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Determination of artemether and its major metabolite, dihydroartemisinin, in plasma using high-performance liquid chromatography with electrochemical detection

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Abstract

A rapid, selective, sensitive and reproducible HPLC with reductive electrochemical detection for quantitative determination of artemether (ART) and its plasma metabolite, dihydroartemisinin (DHA: α and β isomers) in plasma is described. The procedure involved the extraction of ART, DHA and the internal standard, artemisinin (ARN) with dichloromethane–*tert.*-methylbutyl ether (1:1, v/v) or *n*-butyl chloride–ethyl acetate (9:1, v/v). Chromatographic separation was performed with a mobile phase of acetonitrile–water (20:80, v/v) containing 0.1 *M* acetic acid pH 5.0, running through a μBondapak CN column. The method was capable of separating the two isomeric forms of DHA (α, β). The retention times of α-DHA, β-DHA, ARN and ART were 4.6, 5.9, 7.9 and 9.6 min, respectively. Validation of the assay method was performed using both extraction systems. The two extraction systems produced comparable recoveries of the various analytes. The average recoveries of ART, DHA and ARN over the concentration range 80–640 ng/ml were 86–93%. The coefficients of variation were below 10% for all three drugs (ART, α-DHA, ARN). The minimum detectable concentrations for ART and α-DHA in spiked plasma samples were 5 and 3 ng/ml, respectively. The method was found to be suitable for use in clinical pharmacokinetic study.

Keywords: Artemether; Dihydroartemisinin

1. Introduction

Artemether (ART; Fig. 1a) is a semisynthetic antimalarial derived from artemisinin (qinghaosu; ARN, Fig. 1b), a natural product of a Chinese herb *Artemisia annua L.* Artemether and other artemisinin derivatives are now playing critical role in the treatment of *Plasmodium falciparum* malaria in areas with multi-drug resistance such as Thailand [1]. When appropriate dose regimens of ART were used, high clinical efficacy has been shown [2]. Its antimalarial action is very rapid; in most cases parasite and fever are cleared within the first 24 h of drug administration [2,3]. The drug is extensively metabolized in human liver to the active metabolite dihydroartemisinin (DHA; Fig. 1c), which possesses greater antimalarial potency than the parent drug itself [4]. Optimization of oral as well as parenteral

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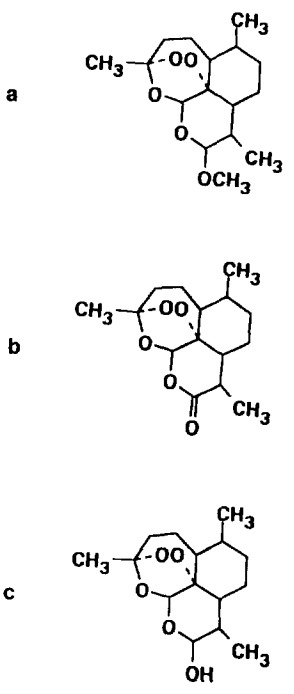

Fig. 1. Chemical structures of (a) artemether (ART), (b) artemisinin (ARN) and (c) dihydroartemisinin (DHA).

dosage regimens for this drug will therefore, only be correctly obtained when more information on the pharmacokinetics of both ART and DHA becomes available. This requires a sensitive and specific drug assay method in biological fluids.

Several methods have been reported for measurement of artemisinin and derivatives in biological fluids including chemical assay [5], thin layer chromatography [6], GC [7], RIA [8] and HPLC [9–17]. HPLC methods involved acid or base hydrolysis to a UV absorbable decomposition product and the assay with reductive electrochemical detection using either thin layer mercury amalgam, dropping mercury or glassy carbon electrode [9–17]. Among the available HPLC methods, HPLC with reductive electrochemical detection has best met either the sensitivity or specificity requirement. The only drawback of the technique is the requirement of rigorous deoxygenation of the system and sample injection. In this report, a relatively simple, rapid, sensitive, accurate and reproducible HPLC method with reductive electrochemical detection for ART and DHA (α and β isomers) in biological fluids is described. Simple sample deoxygenation prior to injection using a modified hypodermic syringe and Rheodyne injector was applied for the system.

