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DETERMINATION OF ARTEETHER IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION AFTER HYDROLYSIS WITH ACID

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for determination of the antimalarial agent arteether in blood plasma based on its decomposition in acidic medium and measurement of the major decomposition product, which has been identified as an α,β-unsaturated decalone. Linear calibration curves were obtained in the range 0–250 ng/ml arteether and the recovery of the drug from plasma was found to be quantitative. There is no interference from desoxyarteether, the putative major metabolite of arteether. The method has been applied to the measurement of arteether in the plasma of rats given 110 mg/kg by intramuscular injection of the drug as a solution in sunflower oil.

INTRODUCTION

The search for new antimalarial drugs to overcome the problem of *Plasmodium falciparum* resistance to most of the available antimalarial agents has led, in recent times, to the discovery of qinghaosu (or artemisinin).

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Qinghaosu (QHS), a sesquiterpene lactone with an endoperoxide function is the active principle of *Artemisia annua* L., a plant which has long been used in antimalarial preparations in China [1]. Although QHS has been shown to be more potent than chloroquine in its antimalarial action, the compound is only sparingly soluble in water and oils and its bioavailability following oral administration is poor [1].

The search for more potent derivatives of QHS with improved bioavailability has focussed on the reduction of QHS to dihydroqinghaosu (DQHS) and subsequent preparation of ether (or ester) derivatives of DQHS [2–4].

Arteether is a very recent semisynthetic ether derivative of QHS which has been reported to be a highly potent antimalarial both in vivo against a drug-sensitive strain of *Plasmodium berghei* in rats and in vitro against *Plasmodium falciparum* [5]. Arteether has, therefore, been selected by the World Health Organisation for clinical evaluation in high-risk patients, in particular in cases of cerebral malaria. In order to use arteether optimally and safely, pharmacokinetic data are necessary, for which a sensitive method for measuring the drug in biological fluids is a primary prerequisite.

QHS and its derivatives have previously been directly measured in biological fluids by thin-layer chromatography in combination with spectrodensitometry [6,7] and by high-performance liquid chromatography (HPLC) in combination with electrochemical detection [8]. While the first method is limited in sensitivity, the latter approach is fairly involved and exacting with regards to the chromatographic conditions necessary for the proper functioning of the electrochemical detector. Since the gas chromatographic approach is precluded by the known heat lability of these compounds [9,10], HPLC in combination with relatively simple spectrophotometric detection seems the most favourable approach to the analysis of arteether and other QHS derivatives. However, arteether like QHS and its other derivatives does not possess any sensitive spectrophotometric characteristics and there is a need to prepare derivatives of the compound which possess suitable UV–visible absorption characteristics. Such an approach to the analysis of QHS has been reported, based on the decomposition of QHS, DQHS and artesunate in an alkaline medium to give an UV-absorbing derivative [11,12]. Reports on the synthesis of some ether derivatives of QHS, however, indicate that these compounds are stable to alkali [4]. During the preparation of arteether it was found that the use of hydrochloric acid in place of boron trifluoride etherate as catalyst in the final etherification step results in a considerably lower yield of the compound [5]. This suggests that arteether may be sensitive to hydrochloric acid as would be expected from the presence of the acetal function in the molecule. The action of aqueous acid on arteether was, therefore, examined for the possible formation of a UV-absorbing derivative, the measurement of which may serve as an indirect method for the analysis of arteether in biological fluids. The following is a report on the HPLC analysis of arteether in blood plasma based on its

decomposition in aqueous–methanolic hydrochloric acid and UV detection of one of the decomposition products.

EXPERIMENTAL

Chemicals

QHS, arteether and desoxyarteether were provided by SAPEC (Switzerland) through the World Health Organisation. The structures of the compounds are depicted in Fig. 1. Progesterone, which was used as the internal standard, was obtained from Sigma (Poole, U.K.) (Lot 87c-0082) and was used without further purification.

Dichloromethane (Hipersolve®, HPLC grade) was obtained from British Drug Houses (Poole, U.K). Acetonitrile (HPLC grade) and concentrated hydrochloric acid (sp. gr. 1.16, analytical grade) were obtained from Fisons Laboratory Supplies (Loughborough, U.K.).

