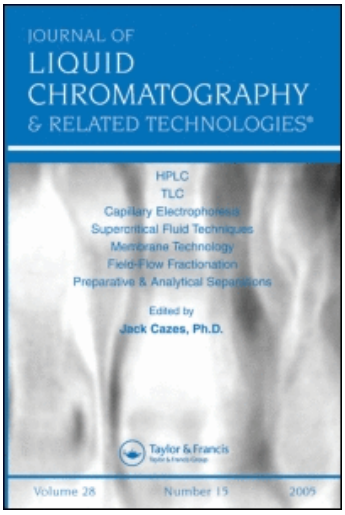

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HPLC with Polarographic Detection of Artemisinin and Its Derivatives and Application of the Method to the Pharmacokinetic Study of Artemether

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HPLC WITH POLAROGRAPHIC DETECTION OF ARTEMISININ AND ITS DERIVATIVES AND APPLICATION OF THE METHOD TO THE PHARMACOKINETIC STUDY OF ARTEMETHER

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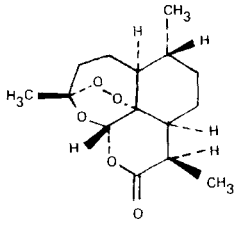
Introduction

Artemisinin (I) is a new sesquiterpene lactone containing a peroxid bridge and has been shown to have remarkable curative effectiveness in various malarias [1]. Its derivatives such as dihydroartemisinin (II), artemether (III) and artesunate (IV) are even more effective than artemisinin itself [2]. It is known that these drugs can be prepared in supporsitories or injection solutions for rectal, intramuscular or intraveinous administrations, respectively, with the advantages of small dosage and high curative effect [3].



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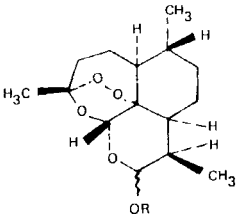

II III IV

(II) R=H
(III) R=CH_3
(IV) R=$COCH_2CH_2COOH$

In order to optimize the clinic uses of these drugs, it is necessary to investigate their pharmacokinetic characteristics. For this, methods for the separations and determinations of both the prototype drugs and their metabolites in blood samples are to be established. Artemisinin and its derivatives contain all a endoperoxide and showed high response on a polarographic detector. Polarographic detector is coming into widespread use in high-performance liquid chromatography [4 - 6]. This paper describes a high-performance liquid chromatographic method with polarographic detecion for the determination of artemisinin and its derivatives and its application to the pharmacokinetic study of the derivative, artemether, in rats and human beings.

Part One

Establishment of the Analytical Method

Experimental

Instrumentation — The instruments used in this work included a HITACHI 635 model high-performance liquid chromatograph, a PAR 310 model polarographic detector, a Model 364 polarographic analyser, a Model 303 static mercury drop electrod and a HITACHI two-pen recorder. a 4 mm i.d. X 15 cm and a 4 mm i.d. X 25 cm

stailess steel columns packed with YWG-$C_{18}H_{37}$ (10 μm) in the author's laboratory were applied.

Standard Materials and Chemicals — Methanol of analytical reagent grad, ammonium sulfate of supper-purity grad and mercury of chemical reagent grad were all obtained from Beijing Chemical Factory (China). The mercury was filtered prior to use. High-purity nitrogen gas was purchased from Beijing Oxygen Gas Plant (China).

Artemisinin and dihydroartemisinin were supplied by Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine (China), and artemether and artesunate by Kunming Pharmaceutical Factory and Guilin Pharmaceutical Factory respectively.

Stock solutions of 1 mg/ml were prepared with methanol as solvent and then stored in a refrigerator. Before use, they were diluted to desired concentrations with methanol.

Deionised, double-distilled water was used for all experiments.

Chromatographic Operation — The mobile phase was deoxidised by boiling under reflux for 1 to 2 hours while purging with nitrogen which had passed in through two wash bottles containing VCl_3-solution to remove the trace oxygen in itself. Samples were purged with a substream of the deoxidised nitrogen for 5 to 10 minutes on line before injection. Injection was performed automatically by forcing the sample solution into a sample loop under the pressure of the nitrogen substream with the aid of a switching valve. Other chromatographic conditions will be given in the related figures and contexts.

