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Journal of Chromatography B, 669 (1995) 289–294

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of artemether and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies

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First received 17 November 1994; revised manuscript received 24 February 1995; accepted 24 February 1995

Abstract

A selective reproducible high-performance liquid chromatographic assay for the simultaneous quantitative determination of the antimalarial compound artemether (ARM), dihydroartemisinin (DQHS) and artemisinin (QHS), as internal standard, is described. After extraction from plasma, ARM and DQHS were analysed using a Lichrocart/Lichrosphere 100 CN stainless-steel column and a mobile phase of acetonitrile–0.05 *M* acetic acid (15:85, v/v) adjusted to pH 5.0, and electrochemical detection in the reductive mode. The mean recovery of ARM and DQHS over a concentration range of 30–120 ng/ml was 81.6% and 93.4%, respectively. The within-day coefficients of variation were 0.89–7.01% for ARM and 3.45–8.11% for DQHS. The day-to-day coefficients of variation were 2.06–8.43% and 3.22–6.33%, respectively. The minimum detectable concentration for ARM and DQHS in plasma was 2.5 and 1.25 ng/ml for both compounds. The method was found to be suitable for use in clinical pharmacological studies.

1. Introduction

The occurrence and spread of resistance to the classical antimalarial drugs in *Plasmodium falciparum* has stimulated the search for alternative medicaments [1]. In this endeavour Chinese scientists isolated and characterized an antimalarial compound from *Artemisia annua* L., a composite plant that was used in traditional medicine against malarial fevers [2]. This compound, qinghaosu or artemisinin (QHS) (Fig. 1), was found to possess high antiplasmodial activity in animal models and the treatment of human malaria, but difficulties were experienced in formulating it for reliable oral or parenteral administration. Semi-synthetic derivatives such as artemether (ARM) (Fig. 1), the methyl ether of QHS, or sodium artesunate, the sodium succinyl ester of QHS, permitted the formulation of parenteral dosage forms [2]. These derivatives have lately also been formulated for oral use [3]. The first metabolite of QHS and its derivatives, dihydroartemisinin (DQHS), is known to exert the highest antimalarial activity in this class.

There is a multitude of different dosage schedules for the QHS-based drugs. This is largely due to the lack of pharmacokinetic information as there is a dearth of sufficiently sensitive and reliable assay methods for these compounds. Recently, the measurement of QHS

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SSDI 0378-4347(94)00109-3

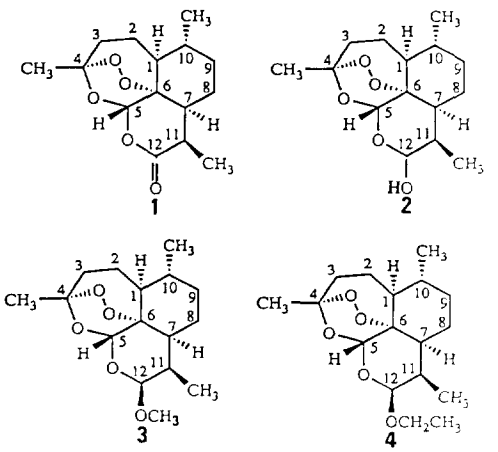

Fig. 1. Structure of artemisinin (1), dihydroartemisinin (2), artemether (3) and arteether (4).

and its derivatives in biological fluids has been reviewed [4]. A sensitive assay method has been described for arteether and DQHS, using HPLC with electrochemical detection (ED) in the reductive mode [5]. This report describes the development of a selective and sensitive method for the simultaneous determination of ARM and DQHS in blood plasma. The assay has been applied for the measurement of ARM and DQHS concentrations in plasma samples obtained from a pilot study involving two healthy Malaysian volunteers.

2. Experimental

2.1. Chemicals

QHS (internal standard) and ARM were obtained from Prof. W.H. Wernsdorfer, Institute for Specific Prophylaxis and Tropical Medicine, University of Vienna, Vienna, Austria. DQHS has been obtained by reduction from QHS, using the method of Brossi et al. [6]. 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, 1-chlorobutane, isooctane, methanol, sodium hydroxide and toluene were purchased from Merck (Darmstadt, Germany), sodium chloride was obtained from Prolabo (Paris, France), ethanol from James Burrough (Witham, UK) and dichlorodimethylsilane from Sigma (St. Louis, MO, USA).