Other reagents were of analytical grade.

Standard solutions

Standard stock solutions of arteether in methanol (100 μg/ml) and of progesterone in methanol (100 μg/ml) were prepared weekly and stored at 5°C. The working solutions, each containing 1.0 μg/ml arteether or 1.2 μg/ml progesterone, were prepared daily by a two-step dilution of the stock solutions using distilled water in place of methanol in the final dilution step.

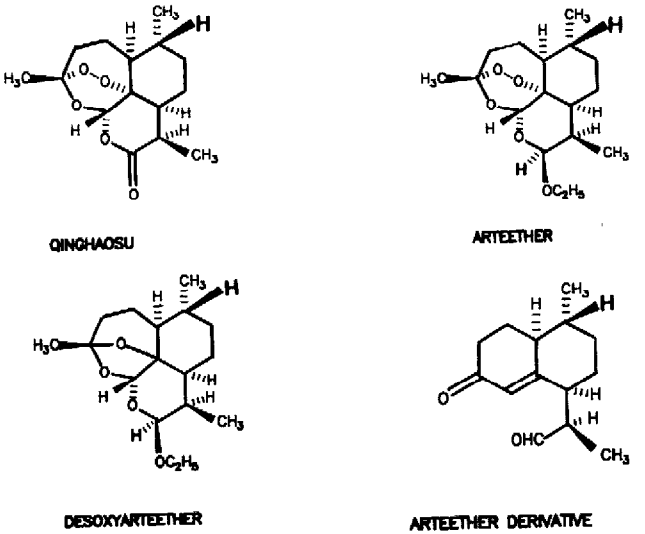

Fig. 1. Structures of qinghaosu, arteether, desoxyarteether and arteether derivative.

Preliminary HPLC

During the preliminary investigations of the reaction of arteether with hydrochloric acid the chromatographic system used consisted of a Spectra-Physics Model SP 8700 solvent delivery system, an SP 8750 organiser module fitted with a Rheodyne Model 7125 injector with a 100-μl sample loop, a Beckman Ultrasphere-ODS C_{18} reversed-phase column (5 μm particle diameter; 15 cm$\times$4.6 mm I.D.) and an SP 8300 fixed-wavelength (254 nm) UV detector. The mobile phase was 0.05 *M* acetate buffer–acetonitrile (60:40, v/v). Before use, the pH of the mobile phase was adjusted to 6.0 using triethylamine. The mobile phase flow-rate was 0.5 ml/min.

Analytical HPLC

For the analysis of arteether in plasma, the chromatographic system was as above except that the mobile phase was acetonitrile–water (60:40, v/v) and the flow-rate was 0.75 ml/min.

Spectroscopic characterisation of the derivative

The ultraviolet spectrum of the reaction mixture was run on a Cecil Instruments CE 5050 double-beam UV–visible spectrophotometer.

The electron-impact (70 eV) mass spectrum of the derivative was obtained on a VG Tritech TS 250 mass spectrometer interfaced to a VG 250-11 data system. Analysis was by direct insertion (source temperature, 180°C) and the accelerating voltage was 4.12 kV.

Chromatographic investigation of the reaction of arteether with hydrochloric acid

To an aliquot of a solution of arteether (1.0 ml; 100 μg/ml) in methanol (99.8%) was added hydrochloric acid solution (1.0 ml; 5 *M*) prepared by mixing equal volumes of concentrated hydrochloric acid and HPLC-grade methanol. The mixture was heated on a water-bath at 70°C for 5 h. After cooling to room temperature, distilled water (1.0 ml) was added and the mixture extracted with dichloromethane (3.0 ml) by shaking on a vortex mixer for 1 min. After removal of the aqueous layer with a Pasteur pipette, the extract was dried over anhydrous sodium sulphate, transferred to another test tube and then evaporated to dryness under nitrogen. The residue was redissolved in 1.0 ml of methanol and an aliquot (50 μl) chromatographed under the conditions outlined for preliminary HPLC above.