2. Experimental

2.1. Chemicals

Standard powder of ART, DHA and ARN were gifts from Arenco (Belgium). All were prepared as 0.5 μg/μl stock solutions in 50% ethanol. Working standard solutions were prepared by diluting the stock standard solution with 50% ethanol to a concentration of 10 ng/μl, and stored as aliquots in 1-ml glass vials. Standard solutions were stored at −70°C until use.

All organic solvents used were of HPLC-grade. Absolute ethanol, and *tert.*-methylbutyl ether were obtained from BDH (Poole, UK); dichloromethane, acetonitrile, ethyl acetate and hexane were obtained from Fisons (Loughborough, UK); *n*-butyl chloride was from Fisher Scientific. Other reagents and solvents (sodium chloride, acetic acid, sodium hydroxide and dimethyldichlorosilane) were of analytical grade quality and supplied by BDH.

2.2. Instrumentation

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

2.3. Chromatography

The separation was carried out on a Waters μBondapak CN column (15 cm×3.9 mm, 10 μm particle size, Waters Associates, USA). The premixed mobile phase consisted of 20% acetonitrile

and 80% water containing 0.1 *M* acetic acid (adjusted to pH 5.0 with sodium hydroxide). 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 1.2 ml/min [back pressure 1800–2200 p.s.i. (1 p.s.i.=6894.76 Pa)]. The system was run continuously with mobile phase recycled back into the reservoir and used for a period of 2 weeks. Detection was performed with a thin-layer dual glassy-carbon electrode run in parallel mode at a potential of −1.0 V versus Ag/AgCl (background currents 70–250 nA). Sensitivity of detector was maintained by a combination of manual (abrasion-based) and electropolishing (−2.0 V versus AgCl for 2 h) techniques. The chromatograms were recorded and analyzed with software provided with the instrument (ChromGraph Report Software).

2.4. Deoxygenated injection

Rigorous sample deoxygenation was done prior to injection [18]. The Rheodyne 7125 valve was used as an integral part of both degassing and injection. The system was fitted with a vent (No.5) which was connected to the helium supply. It was operated in the "injection" position. Condition for a proper deoxygenation was the use of the needle port cleaner (No. 7125-054) and the use of a 1-ml hypodermic syringe with a small hole for letting the helium out at the top end. To deoxygenate, sample was sparged with inert gas as it stood in the syringe in the needle port of the injection valve. In the injection position, valve port No. 5 acted as an inlet for inert gas to flow into the syringe. With the syringe standing vertically in the horizontal injection valve, the sample was sparged as inert gas flowing through the injection port, bubbling up through the sample in the syringe, and then vented out the small hole made in the top of the syringe. After adequate sparging (4–5 min), the injection valve was switched to "load" and the sample loop was filled with deoxygenated sample. The sample was injected by switching from "load" to "injection".

2.5. Sample extraction procedure

In order to minimize drug to glass adsorption, extraction was carried out in 15-ml screw-cap glass test tubes precoated with dimethyldichlorosilane in toluene (5%, v/v). To a 1-ml plasma sample, was added an internal standard ARN (100 ng), followed by vortex-mixing for 30 s. The resultant mixture was extracted twice with 5 ml of extraction solvent (described below) by mechanical tumbling for 10 min. After centrifugation at 1200 *g* for 10 min, 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 isomers of DHA [11]. After rigorous deoxygenation, 20 μl were injected onto the column.

Seven distinct extraction solvents were compared for their extraction efficiencies i.e., dichloromethane, *tert.*-methylbutyl ether, hexane, ethyl acetate, dichloromethane–*tert.*-methylbutyl ether (8:2, v/v), dichloromethane–*tert.*-methylbutyl ether (6:4, v/v), dichloromethane–*tert.*-methylbutyl ether (1:1, v/v), *n*-butyl chloride–ethyl acetate (9:1, v/v).

