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Simultaneous determination of artesunic acid and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies

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Abstract

A selective reproducible high-performance liquid chromatographic assay for the simultaneous quantitative determination of the antimalarial compound artesunic acid (ARS), dihydroartemisinin (DQHS) and artemisinin (QHS), as internal standard, is described. After extraction from plasma, ARS and DQHS were analysed using an Econosil C_8 column and a mobile phase of acetonitrile–0.05 *M* acetic acid (42:58, v/v) adjusted to pH 5.0 and electrochemical detection in the reductive mode. The mean recovery of ARS and DQHS over a concentration range of 50–200 ng/ml was 75.5% and 93.5%, respectively. The within-day coefficients of variation were 4.2–7.4% for ARS and 2.6–4.9% for DQHS. The day-to-day coefficients of variation were 1.6–9.6% and 0.5–8.3%, respectively. The minimum detectable concentration for ARS and DQHS in plasma was 4.0 ng/ml for both compounds. The method was found to be suitable for use in clinical pharmacological studies.

Keywords: Artesunic acid; Dihydroartemesin

1. Introduction

Artesunic acid (dihydroartemisinin 12α–succinate, Fig. 1) (ARS) is one of the water soluble derivatives from the artemisinin family of antimalarial drugs. This group of compounds has proved particularly effective in the treatment of severe and multi-drug resistant malaria caused by *P. falciparum* [1]. Comparative studies of patients with different disease severity have been undertaken. Artemisinin (QHS, Fig. 1) compounds cure malaria more rapidly than all other antimalarial drugs [2]. Recently, ARS has been formulated for oral and rectal administrations and has proven to be rapidly effective and safe [3,4]. There is inadequate data on the comparative pharmacokinetics as well as bioavailability of this compound in the literature. One contributing factor maybe the lack of sufficiently sensitive and reliable assay methods for the compounds [5].

A few chromatographic techniques with UV and electrochemical detections for quantification of ARS have been published [6–8]. However, these methods lack the required sensitivity to be used for measurement of blood samples generated from clinical pharmacokinetic studies. GC-mass spectrometry has been reported to quantitate dihydroartemisinin (DQHS, Fig. 1) in blood samples collected from animals administered with ARS [9]. This approach is not practical for routine pharmacokinetic studies because of expense and complexity of operation and

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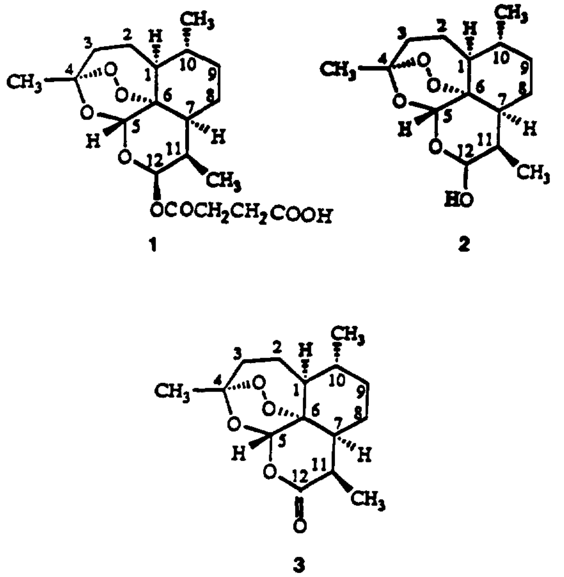

Fig. 1. Structure of artesunic acid (1) dihydroartemisinin (2) and artemisinin (3).

maintenance of these instruments. In addition this method also employs pyrolysis during sample preparation and thus lacks in specificity of compounds analysed. This paper describes a rapid, sensitive and selective chromatographic procedure to quantitate ARS and its principal active metabolite, DQHS and its application to a clinical pharmacokinetic study.

2. Experimental

2.1. Chemicals

QHS (internal standard), DQHS and ARS were obtained from The World Health Organization. The identity of the three compounds has been confirmed by mass spectrometry and infrared spectrometry. All chemicals and solvents used in the assay procedure were of analytical/chromatographic grade. Acetonitrile, acetic acid, ethanol methanol, sodium hydroxide and toluene were purchased from Merck (Darmstadt, Germany) and dichlorodimethylsilane from Sigma (St. Louis, MO, USA). The solid-phase extraction (SPE) cartridges used were Supel Clean C_{18} (Supelco, Bellefonte, PA, USA) with a 1-ml capacity and containing 100 mg sorbent.