Optimisation of the conditions of the reaction of arteether with hydrochloric acid

Acid concentration. Aliquots of arteether solution (1.0 ml; 100 μg/ml) were placed in a series of test tubes. Concentrations of hydrochloric acid (5, 1 and 0.1 *M*) in methanol were prepared by dilution of concentrated hydrochloric acid with methanol and the arteether solutions were separately treated with 1.0-ml portions of the different solutions of hydrochloric acid. The mixtures

were then warmed in a water-bath at 53°C for 15 min. After cooling, 3.0 ml of dichloromethane were added. For the experiments with the lower concentrations of acid, homogeneous mixtures were obtained, and drops of water had to be added to the mixture until the dichloromethane separated as the lower phase. The mixtures were then shaken on a vortex mixer for 1 min, the aqueous layers removed and the extracts dried over anhydrous sodium sulphate. The dry extracts were evaporated in a water-bath at 40°C under a stream of nitrogen. The residues were redissolved in 2.0 ml of methanol, and an aliquot (30 μl) was chromatographed under the conditions described for preliminary HPLC. The yield of the reaction was estimated by measuring the peak area of the largest peak in the chromatogram which is potentially the most analytically useful peak.

Time. A series of 1.0-ml aliquots of arteether solution was treated with 5 *M* (or 1 *M*) hydrochloric acid in methanol at 53°C for different periods of time ranging from 15 min to 3 h. After each reaction period the reaction mixtures were extracted and the extracts chromatographed as described in the preceding section.

The experiment was also repeated using aqueous solutions in hydrochloric acid (5 or 1 *M*) in place of the methanolic acid solutions.

Spectroscopic investigation of the reaction of arteether with hydrochloric acid

To identify the most prominent peak in the chromatogram a 2.0-ml aliquot of the arteether solution (100 μg/ml) was treated with 5 *M* aqueous hydrochloric acid (2.0 ml) at 53°C for 15 min (these conditions were established as being optimum for the reaction in terms of the yield of the major product and the limited number of other products; see above). After extraction of the reaction mixture with dichloromethane, drying and removal of the solvent, the residue was redissolved in 50 μl of acetonitrile. The solution was chromatographed in five 10-μl portions under the conditions described for analytical HPLC except that the mobile phase flow-rate was 0.50 ml/min. During each chromatographic run, the eluate fraction corresponding to the most prominent peak (retention time = 13 min) was collected as it emerged from the detector. The pooled eluate fractions were extracted with dichloromethane (approximately 1.5 ml of extractant to 5 ml of eluate) for 1 min on a vortex mixer. The extract was dried over anhydrous sodium sulphate, transferred to a tapered glass tube and the solvent evaporated under nitrogen. The procedure was repeated five times (equivalent to overall reaction of 1.0 mg of arteether) using the same tapered glass tube in the last evaporation step. The residue obtained finally was redissolved in 100 μl of methanol and the solution examined by mass spectrometry.

Another portion (2.0 ml) of the arteether solution was reacted with 5 *M* aqueous hydrochloric acid as above. After cooling, the mixture was extracted with dichloromethane, and the extract dried and evaporated as above. The

residue was redissolved in 4.0 ml of methanol and the UV–visible spectrum of the solution measured using methanol as reference.

Determination of arteether in plasma

Calibration. Using 10-ml glass culture tubes, standard solutions of arteether in the range 50–250 ng/ml (0.05–0.25 ml of a 1 μg/ml solution) were prepared by adding known quantities of the drug to drug-free human plasma (1.0 ml) containing 48 ng of progesterone (0.04 ml of a 1.2 μg/ml solution) as internal standard. After addition of dichloromethane (3.0 ml) the plasma solutions were extracted by shaking on a vortex mixer for 1 min and the mixture then centrifuged at 500 *g* for 15 min. The aqueous layer was removed with a Pasteur pipette, the dichloromethane extract transferred to a clean extraction tube and evaporated under nitrogen at 37°C. After adding methanol (1.0 ml) to the residue and shaking for a few seconds on the vortex mixer, 5 *M* aqueous hydrochloric acid solution (1.0 ml) was added. The mixture was warmed for 15 min at 53°C, allowed to cool to room temperature and then extracted with 2.5 ml of dichloromethane by shaking on a vortex mixer for 1 min. The acidic aqueous layer was removed and the extract shaken with sodium hydroxide solution (1.0 ml; 5 *M*) for about 30 s on the vortex mixer. After removing the alkaline aqueous layer the extract was further shaken with distilled water (1 ml) for 10–15 s. The aqueous layer was discarded and a little anhydrous sodium sulphate added to the extract to dry it.