2.2. Chromatography

The analytical instrument used was a Model BAS 200A liquid chromatograph with Rheodyne 7125 injector, coupled to an electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA). The instrument was operated in the reductive mode as a closed system under chromatography grade helium to exclude any access oxygen at the detector's electrodes. The ED apparatus was equipped with glassy carbon electrodes and an Ag/AgCl reference electrode. When required, electropolishing and wiping of the electrode was carried out according to the manufacturer's instructions (Reference Manual BAS, Sections 4–9). The chromatograms were recorded and analyzed with software provided with the instrument. Chromatographic separations were obtained with a Lichrocart/Lichrosphere 100 CN stainless-steel column 250 × 4.0 mm I.D., 5 μm particle size (Merck) maintained at 30°C. The mobile phase consisted of acetonitrile–0.05 *M* acetic acid (15:85, v/v) adjusted to 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, extraction was carried out in 15-ml glass test tubes pretreated with dichlorodimethylsilane in toluene (5%, v/v). The internal standard QHS (10 μl, 10 ng/μl) was added to 1 ml of plasma, followed by vortex-mixing for 30 s. Saturated sodium chloride solution, 250 μl, was added and the mixture vortex-mixed for 5 s. Then, 5 ml of the extracting solvent, isooctane-1-chlorobutane (45:55, v/v), were added, followed by vortex-mixing for 3 min. The sample was then centrifuged at 1440 *g* for 15 min, and the organic layer transferred to another tube and dried in a

gentle stream of nitrogen at 45°C. The residue was reconstituted in 50 μl of ethanol–water (50:50, v/v) and left for 18 h at 4°C in order to allow stabilisation of the ratio of the α and β isomers of DQHS. After deoxygenation, 20 μl were injected in the column, using the technique of Lloyd [7].

2.4. Calibration

Solutions of QHS, ARM and DQHS in ethanol–water (50:50, v/v), ranging from 6.25 to 100 ng/ml and 75 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, ARM and DQHS were all linear ($r > 0.999$) in the range 6.25 to 100 ng/ml and 75 to 500 ng/ml. Subsequently, stock solutions of ARM 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 15–240 ng/ml of ARM 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 $r = 0.99983$ for DQHS and $r = 0.99962$ for ARM. The equation of the calibration plots ($n = 5$) for ARM was $y = 0.008x - 0.0102$ and for DQHS $y = 0.0058x + 0.0045$.

2.5. Analytical recovery and assay precision

The analytical recoveries of the extraction procedure for ARM and DQHS were determined by comparing the peak heights obtained from plasma samples containing known amounts of the two compounds in the range of 30 to 120 ng/ml with those measured with equivalent amounts of the compounds in ethanol–water, (50:50, v/v). The mean recovery for ARM and DQHS were 81.6 ± 4.7% with a C.V. of 5.8%, and 93.4 ± 6.0% with a C.V. of 6.4%, respectively.

The within-day precision was determined at 5 concentrations by replicate assays of samples from pools of plasma spiked with 15, 30, 60, 120 and 240 ng/ml. The day-to-day assay variation was assessed at 3 concentrations over a period of 4 consecutive days at the same concentration range with 3 replicates at each level (Table 1). As to be expected the C.V. values for within-day and day-to-day variation were highest at the lower concentrations. The overall values indicate good reproducibility of the assay method. The minimum detectable concentrations of ARM and DQHS corresponding to a peak three times baseline noise at 0.005 a.u.f.s. were 2.5 and 1.25 ng/ml, respectively.

2.6. Tautomerism of DQHS

As earlier observed by Melendez et al. [5], DQHS shows tautomerism. Immediately after dissolution, the proportion of the α:β tautomers is close to 1:1, but dissolved in ethanol–water

Table 1
Within-day, and day-to-day variation of assay for artemether and dihydroartemisinin

Compound	Concentration added (ng/ml)	Concentration measured (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Within-day variation			
ARM	15	14.32 ± 0.85	5.93
	30	28.67 ± 2.01	7.01
	60	62.10 ± 1.04	1.67
	120	121.91 ± 1.81	1.48
	240	238.54 ± 2.11	0.89
DQHS	15	13.69 ± 1.11	8.11
	30	29.15 ± 1.55	5.33
	60	61.75 ± 1.78	2.89
	120	121.29 ± 5.79	4.77
	240	239.08 ± 8.24	3.45
Day-to-day variation			
ARM	30	30.55 ± 2.57	8.43
	60	61.63 ± 2.39	3.87
	120	119.12 ± 2.46	2.06
DQHS	30	29.54 ± 1.87	6.33
	60	60.92 ± 3.62	5.93
	120	121.90 ± 3.93	3.22

(50:50, v/v), the α:β ratio increases, to stabilise after about 18 h at $\alpha:\beta = 4.5:1$. In order to achieve uniform assay results for DQHS it was decided to run the assays at a time when the ratio is stabilised, i.e. after holding the reconstituted extracted residue for 18 h prior to injection.