2.2. Chromatography

The analytical instrument used was a Model BAS 200A liquid chromatograph with Rheodyne 7125 injector (Bioanalytical Systems, West Lafayette, IN, USA). The instrument was operated in the reductive mode (−1000 mV) as a closed system under chromatography grade helium to exclude any access oxygen at the detectors electrodes. The electrochemical detection (ED) apparatus was equipped with glassy carbon electrodes and an Ag/AgCl reference electrode. When required, polishing of the electrode was carried out by scrubbing the glassy carbon electrode on the pad treated with alumina or diamond polish (Reference Manual BAS, Sections 4–9).

The chromatograms were recorded and analyzed with software provided with the instrument. Chromatographic separations were obtained with Econosil C_8 column 250×4.6-mm I.D., 10-μm particle size (Alltech, Deerfield, Illinois, USA) maintained at 35°C. An Econosil guard cartridge CN 10×4.6-mm I.D., 10-μm particle size (Alltech, Deerfield USA) was used to protect the column. The mobile phase consisted of acetonitrile–0.05 *M* acetic acid (42:58, v/v) adjusted to an apparent pH 5.0 with 1.0 *M* NaOH. The flow-rate was 1.5 ml/min.

2.3. Extraction procedure and sample preparation

In order to minimize eventual drug adsorption, all glassware was silanized before use with dichlorodimethylsilane in toluene (5%, v/v). SPE extraction was carried out using a vacuum manifold (Supelco). Plasma (0.5 ml) and internal standard QHS (10 μl, 10 ng/μl) were diluted to 1 ml with water and loaded onto an SPE cartridge already conditioned with acetonitrile and water (each 1 ml×2). Each cartridge was then washed with 1 ml water. Elution was carried-out with 3 sequential applications of 0.5 ml acetonitrile. The residue was collected into a test tube and dried in a gentle stream of nitrogen at room temperature. The residue was reconstituted in 100 μl of ethanol–water (50:50, v/v) and left for 18 h at 4°C in order to allow stabilization of the ratio of the α and β isomers of DQHS. Immediately after dissolution the proportion of α:β tautomers is close to 1:1 but this ratio increases, to stabilise after 18 h at $\alpha:\beta=4.5:1$ (Table 1). This technique has been employed successfully in the simultaneous determination of artemether and DQHS [10]. Sample was injected onto the column using the modified technique of Lloyd [11]. Essentially a gas-tight syringe containing the reconstituted sample was placed in the injector port and the sample was degassed using purified helium (purity 99.999) for 5 min prior to injection into the HPLC.

2.4. Calibration

Solutions of QHS, ARS and DQHS in ethanol–water (50:50, v/v), ranging from 12.5 to 500 ng/ml were injected into the HPLC–ED apparatus in order to assess detector linearity. Peak height was plotted against the amount of compound injected. QHS, ARS and DQHS were all linear ($r>0.999$) in the range 12.5 to 500 ng/ml. Subsequently, stock solutions of ARS and DQHS as well as internal standard (QHS) were prepared. Calibration curves were obtained by spiking drug-free plasma samples with standard solutions to produce concentrations of 12.5–200 ng/ml of ARS and DQHS. Internal standard (10 μl, 10 ng/μl) was also added. The samples were taken through the extraction and assay procedure and the peak heights were plotted against the corresponding drug concentrations. Linear regression analysis yielded the correlation coefficients $r\geq0.998$ for both DQHS and ARS. The equation of the calibration plots ($n=5$) for ARS was $y=0.00683x-0.02134$ and for DQHS $y=0.00833x-0.04470$.

2.5. Analytical recovery and assay precision

The analytical recoveries of the extraction procedure for ARS and DQHS were determined by comparing the peak heights obtained from plasma samples containing known amounts of the two compounds in the range of 50 to 200 ng/ml with those measured with equivalent amounts of the compounds in ethanol–water, (50:50, v/v). The mean recovery ($n=5$) for ARS and DQHS were 75.5±4.9% with a C.V. of 6.5% and 93.1±1.8% with a C.V. of 1.9%, respectively. With respect to QHS (internal standard) an analytical recovery of 100 ng/ml was determined in five replicates which resulted in a mean recovery of 94.8±4.1% with a C.V. of 4.3%.

Table 1
Ratio of α and β isomers of dihydroartemisinin in ethanol–water (50:50 v/v) at 4°C over time

Time (h)	Ratio of α/β isomers
0	1.1
4	2.0
8	2.7
12	3.5
16	4.1
18	4.5
22	4.5
26	4.4

The within-day precision was determined at three concentrations by replicate assays ($n=5$) of samples from pools of plasma spiked with 30, 60 and 110 ng/ml. The day-to-day assay variation was assessed at concentration of 30, 60, 100 and 200 ng/ml over a period of 5 consecutive days with five replicates at each level (Table 2). The overall values indicate good reproducibility of the assay method. The minimium detectable concentration of ARS and DQHS in plasma corresponding to a peak three times baseline noise at 0.005 a.u.f.s. was 4.0 ng/ml for both.