2.6. Calibration curves

Solutions of ART and DHA in 50% ethanol, ranging from 10 to 640 ng/ml, were injected into the HPLC system in order to assess detector linearity. Peak heights were plotted against the quantities of ART and DHA injected. ART and DHA were linear ($r \geq 0.9999$) in the concentration range observed.

Calibration curves were prepared by triplicate analysis of 1-ml plasma samples spiked with concentrations of DHA and ART in the range 10–640 ng/ml, with a fixed concentration of internal standard ARN (100 ng). Samples were analyzed as described above (Section 2.5), and the peak-height ratios of ART 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. As the ratio of α- and β-DHA in spiked samples was stable, as judged from performing the injection 18 h after reconstitution, and as the

α-DHA was the predominant isomer, quantification of DHA was assessed from only the α-isomer.

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

The analytical recoveries of the extraction procedure for ART, DHA (α plus β) and ARN were estimated by comparing the peak heights obtained from an extracted sample with those measured with equivalent amounts of each compound in 50% ethanol. The concentrations used were 10, 80, 240 and 640 ng/ml for DHA and ART, and 10, 80, 640 ng/ml for ARN.

The precision of the method based on within-day repeatability was determined by replicate analysis of three samples spiked with four different concentrations of ART, DHA and ARN (10, 80, 240 and 640 ng/ml for ART, DHA and 10, 80, 640 ng/ml for ARN). The reproducibility (day-to-day variation) of the method was established using the same concentration range as above, but only a single determination of each concentration was made on three different days. Coefficients of variation (C.V.) were calculated from the ratios of standard deviation (S.D.) to the mean.

Accuracy was determined by replicate analysis of four different levels (10, 80, 240, 640 ng/ml for ART, DHA and 10, 80, 640 ng/ml for ARN) and comparing the difference between spiked value and that actually found.

The stabilitys of each compound was determined by storing plasma standard and working standard solution for 6 months at −70°C. Concentrations were measured periodically (2 weeks, 1, 2, 4 and 6 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) after subjecting them to the extraction procedure.

2.8. Application of the method to biological samples

The method was applied to the investigation of the pharmacokinetics of ART and DHA (α-isomer) in a male Thai volunteer (aged 23, weighing 56 kg) following the administration of a single oral dose of 300 mg ART (Artenam, Arenco; 50 mg per tablet). The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. The subject was fasted overnight. Venous blood samples (3 ml) were collected into sodium heparinized plastic tubes at 0 (pre-dose), 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 done by a model-independent method [19]. 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 elimination plasma concentration–time data, and the terminal phase elimination half-life ($t_{1/2z}$) from the ratio $0.693/\lambda_z$. The area under the plasma concentration–time curve (AUC) was calculated by 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.

3. Results and discussion

A number of HPLC chromatographic systems were studied to optimize the simultaneous, isocratic separation of DHA, ARN and ART. Waters μBondapak CN reversed-phase column was shown to give optimal separation with a 12-min run time. ARN was used as an internal standard. ARN and ART had similar retention times and assay recovery behavior. With the use of this procedure, excellent precision and accuracy were obtained over the range 10–640 ng/ml in plasma samples.

The chromatographic separation of the standard solutions of DHA, ARN, and ART (in 50% ethanol) are shown in Fig. 2a. Fig. 2b–e illustrate typical chromatograms for blank plasma, spiked samples with ART, DHA, ARN and plasma obtained from a healthy Thai male at 30 min post-dosed with 300 mg ART. Endogenous peaks from extracted drug-free plasma did not interfere with drug analysis. The two isomers (α and β) of DHA eluted at 4.6 and 5.9 min,