Results and Discussion

Dynamic E - I Characteristics of Artemisinin and Its Derivatives — The dynamic E - I characteristical curves of

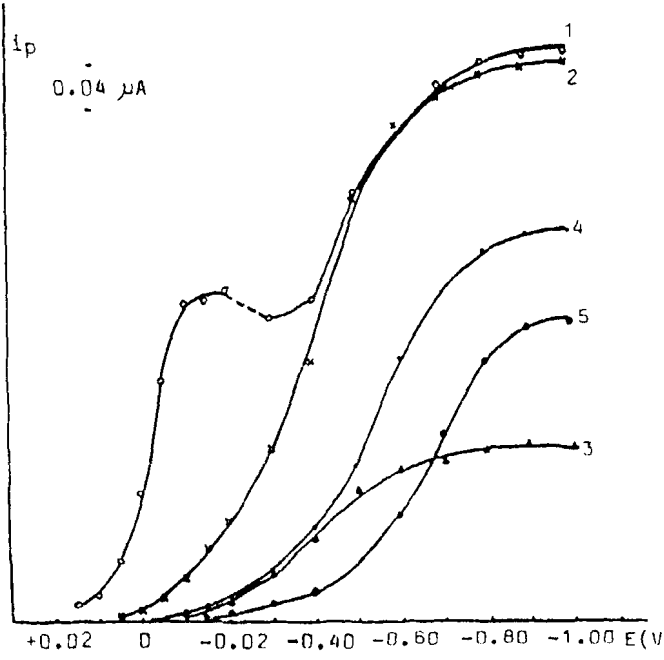

Figure 1. Dynamic E ~ I characteristics of artemisinin aand its derivatives

mobile phase, methanol : 0.02 M ammonium sulfate solution = 75:25; flow rate, 1 ml/min; 1 — artesunate; 2 — α-dihydroartemisinin; 3 — β-dihydroartemisinin; 4 — artemisinin; 5 — artemether

artemisinin and its derivatives under given conditions are illustrated in figure 1. It can be seen in this figure that artemisinin, dihydroartemisinin, artemether and artesunate have their sticking potentials at about -0.90 Volt v.s. Ag/AgCl. Therefore, -0.90 Volt was selected to be the working potential, while the polarographic analyser operated in the mode of sampled

direct-current (SDC). Experimental observations indicated that the operation mode of differential puls (DP) showed greater background current than SDC did and that the former mode was not advanced over the latter one in detection sensitivity on the flow-through polarographic detector.

Selection of the Separation Conditions — Both the ratio between methanol and ammonium sulfate solution and the pH value of the mobile phase affected the retention times of artemisinin and its derivatives to different extents (see figures 2 and 3).

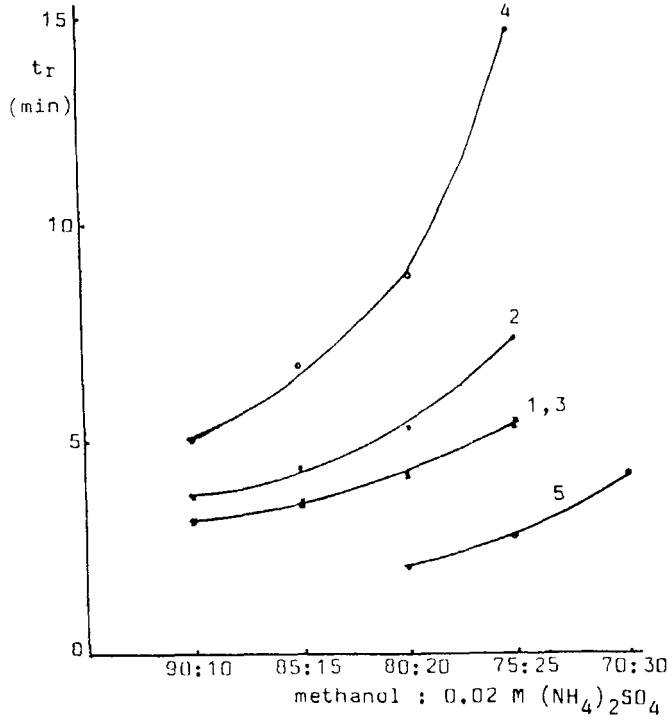

Figure 2. Dependance of retention time on the composition of mobile phase

1 — α-dihydroartemisinin; 2 — β-dihydroartemisinin; 3 — artemisinin; 4 — artemether; 5 — artesunate