The extract was then transferred to a tapered glass centrifuge tube and the solvent evaporated down to about 0.2 ml under nitrogen at 37°C. The tube was allowed to cool to room temperature and then shaken briefly on the mixer. The solution was then evaporated to dryness by placing the tube in a breaker of warm water (45°C) and blowing gently in a hand pump. This last evaporation step was carried out immediately before chromatographic analysis.

The residue was redissolved in acetonitrile (30 μl) and 20–25 μl aliquots were chromatographed under the conditions described for analytical HPLC above. The peak-height ratio of arteether to progesterone was plotted against the corresponding weight of arteether. The calibration procedure was repeated using distilled water in place of drug-free plasma. All the test tubes used were pretreated with dichlorodimethylsilane–toluene (5:95, v/v) to prevent adsorption of arteether to glass.

Analytical precision and recovery

On different days, a series of solutions of arteether containing 60 ng/ml (or 120 ng/ml) of the drug in plasma were prepared and treated as unknowns which were determined by running appropriate standard curves at the same time as the analysis of these solutions.

Furthermore, a 100 ng/ml solution of arteether in plasma was prepared and stored, in a previously silanised glass test tube, at −20°C. Aliquots (1.0 ml)

of this solution were analysed as unknowns over a one-week period using standard curves prepared on the same day.

Animal studies

Four male Wistar rats were anaesthetised with pentobarbitone sodium (60 mg/kg) and the trachea, femoral vein and carotid artery exposed and cannulated. Heparin was then administered (200 U in saline, intravenously). The animals then received arteether (110 mg/kg, intramuscularly) as a solution in sunflower oil. Blood samples (~ 750 μl) were removed from the carotid artery at 10, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min into Microcap tubes (L.I.P. Equipment and Services, Shipley, U.K.) containing 20 μl of 1000 U/ml heparin. The blood samples were centrifuged immediately at 1000 *g* for 10 min and the plasma transferred to glass micro tubes which had been treated previously with dichlorodimethylsilane–toluene (5:95, v/v). Saline (0.45%) was added to the plasma to a volume of 0.5 ml. The tubes were sealed with Parafilm and the samples stored at −20°C until analysis.

For analysis, each plasma sample was transferred quantitatively into 10-ml glass culture tubes containing a solution of progesterone, the internal standard. The samples were then treated as described for standard solutions above.

RESULTS AND DISCUSSION

Reaction of arteether with hydrochloric acid

HPLC of an extract of the reaction mixture shows that heating arteether with hydrochloric acid results in a plethora of decomposition products which are responsive to UV detection. The chromatogram of an extract of the reaction mixture obtained after heating arteether with 5 *M* hydrochloric acid at 70°C for 5 h is shown in Fig. 2A.

To be analytically useful, the reaction had to be controlled to limit the number of products and also to optimise the yield of one of the products. When the acid concentration was reduced to 1 *M*, the reaction temperature reduced to 53°C and the reaction time shortened to 15 min, the reaction was found to result in only one major product (retention time, 47 min) with four or five minor side-products as shown in the chromatogram in Fig. 2B. Since the reaction is obviously sensitive to these factors, an attempt was made to optimise these conditions to obtain the maximum yield of the product responsible for the major peak in chromatogram. The reaction was found to be very slow at room temperature though the peak eluting at 47 min was detectable (peak height about 2 cm) following chromatography of a reaction mixture of arteether and 5 *M* hydrochloric acid kept at room temperature for 1 h. The reaction temperature was, therefore, raised to 53°C and the effect of other factors — time and acid concentration — was studied at this temperature. The yield of the reaction was measured in terms of the peak area of the peak eluting

