clinical applicability of the method, the pharmacokinetics of ARS and α-DHA were investigated in two healthy male Thais following oral administration of ARS or DHA. Plasma concentration–time profiles of ARS and α-DHA following administration of both drugs are shown in Fig. 3a,b. The decline of drug concentration can adequately be described by a one-compartment

Table 1
Precision (within-day and day-to-day) of the assay

Drug	Amount added (ng/ml)	Within-day		Day-to-day	
		Amount measured (ng/ml)	C.V. (%)	Amount measured (ng/ml)	C.V. (%)
ARS	10	10.4	15.2	9.8	16.7
	50	52.1	2.9	54.2	7.0
	200	203.1	2.5	205.1	6.5
	400	403.3	1.6	416.1	8.3
	800	801.8	1.6	799.9	2.5
DHA	10	10.2	12.8	10.8	7.75
	50	50.3	2.9	48.4	4.6
	200	200.0	3.9	198.8	3.2
	400	418.3	4.2	386.9	6.1
	800	805.2	0.6	806.3	3.9
ARN	10	10.6	16.1	10.5	17.0
	50	49.4	2.8	49.5	4.3
	200	199.3	3.5	195.6	3.4
	400	403.2	3.0	398.9	3.1
	800	804.6	2.1	802.1	2.6

$n=5$ for each point.

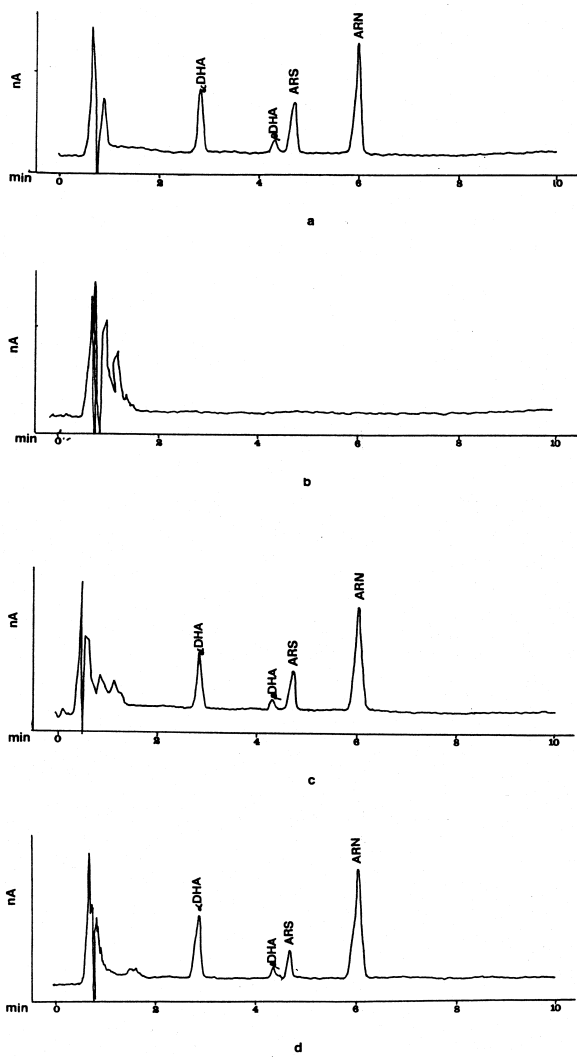

Fig. 2. Chromatograms of (a) standard solutions (100 ng of DHA, ARS and ARN), (b) blank plasma extracted with dichloromethane–*tert*.-methyl butyl ether (8:2, v/v), (c) plasma spiked with 400 ng of DHA, ARS and 300 ng of ARN and (d) plasma obtained from a healthy male Thai 1 h after a single oral dose of 300 mg of ARS (retention times for α-, β-DHA, ARS and ARN were 2.9, 4.2, 4.5 and 6.0 min, respectively).

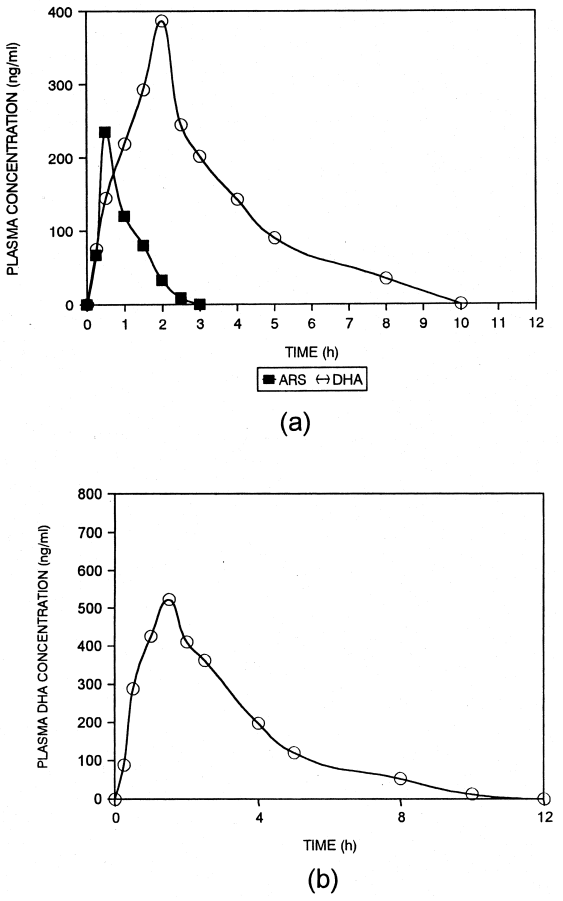

Fig. 3. Plasma concentration–time profiles for ARS and DHA in one healthy male Thai following a single oral dose of (a) 300 mg of ARS and (b) 300 mg of DHA.

model. In all cases, the concentrations of ARS and α-DHA fell below the limit of detection 12 h after administration of the drug. After a single oral dose containing 300 mg of ARS, the drug was detectable in plasma within 15 min. A C_{max} of 335 ng/ml was rapidly achieved at 0.5 h (t_{max}); the absorption half-life ($t_{1/2a}$) was 0.28 h. As previously reported, ARS was rapidly and extensively biotransformed to DHA and concentrations of the parent drug were measurable only for 3 h following drug intake. The AUC, $t_{1/2z}$, V_z/F and Cl/F were 229 ng·h/ml, 0.41 h, 14 l/kg and 396 ml/min/kg, respectively. The metabolite, α-DHA, reached its C_{max} later, i.e. 2 h after dosing with ARS, but had a higher C_{max} value and systemic exposure (C_{max}, 387 ng/ml; AUC, 1178 ng·h/ml). In addition, the $t_{1/2z}$ was longer (1.57 h). When DHA was given as formulated drug in capsules at a dose of 300 mg, the kinetics of DHA appear to be similar to those following the administration of ARS (C_{max}, 523 ng/ml; t_{max}, 1.5 h; AUC, 1967 ng·h/ml; $t_{1/2a}$, 1.66 h; Cl/F 50.6 ml/min/kg; V_z/F, 72.8 l/kg). Further investigations with large

number of subjects are required to accurately describe the pharmacokinetics of both drugs.

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