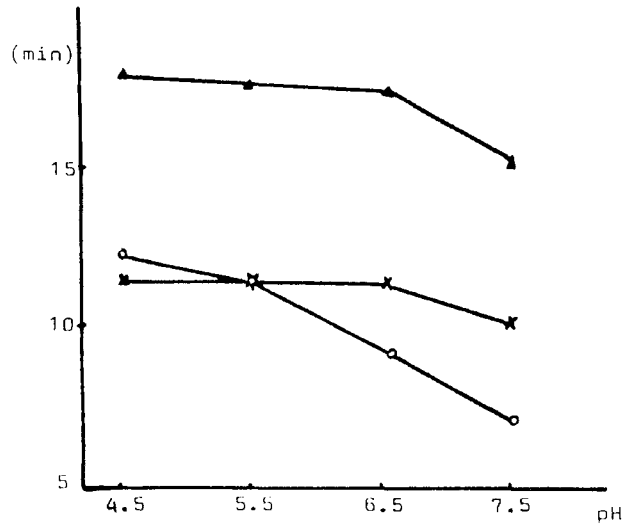

Figure 3. Dependance of retention time on the pH value of mobile phase

mobile phase, methanol : 0.02 M ammonium sulfate solution = 75:25, adjusted to desired pH values with tetrabutylammonium hydroxide; 1 — artesunate; 2 — α-dihydroartemisinin; 3 — β-dihydroartemisinin

It can be seen in these figures that good separation of both artemisinin and dihydroartemisinin from artemether can be achieved with a mixture of methanol and 0.02 M ammonium sulfate solution having the volume ratio of 8:2 as mobile phase, while artesunate can be easily separated from artemisinin and dihydroartemisinin with a mixture of methanol and 0.02 M ammonium sulfate (75:25) as mobile phase. However, the pH value of the mobile phase should be adjusted to 7.5 with tetrabutyl-ammonium hydroxide.

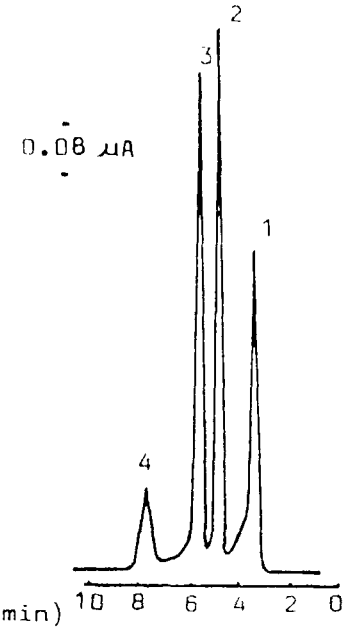

Figure 4. HPLC-chromatogram of a mixture of artesunate and dihydroartemisinin

mobile phase, methanol : 0.02 M ammonium sulfate solution = 75:25, pH = 7.5; flow rate, 1 ml/min; potential setting, - 0.9 Volt v.s. Ag/AgCl; 1 — Oxygen peak; 2 — artesunate; 3 — α-dihydroartemisinin; 4 — β-dihydroartemisinin

In accordance with the conditions in clinical analysis, two prepared samples, one contained artesunate and dihydroartemisinin and the other contained artemether and artemisinin, were chromatographed. The two components in the latter prepared sample can be utilized as internal standard of each other. The obtained chromatograms are shown in fig. 4 and 5, respectively.

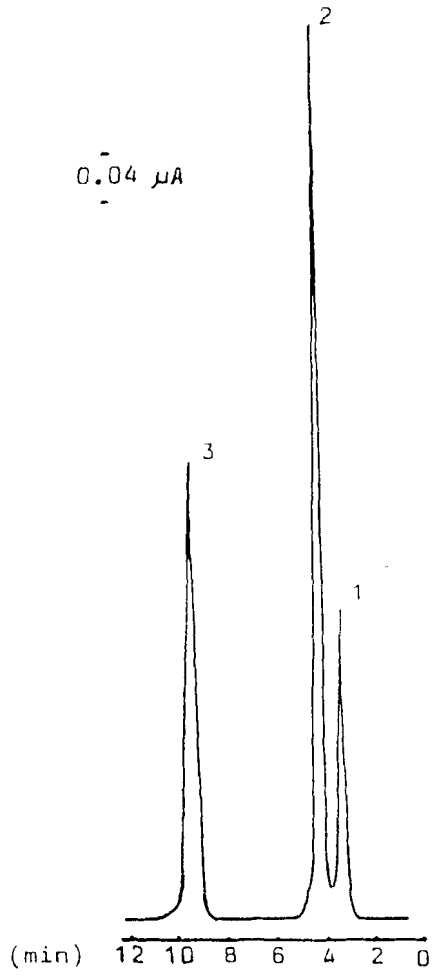

Figure 5. HPLC-chromatogram of a mixture of artemisinin and artemether

mobile phase, methanol : 0.02 M ammonium sulfate solution = 80:20; flow rate, 1 ml/min; potential setting, - 0.9 Volt v.s. Ag/AgCl; 1 — oxygen; 2 — artemisinin; 3 — artemether