2.6. Study on a healthy volunteer

A male volunteer age 40 years and weighing 57 kg was selected for the study. The study protocol was approved by the Institutional Ethics Committee. The investigations were carried-out in accordance with the principles laid down by the World Medical Assembly of 1975 on Ethics in Human Experimentation and informed written consent was obtained from the subject. No other drugs or any alcohol were taken 7 days prior to or during the clinical trial. The subject was given 200 mg of ARS tablet (Plasmotrim^R-Lactab^R, Mepha, Basle, Switzerland) with 150 ml of water following an overnight fast. A normal breakfast was served 3 h later. Venous blood samples (10 ml) were taken pre-dose, then after 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0 and 72.0 h. Blood was collected in heparinized vacutainers and then was centrifuged (2073*g* for 20

Table 2
Within-day ($n=5$) and day-to-day ($n=5$) variation of assay for artesunic acid (ARS) and dihydroartemisinin (DQHS)

Compound	Concentration spiked (ng/ml)	Concentration measured (mean±S.D.) (ng/ml)	Coefficient of variation (%)
Within-day variation			
ARS	30	33.5±1.4	4.2
	60	64.8±4.8	7.4
	110	112.2±3.8	3.4
DQHS	30	35.0±0.9	2.6
	60	63.7±3.1	4.9
	110	105.4±2.9	2.8
Day-to-day variation			
ARS	30	32.3±1.0	3.1
	60	63.8±6.1	9.6
	100	99.8±8.0	8.0
	200	200.0±3.2	1.6
DQHS	30	32.6±2.7	8.3
	60	62.9±3.7	5.9
	100	94.6±6.5	6.9
	200	200.3±1.1	0.5

min) and the plasma was removed and stored at −70°C until analysis.

2.7. Pharmacokinetic analysis

The elimination half life was calculated by regression analysis of the log-linear portion of the plasma concentration versus time curve. The area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal rule. Other pharmacokinetic parameters (plasma clearance and apparent volume of distribution) were calculated using standard model-independent formulae. Maximum concentration and time to reach maximum concentration are the observed values.

3. Results and discussion

The above described analytical method for the simultaneous determination of ARS and DQHS meets the criteria for use in clinical pharmacological studies. The solid-phase extraction procedure is simple when compared to the differential extraction approach reported by Zhou et al. [6]. The minimum detectable concentration of ARS and DQHS in plasma was 4.0 ng/ml for both compounds. The assay method developed is superior in terms of sensitivity and simplicity to that of Edlund et al. employing base hydrolysis after HPLC separation of the analytes [8]. In addition our method permits specific quantification of ARS and DQHS in plasma which is needed in conducting pharmacokinetic studies. This is the major limitation to GC/MS approach reported by Theoharides et al. [9] as quantification of DQHS was based on its pyrolysis product. The analytical method reported here requires meticulous operation as chromatographic resolution and sensitivity depend on oxygen-free conditions in the mobile phase and samples. However, once the technique is established, the method is relatively fast and easy. The need for maintaining the system in a closed helium-atmosphere is a financial and in some areas, also a logistic constraint, but none of the more economic methods has so far achieved the degree of selectivity, sensitivity and reproducibility that is required for clinical pharmacokinetic studies. Fig. 2 illustrates the chromatograms obtained typically from a standard mixture (a), from a healthy volunteer having received 200 mg of oral ARS (b), and from drug-free plasma (c). The method yields clean chromatograms, with baseline resolution of ARS, DQHS and the internal standard at the retention times of 6.6, 8.7 and 17.7 min, respectively.

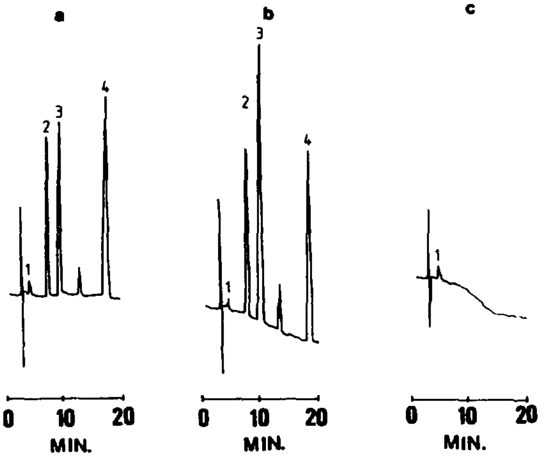

Fig. 2. Typical chromatogram showing (a) a standard mixture of artesunic acid (100 ng), dihydroartemisinin (100 ng) and the internal standard (100 ng), (b) plasma obtained from a healthy volunteer following oral administration of 200 mg artesunic acid showing levels of artesunic acid (106.0 ng/ml) and dihydroartemisinin (172.8 ng/ml) and (c) extraction of drug-free plasma. Peaks: 1=oxygen peak, 2=artesunic acid, 3=dihydroartemisinin, 4=artemisinin (internal standard).