2.7. Study in healthy volunteers

Two male volunteers age 28 and 29 years and both weighing 48 kg were 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 subjects. No other drugs or any alcohol were taken 7 days prior to or during the clinical trial. The subjects were given either 120 mg or 240 mg of ARM 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, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.0, 5.0, 8.0, 12.0, 24.0, 36.0, 48.0 and 72.0 h. Blood was centrifuged (2073 *g* for 20 min) and the plasma was removed and stored at −70°C. Analysis was done 24 h after the last blood sample was taken.

2.8. Pharmacokinetic analysis

Data in the text are presented as mean ± S.D. values. 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 determination of ARM and DQHS meets the criteria for use in clinical pharmacological studies. The extraction procedure is simple and economical when compared to that reported by Thomas et al. [8]. In addition, our method can quantitate ARM and DQHS in plasma down to a concentration of 2.5 and 1.25 ng/ml for both compounds, respectively. Therefore the assay method developed is more sensitive to that of Muhia et al. [9] to determine ARM and DQHS in plasma samples. The use of the HPLC–ED system in the reductive mode required meticulous operation as chromatographic resolution and sensitivity depend on oxygen-free conditions. However, once the routine is acquired, the method is relatively fast. 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 sensitivity and reproducibility that is required for pharmacokinetic investigations. Fig. 2 illustrates the chromatograms obtained typically from drug-free plasma (A), a standard mixture (B), and from a healthy volunteer having received 240 mg of ARM by the oral route (C). The method yields clean chromatograms, with baseline resolution of DQHS, the internal standard and ARM, at the retention times of 8.2, 16.1 and 18.2 min, respectively.

The validated method for plasma was used to study the pharmacokinetics of ARM in two healthy volunteers after a single oral 120 or 240 mg dose of the drug. The corresponding plasma concentration–time profiles over the period of 0–8 h are shown in Figs. 3 and 4. Plasma concentrations of ARM and DQHS were measurable in both subjects up to 8 h of the study. In the first subject, who received 120 mg of ARM, the maximum blood concentration of DQHS (C_{max}) of 570.7 ng/ml was reached 1.75 h post-dose, and the AUC_{0-t} was 747.4 ng h/ml. The maximum blood concentration of ARM (C_{max}) of 572.7 ng/ml was reached 1.75 h post

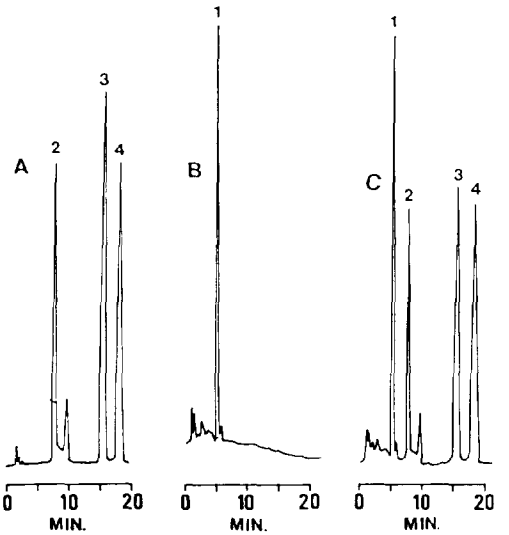

Fig. 2. Chromatograms of (A) extraction of drug-free plasma, (B) a standard mixture of artemether (200 ng) dihydroartemisinin (200 ng) and the internal standard (100 ng), and (C) plasma obtained from a healthy volunteer following oral administration of 240 mg artemether showing levels of artemether (195.4 ng/ml) and dihydroartemisinin (289.0 ng/ml). Peaks: 1 = plasma peak, 2 = dihydroartemisinin, 3 = artemisinin (internal standard), 4 = artemether.