Linearity and Precision — The peroxide bridge, -O-O-, is an electrochemically active functional group which can be easily reduced at a mercury cathode. On the basis of this, its polarographic detection is rather highly selective and specific. The relative standard deviation of ten replicate measurements of a standard solution was less than 7% for artemisinin and dihydroartemisinin (α and β species) and less than 8% for artesunate and artemether, respectively. The external standard calibration graphs of artesunate and dihydroartemisinin are shown in figure 6. They show good linearity over the mass range from 10 ng to 1.6 μg. Artemether was assaied by both internal and external methods. The calibration curves are depicted in figures 7 and 8. The linearity is satisfactory, too, over the range of 10 to 120 ng.

Taking peak height in milimeter as function y and injected amount as variable x respectively, linear regression treatment of the experimental data by the least square method yielded the regression equations, namely the best straight lines, listed in table I.

Table I. The Best Straight Lines Fit to the Calibration Curves

Analyte	Range ng	Regression Equation	Correlation coefficient
Artesunate	100-1600	y=0.137x - 6.96	0.999
	10- 160	y=0.428x + 2.33	0.998
α-Dihydroartemisinin	100-1600	y=0.095x - 9.58	0.999
	10- 160	y=0.373x + 3.13	0.997
β-Dihydroartemisinin	100-1600	y=0.018x - 0.188	0.999
	10- 160	y=0.059x + 0.199	0.997
Artemether	10- 120	y=0.012x - 0.017	0.999

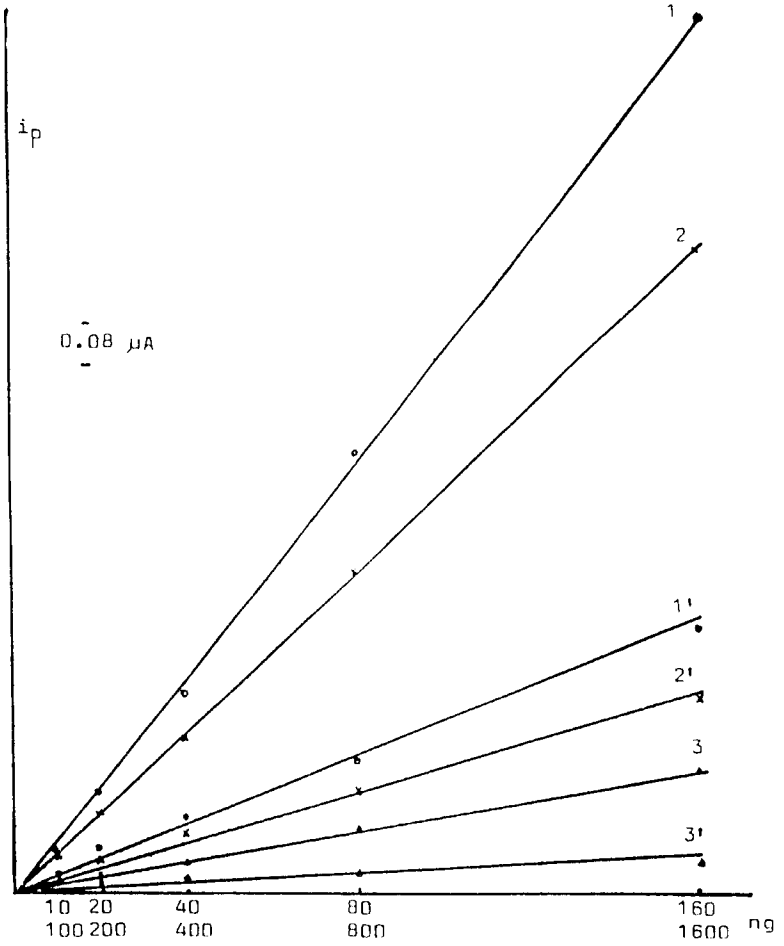

Figure 6. Calibration curves of artesunate and dihydroartemisinin in external standard method

mobile phase, methanol : 0.02 M ammonium sulfate solution = 75:25 , pH = 7.5; flow rate, 1 ml/min;