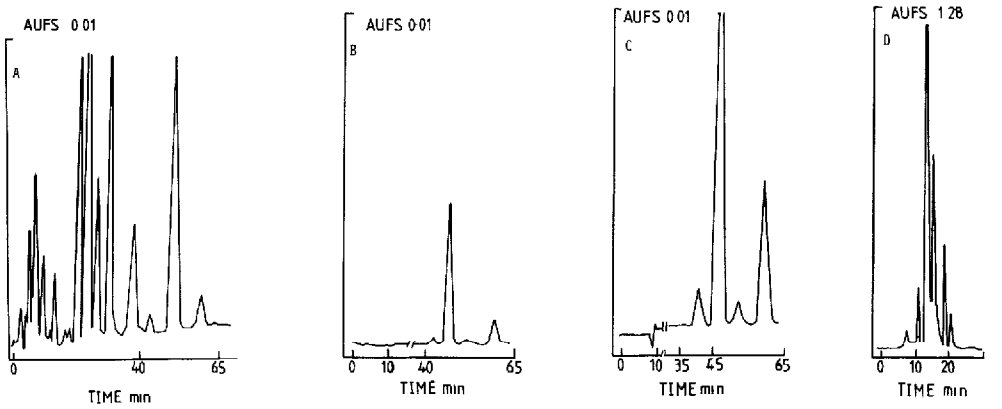

Fig. 2. Chromatograms of the reaction mixture from heating arteether with hydrochloric acid under the following different reaction conditions: (A) 5 *M* HCl for 5 h at 70°C, (B) 1 *M* HCl for 15 min at 53°C, (C) 5 *M* HCl for 15 min at 53°C, (D) condition as in C but mobile phase is acetonitrile–water (60:40, v/v); flow-rate 0.50 ml/min.

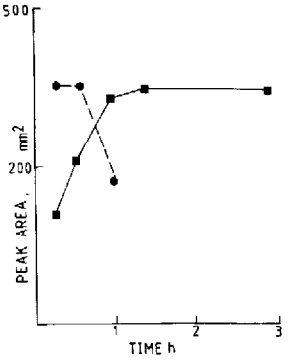

Fig. 3. Variation of yield of arteether derivative with acid concentration and time: (●) 5 *M* hydrochloric acid; (■) 1 *M* hydrochloric acid.

at 47 min following chromatography of the reaction mixture. The results, which are depicted in Fig. 3, show that the reaction is faster and the yield slightly higher in 5 *M* hydrochloric acid than in 1 *M* hydrochloric acid. The product eluting at 47 min seems less stable in the higher concentration of acid. The yield in 5 *M* acid is, however, stable for 15 min after the optimum has been attained. The use of 5 *M* hydrochloric acid at 53°C for 15 min was regarded as the optimum condition for the transformation of arteether to this UV-absorbing derivative. The chromatogram of the reaction mixture under these conditions is shown in Fig. 2C. On changing the mobile phase to acetonitrile–water (60:40, v/v) and using a flow-rate of 0.50 ml/min the pattern of peaks obtained is shown in Fig. 2D, with the major peak now eluting at about 13.5 min.