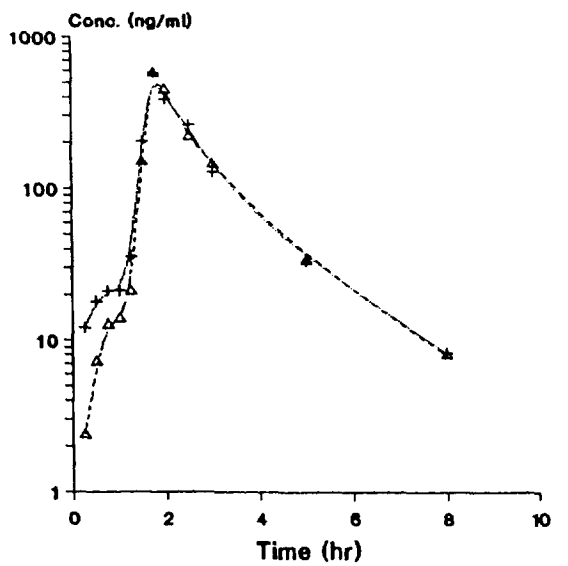

Fig. 3. Plasma concentrations of (+) artemether (ARM) and (△) dihydroartemisinin (DQHS) in a healthy Malaysian volunteer following the oral administration of a single dose (120 mg) of artemether.

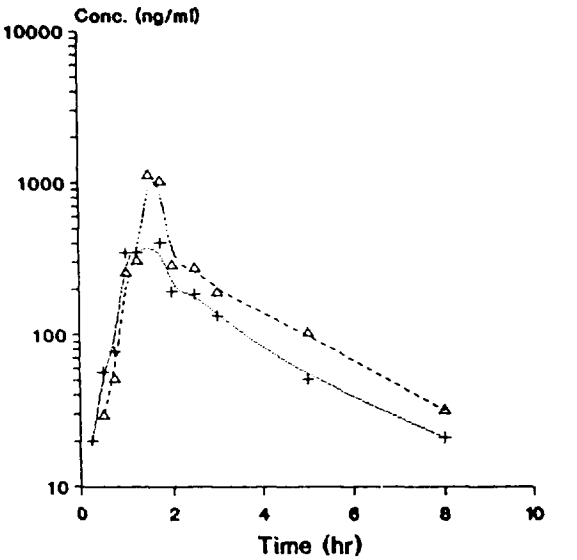

Fig. 4. Plasma concentrations of (+) artemether (ARM) and (△) dihydroartemisinin (DQHS) in a healthy Malaysian volunteer following the oral administration of a single dose (240 mg) of artemether.

dose, and the $AUC_{0-\infty}$ was 765.7 ng h/ml. The pharmacokinetic parameters of ARM in this subject such as the plasma clearance (Cl), apparent volume of distribution (V_d) and elimination half-life ($t_{1/2}$) were 54.4 ml/min/kg, 5.1 l/kg and 1.1 h, respectively. With respect to the second subject who received 240 mg of ARM, the metabolite DQHS reached a C_{max} (1127.0 ng/ml) at 1.5 h post-dose, with an AUC_{0-t} of 1430.1 ng h/ml. ARM was found in blood 0.25 h post-dose with a maximum concentration (408.2 ng/ml) and at 1.75 h post dose with $AUC_{0-\infty}$ 960.7 ng h/ml. Cl, V_d and $t_{1/2}$ values of ARM in this subject were 86.7 ml/min/kg, 13.3 l/kg and 1.8 h. The above described analytical method for the determination of ARM and DQHS fulfils all the criteria required for an assay to be suitable for clinical pharmacokinetic studies.

Acknowledgements

The authors are grateful to Prof. W.H. Wernsdorfer for his review of this paper.

References

[1] World Health Organization. Advances in Malaria Chemotherapy, WHO Tech. Rep. Ser. No. 711, WHO, Geneva, 1984.
[2] China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials. J. Tradit. Chin. Med., 2 (1982) 45.
[3] World Health Organization. Practical Chemotherapy of Malaria, WHO Tech. Rep. Ser. No. 805, WHO, Geneva, 1990.
[4] G. Edwards, Trans. R. Soc. Trop. Med. Hyg., 88 (Suppl. 1) (1994) 37.
[5] V. Melendez, J.O. Peggins, T.G. Brewer and A.D. Theorides, J. Pharm. Sci., 80 (1991) 132.
[6] A. Brossi, B. Venugopalan, L.D. Gerpe, H.J.C. Yeh, J.L. Flippen-Anderson, P. Buchs, X.D. Luo and W. Peters, J. Med. Chem., 31 (1988) 645.
[7] J.B.E. Lloyd, J. Chromatogr., 256 (1983) 323.
[8] C.G. Thomas, S.A. Ward and G. Edwards, J. Chromatogr., 583 (1992) 131.
[9] D.K. Muhia, E.K. Mberu and W.M. Watkins, J. Chromatogr. B, 660 (1994) 196.