1, 1' — artesunate; 2, 2' — α-dihydroartemisinin;

3, 3' — β-dihydroartemisinin

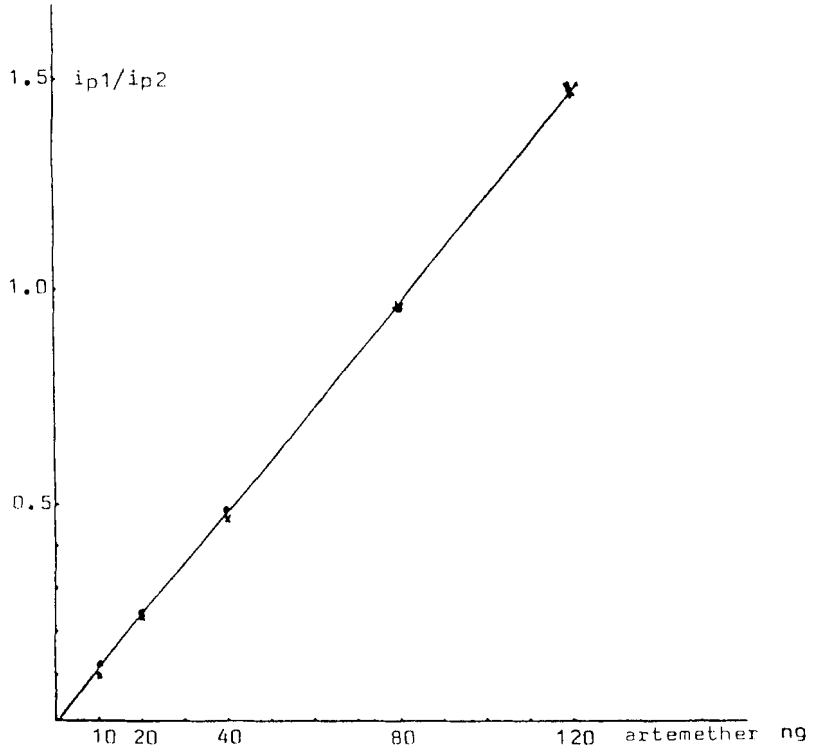

Figure 7. Internal standard calibration graph of artemether mobile phase, methanol : 0.02 M ammonium sulfate solution = 80:20; flow rate, 1.2 ml/min; potential setting, - 0.90 Volt v.s. Ag/AgCl; o——o, standard solutions; x——x, extracted blood samples of 1 ml which were spiked with artemether standard.