The validated method for plasma was used to study the pharmacokinetics of ARS in a healthy volunteer following a single oral 200 mg dose of the drug. The plasma concentration–time profile over the period 0–6 h is presented in Fig. 3. Time lapse until detectability for both ARS and DQHS was 0.25 h. Plasma concentrations were measurable up to 3 h for ARS and 6 h for DQHS. The maximum blood concentration of ARS (C_{max}) of 577.2 ng/ml was reached 0.5 h post-dose, and the $AUC_{0-\infty}$ was 312 ng h/ml. The plasma clearance (*Cl*), apparent volume of distribution (V_d) and elimination half life ($t_{1/2}$) were 187.7 ml/min per kg, 6.4 l/kg and 0.39 h respectively. With respect to DQHS, the maximum blood concentration (C_{max}) of 1141.4 ng/ml was reached 1 h post-dose, and the AUC_{0-t} was 1340 ng h/ml. The above described analytical method for the quantification of ARS and DQHS meets the criteria for use in clinical pharmacokinetic studies.

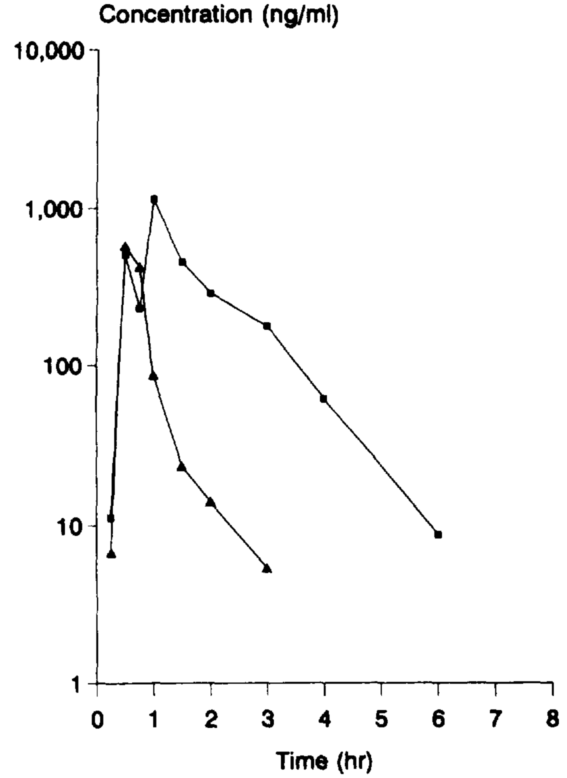

Fig. 3. Plasma concentrations of artesunic acid (▲) and dihydroartemisinin (■) in a healthy Malaysian having received a single oral dose of 200 mg artesunic acid.

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References

[1] China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, J. Tradit. Chin. Med., 2 (1982) 45.
[2] T.T. Hien and N.J. White, Lancet, 341 (1993) 603.
[3] S. Looareesuwan, P. Wilairatana, S. Vanijanonta and C. Viravan, Ann. Trop. Med. Hyg., 89 (1995) 469.
[4] S. Looareesuwan, Trans. R. Soc. Trop. Med. Hyg., 88 (Suppl. 1) (1994) 9.
[5] G. Edwards, Trans. R. Soc. Trop. Med. Hyg., 88 (Suppl. 1) (1994) 37.

[6] S.M. Zhou, J.C. Anders, H. Chung and A.D. Theoharides, J. Chromatogr., 414 (1987) 77.

[7] S.D. Yang, J.M. Ma, J.H. Sun and Z.Y. Sang, Acta Pharm. Sin., 20 (1985) 457.

[8] P.O. Edlund, D. Westerlund, J. Carlquisk, W. Bo-Liang and J. Yunhua, Acta Pharm. Suec., 21 (1984) 223.

[9] A.D. Theoharides, M.H. Smyth, R.W. Ashmore, J.M. Halverson, Z.M. Zhou, W.E. Ridder and A.J. Lin, Anal. Chem., 60 (1988) 115.

[10] V. Navaratnam, S.M. Mansor, L.K. Chin, M.N. Mordi, M. Asokan and N.K. Nair, J. Chromatogr., 669 (1995) 289.

[11] J.B.E. Lloyd, J. Chromatogr., 256 (1983) 323.