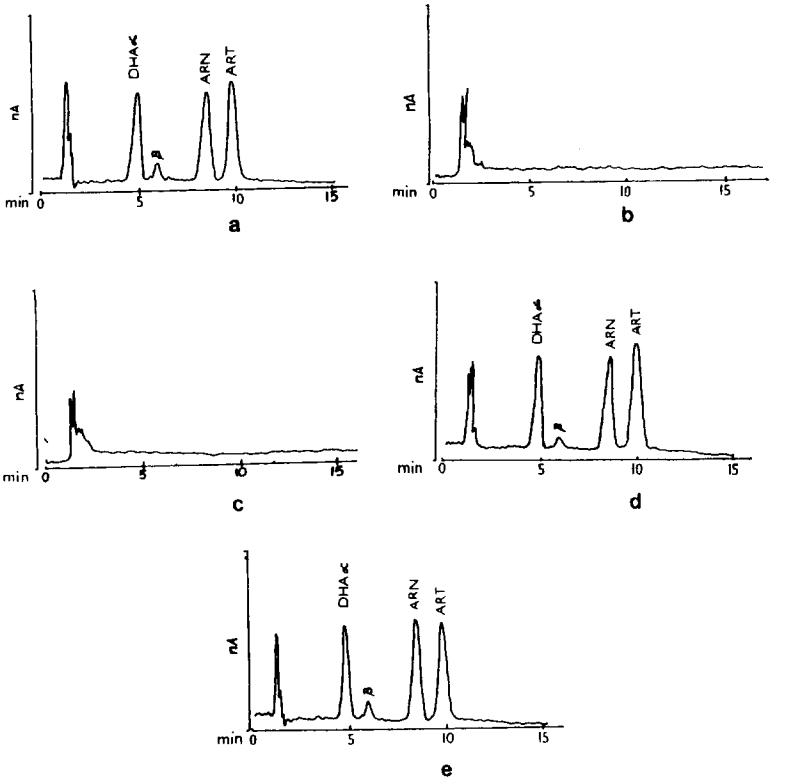

Fig. 2. Chromatograms of (a) standard solutions (100 ng DHA, ARN, ART), (b) blank plasma extracted with dichloromethane–*tert.*-methylbutyl ether (1:1, v/v), (c) blank plasma extracted with *n*-butyl chloride–ethyl acetate (9:1, v/v), (d) spiked plasma (80 ng DHA, ART and 100 ng ARN) and (e) plasma obtained from a healthy male Thai 30 min after a single oral dose of 300 mg artemether [extraction solvent for (d) and (e) was dichloromethane–*tert*-methylbutyl ether (1:1, v/v); retention times for α-DHA, β-DHA, ARN and ART=4.6, 5.9, 7.9, 9.6 min, respectively].

followed by the internal standard ARN (7.9 min) and ART (9.6 min). The method was free from chromatographic interference from endogenous compounds and the commonly used antimalarials (mefloquine, primaquine, chloroquine, pyrimethamine, quinine).

Seven extraction solvents were tested for their extraction efficiency. The mixture of dichloromethane–*tert.*-methylbutyl ether (1:1, v/v) and of *n*-butyl chloride–ethyl acetate (9:1) resulted in a clean extract (Fig. 2b,c). Average recoveries for ART, DHA and ARN in plasma over the concentration range 10–640 ng/ml were 91.6, 93.2 and 86.1%, respectively. The recoveries were similar for the two extraction systems and therefore both extraction systems were employed in the validation of ART, ARN and α-DHA assay in plasma. Stability studies using both extraction solvents showed that DHA, ARN and ART were stable in plasma when samples were stored at −70°C for 6 months.

Calibration curves for ART and DHA were linear over the range of 10–640 ng/ml, with correlation coefficients of 0.9999 or better for both extraction methods. There was very little variation in ART and DHA assays; coefficients of variation in all cases were below 10%. The inter-assay (day-to-day) precision, intra-assay (within-day) precision and the coefficients of variation (C.V.) for ART and DHA at four different concentrations are given in Table 1.

The minimum detectable concentration, defined as a peak 3 times the baseline noise at a sensitivity of −20 nA in a 1-ml plasma sample were 5 and 3 ng/ml, respectively for ART and α-DHA.