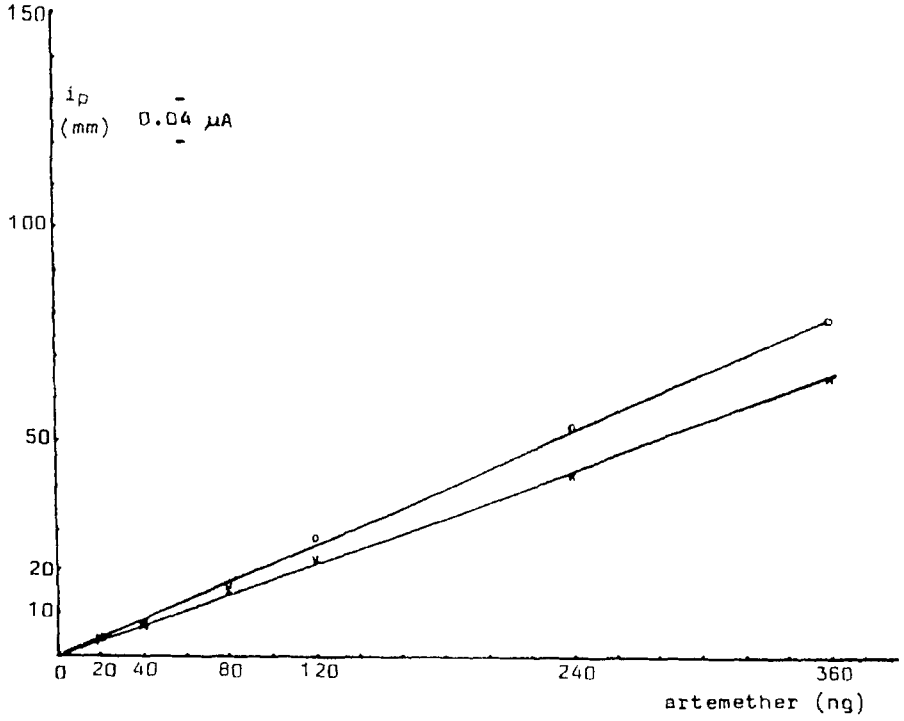

Figure 8. External standard calibration curves of artemether

o—o standard solutions of artemether prepared in in methanol-water mixture (1:1 V/V); x—x blank plasma samples which were spiked with different amounts of artemether standard and extracted as described in the text

mobile phase, methanol : 0.03 M ammonium sulfate solution = 87:13; flow rate, 1 ml/min; mode, sampled direct-current; working electrode, hanging mercury drop electrode; potential setting, -0.90 Volt v.s. Ag/AgCl

Part Two

Pharmacokinetics of Artemether in Rats and Human Beings

Experimental

Artemether Preparations and Chemicals — The injection solutions of artemether used were Injectio Artemetheri produced by Kunming Pharmaceutical Factory.

All chemicals have been given in part one.

Animal Experiment — 4 male Wistar rats weighing 469 ± 21 g were used as the subjects. Before the experiments they had been fasted overnight. Each rat was given a single dose containing artemether at 80 mg/kg by im. injection of the preparation, Injectio Artemetheri. Blood samples of 1.1 ml were collected from a tail vein by a new depression method developed by Zhou [7] at regular time intervals after the drug administration. The blood samples were immediately centrifuged and plasma samples were then taken and transferred into 10 ml test tubes. They were afterwards extracted and chromatographed according to the preparation procedure which will be described in a following section.

Human Experiment — 6 male healthy volunteers, who were at the ages of 25 ± 3 and weighed from 50 to 60 kg, received a single dose of artemether at 6 or 10 mg/kg by im. injection of Injectio Artemetheri. Blood samples were then drawn from a brachials vein into a heparinised syringe at frequent time intervals. The blood samples were transferred into centrifuge tubes and centrifuged immediately to separate plasma. The plasma samples obtained were stored at -40 °C till taken for analysis. The preparation of the human plasma samples for chromatography was accomplished according to the procedure given in the following section.

Preparation of Plasma Samples — 0.5 ml of rat plasma or 1.0 ml of human plasma was pipetted into a 10 ml centrifuge tube. The rat plasma sample and the human plasma sample were extracted with 1.5 ml and 2.5 ml of ethyl acetate, respectively, on a vortex mixer for about one minute. After being centrifuged at 3000 rev./min for 10 minutes, the supper organic layer was transferred into a small test tube. The plasma layer was then extracted twice more with as a same volume of ethyl acetate as that used in the first extraction, and the extracts were combine with the first one in the test tube. After the evaporation of the extractant by compressed air, the test tube with sample residue was stored in a refrigerator till taken for chromatography. Prior to being injected, the residue was prepared in 0.3 ml of methanol-water mixture (1:1 v/v). A aliquot of this solution was injected onto the column for chromatographic determination.

Test of Recovery and Precision of the Determination of Artemether in Plasma — In order to study the recovery of the proposed method, different amounts of methanolic solution of artemether were added to separate test tubes to give 20 to 480 ng per tube. After the solutions were evaporated to dryness 0.5 or 1 ml of blank plasma sample was pipetted into each tube and vortexed thoroughly. The samples were then extracted and chromatographed according to the preparation procedure of plasma samples. Recovery ratios were obtained by comparing the peak area of each test solution with that of a standard one with a similar concentration.

For the examination of precision, 5 aliquots of each of plasma samples were simultaneously assaied.

Calibration — The calibration curves were accomplished by external standard method. Different amounts of artemether

standard, which covered the rang of the concentrations of the plasma samples being analysed, were added to separate test tubes. After the addition of 0.5 ml of blank rat plasma or 1 ml of blank human plasma to each tube,the samples were extracted and chromatographed according to the preparation procedure of plasma samples described above.

Results and Data Analysis

Recovery and Precision of the method — The recovery ratio of artemether in plasma was 71 - 100% at different concentration levels. It was inversely proportional to concentration. Therefore, linear calibration curves were obtained in the calibration procedure (see also figure 8).

The relative standard deviation was less than 8%.

Data Analysis — Plasma concentration-time curves were analysed according to the nonlinear least squares regression analysis program, MCPKP [8], at a Great Wall 0520 model microcomputer (China). The significant difference in pharmacokinetic parameters of artemether was assessed by means of Student's t-test.

Figure 9 shows the mean semilogarithmic values of plasma concentration - time plots of artemether in rats which received all a single dose at 80 mg/kg via im. administration. The data fit in with the one-compartment model expressed by the formula

$$C_p = B \cdot e^{-kt} - A \cdot e^{-k_a t}$$

The pharmacokinetic parameters are listed in table II. It can be seen that the k_a value is about 10 times the k value. The elimination half - life of artemether was 15.8 hours. However, artemether could be found at the injection site for

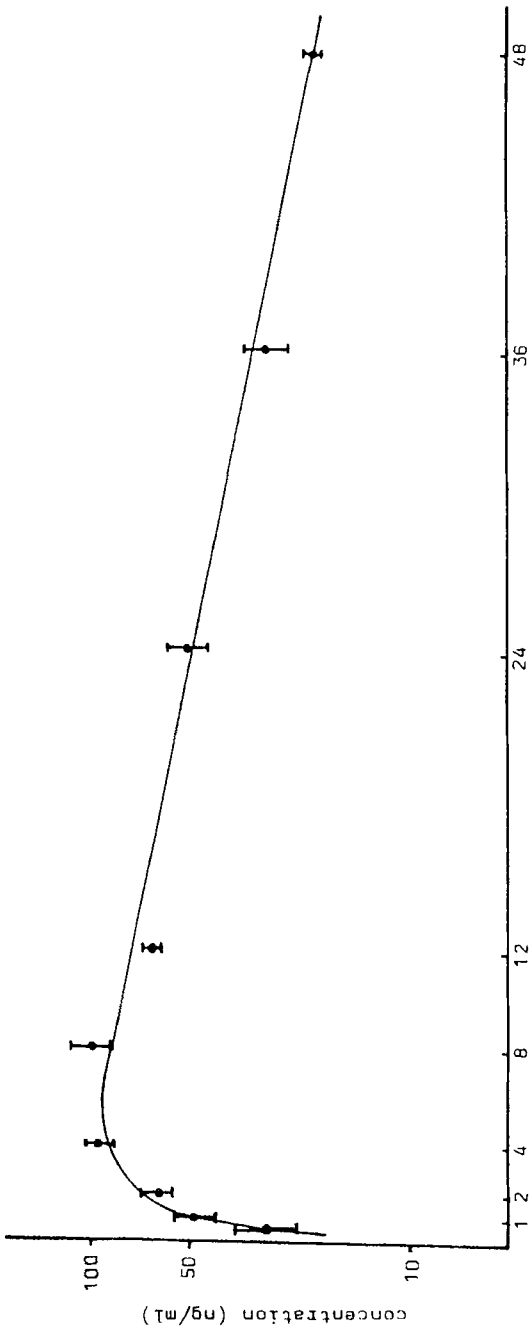

Figure 9. Plasma concentration - time plots of artemether in rats after the intramuscular administration of a dose at 80 mg/kg

The line was obtained by methmatical fitting treatment by the nonlinear least squares method

Each point represents the arithmetical mean value +/- SD of the concentrations in 4 rats at a time interval

Table II. Pharmacokinetic parameters of artemether in 4 rats after im. injection of Injectio Artemetheri (8o mg/kg)

Parameters	Units	Mean +/- SD
Do	mg/kg	80
Co	ug/ml	0.1358 +/- 0.0433
Ka	/h	0.4761 +/- 0.1795
K	/h	0.0443 +/- 0.0046
Ka t1/2	h	1.7021 +/- 0.8917
K t1/2	h	15.7553 +/- 1.5829
AUC	ug.h/ml	3.0210 +/- 0.6587
Tmax	h	5.9456 +/- 1.7738
Cmax	ug/ml	0.1022 +/- 0.0218