To demonstrate the clinical applicability of the

Table 1
Precision, accuracy of the assay ($n=3$ for each point)

Extraction solvent	Drug	Amount added (ng/ml)	Within-day			Day-to-day		
			Amount measured (mean) (ng/ml)	C.V. (%)	Accuracy (%)	Amount measured (mean) (ng/ml)	C.V. (%)	Accuracy (%)
Dichloromethane–*tert.*-methylbutyl ether (1:1, v/v)	ART	10	10.4	8.6	0	10	7.0	+0.1
		80	79.5	2.8	−3.1	80	4.4	−0.2
		240	250	6.8	+3.2	246	5.7	+2.3
		640	640.4	1.6	+0.16	649	1.6	+0.2
	α-DHA	10	10.1	8.9	+4	10.9	1.9	+1.9
		80	81.5	3.8	+1.25	82.6	1.1	+2.7
		240	236	4.2	−2.9	252	3.6	3.1
		640	643	0.6	+0.16	644	1.0	+3.1
	ARN	10	10.7	8.4	+2	10.1	4.0	+0.8
		80	82.9	1.1	+1.25	83.1	1.3	+1.1
		640	645	1.1	+0.78	644	0.1	+1.9
n-Butyl chloride–ethyl acetate (9:1, v/v)	ART	10	10.5	9.5	0	10	8.0	+0.1
		80	80.2	3.9	+1.88	80.3	3.2	+1.9
		240	235	5.1	−1.9	241	2.1	+0.7
		640	644	0.8	+1.87	645.2	1.5	+2.1
	α-DHA	10	11.7	7.7	+2	10.9	8.3	+0.4
		80	80.9	1.4	+4.38	79	1.4	+0.9
		240	243	6.2	+2.31	245	2.9	+0.9
		640	638	1.1	−0.31	638	1.3	−1.8
	ARN	10	8.9	5.6	−6	10.3	6.8	−0.6
		80	78	2.7	−2.5	82	2.3	−1.1
		640	641	1.1	+2.4	645	1.1	+2.4

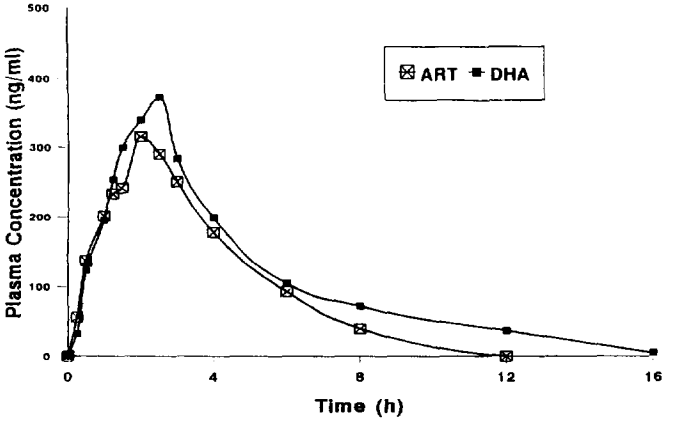

Fig. 3. Plasma concentration–time profiles of ART and α-DHA in healthy male Thai following a single dose of 300 mg ART.

method, the pharmacokinetics of ART and DHA were investigated in a healthy Thai males following an oral administration of 300 mg ART. Plasma concentration–time profiles of DHA and ART are shown in Fig. 3. The concentrations of the parent drug and its major plasma metabolite were measurable up to 12 h. ART was rapidly absorbed. C_{max} of 315 ng/ml was reached at 2 h (t_{max}). AUC, $t_{1/2z}$, V_z/F and Cl/F were 1350 ng h/ml, 1.91 h, 10.2 l/kg and 61.7 ml/min/kg, respectively. The corresponding kinetic parameters for DHA were 373 ng/ml, 2.5 h, 1,790 ng h/ml, 3.31 h, 13.4 l/kg and 10.2 ml/min/kg, respectively.

The analytical method for the determination of ART and DHA described in this paper meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study. The advantage of the method over that previously reported is basically, its high sensitivity and selectivity.

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