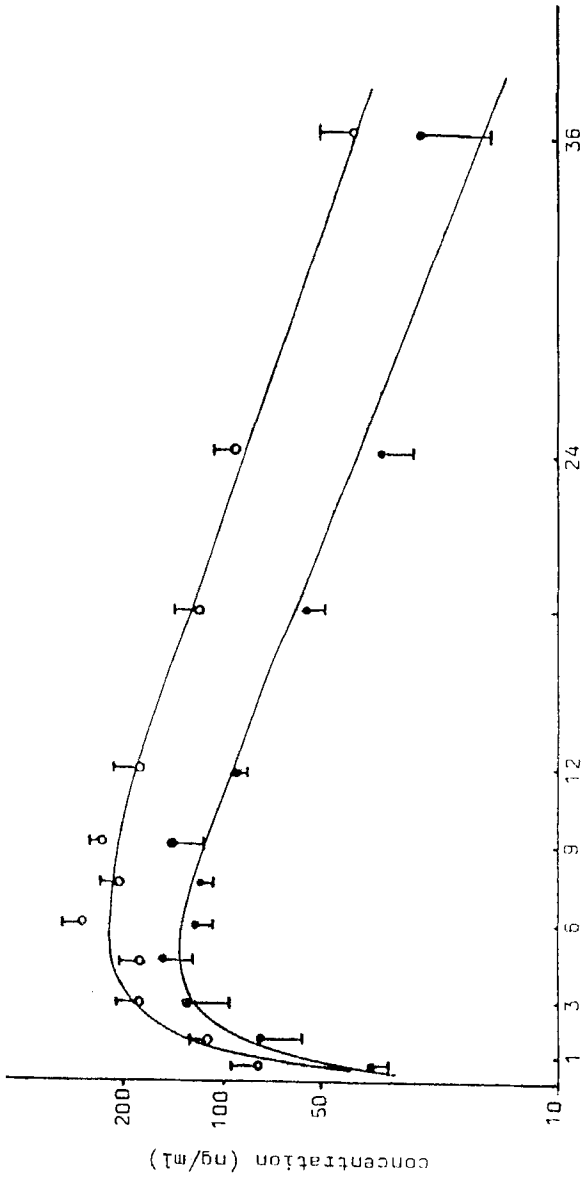

Figure 10. Plasma concentration of artemether in human subjects after intramuscular administration of a dose at 10mg/kg (o——o) and 6 mg/kg (●——●)

Lines were obtained by the nonlinear least squares method

Each point represents the mean concentration +/- SD of 3 subjects at a time interval

Table III. Pharmacokinetic parameters of artemether in 6 men after im. injection of Injectio Artemetheri (6 or 10 mg/kg)

Parameter	Unit	Mean +/- SD (n =3)	
		6	10
Do	mg/kg	0.2903 +/- 0.1250	0.3260 +/- 0.0322[a)]
Co	ug/ml	0.3547 +/- 0.2290	0.4268 +/- 0.2541[a)]
Ka	/h	0.1190 +/- 0.0624	0.0646 +/- 0.0157[a)]
K	/h	2.6351 +/- 1.7021	2.0805 +/- 1.2225
Ka t1/2	h	7.7215 +/- 5.6269	11.1163 +/- 2.4552
K t1/2	h	2.5690 +/- 0.4377	5.1580 +/- 0.7512[c)]
AUC	ug.h/ml	5.2358 +/- 1.2988	6.2727 +/- 2.5687[a)]
T_{max}	h	0.1448 +/- 0.0381	0.2237 +/- 0.0262[b)]
C_{max}	ug/ml		

a). $p \geq 0.05$; b). $p \leq 0.05$; c). $p \leq 0.01$ in comparison with the 6 mg/kg-group

72 hours, the concentrations being as high as that of the preparation.

Figure 10 presents the mean plasma concentration - time profiles of artemether in the 6 human subjects after im. administration of a single dose at 6 and 10 mg/kg, respectively. The pharmacokinetic parameters which resulted from the methmatical fitting treatment of these profiles are given in table III. The AUC and C_{max} wre proportional to the administrated doses, and different dosages could cause significant difference in these values. According to the effective concentration of artemehter on erythrocytic forms of FCcl/HN strain of P. falciparum in vitro, EC_{90} - the drug concentration which causes 90% reduction of the parasite density - is equal to 4.12 ng/ml [10]. The experiment observations showed that the im. administration of a single dose of artemether at 6 mg/kg could keep its plasma concentration at a level over 4.12 ng/ml.for 50 hours. This indicates that Injectio **Artemetheri** is a long-acting preparation and suggests that one-daily dosing should be appropriate for maitaining therapeutic concentration.

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