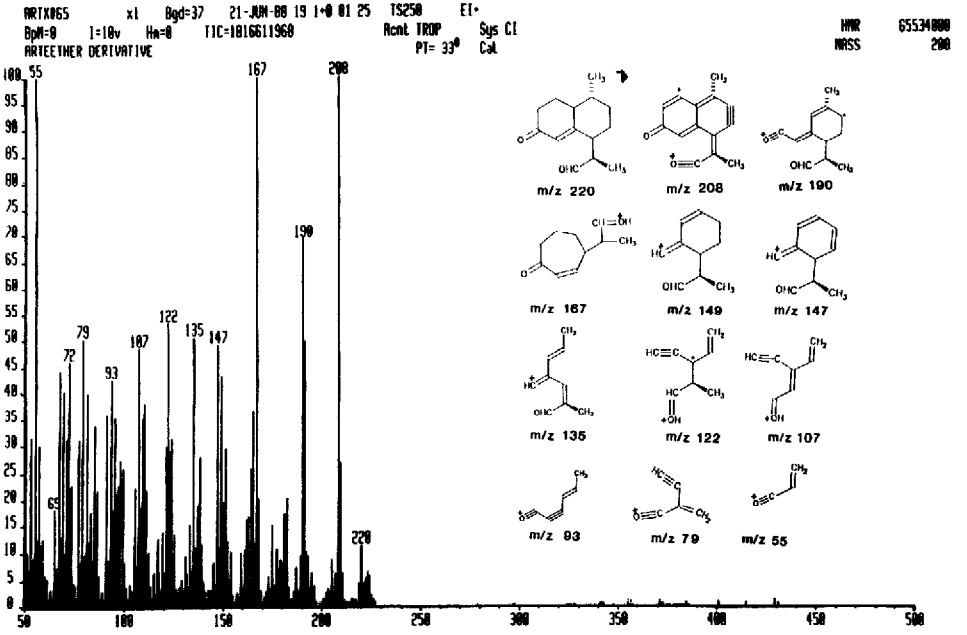

Fig. 4. Mass spectrum of arteether derivative.

Spectrophotometric examination of an extract of the reaction mixture shows an absorption spectrum with a maximum at 254 nm. The fixed-wavelength (254 nm) detector is therefore suitable for the HPLC analysis of arteether based on its decomposition in acid. The compound responsible for the most prominent peak in the chromatogram (Fig. 2D) was tentatively identified by its mass spectrum to be the α,β-unsaturated decalone, 8-methyl-5-(2-propanalyl)decalin-4-ene-3-one, the structure of which is depicted in Fig. 1. The mass spectrum is shown in Fig. 4, together with the possible structures of the important fragment ions.

Determination of arteether in plasma after reaction with acid

Standard curves were linear in the range 0–250 ng ($r=0.978$; $p\leqslant 0.05$) for drug solutions prepared either in drug-free plasma or prepared in distilled water. An advantage of the method is that there was no significant difference between standard curves prepared using solutions prepared in plasma and those prepared using solutions in distilled water and standard solutions of the drug in distilled water may, therefore, be used to determine unknown plasma samples (see Table I).

The extraction procedure resulted in quantitative recovery of arteether from plasma as determined by comparison of the slopes of calibration curves obtained for methanolic solutions of arteether determined directly and those ob-

TABLE I

VALIDATION DATA FOR THE ANALYSIS OF ARTEETHER IN PLASMA

Actual concentration (ng/ml)	Intra-assay			Inter-assay		
	Concentration found (mean ± S.E.M.[a]) (ng/ml)	n	C.V.[b] (%)	Concentration found (mean ± S.E.M.[a]) (ng/ml)	n	C.V.[b] (%)
60	65.26 ± 4.19	4	12.85	72.2 ± 3.54	9	14.71
	77.74 ± 4.17	5	12.00			
100	88.4 ± 6.62	5[c]	16.75	107.8 ± 7.24	10	21.23
	117.5 ± 9.57	5[c]	18.21			
	95.56 ± 2.20	5[c]	5.16			
	110.26 ± 8.5	5	17.27			
120	106.55 ± 4.47	5	9.38	120.2 ± 5.98	10	15.76
	133.84 ± 7.18	5	11.99			

[a]Standard error of the mean.
[b]Coefficient of variation.
[c]Values obtained using standard aqueous solutions of arteether for calibration.

tained following analysis of extracted aqueous or plasma standards. Further, comparison of the estimates obtained for the validation samples (containing 60 or 120 ng of arteether) from the standard curves with the amount of arteether actually present shows the recovery to be 110.8 ± 19.3% (n = 18). Ethyl acetate or methyl *tert.*-butyl ether were less satisfactory for the extraction of arteether from plasma. With ethyl acetate, many endogenous substances were co-extracted with the arteether, while methyl *tert.*-butyl ether gave a much lower recovery of arteether than dichloromethane.

The precision and accuracy of the method were determined by replicate analysis of spiked samples. The results are shown in Table I for plasma samples spiked with 60 or 120 ng of arteether and treated as unknowns. Table I also shows the results obtained for the replicate analysis of a plasma solution of arteether (100 ng/ml) stored at −20 °C and analysed over a one-week period. There is no difference between the results obtained when standard curves were prepared using aqueous or plasma solutions of the drug.

Storage of arteether solutions in plasma using plastic containers was found to result in a considerable loss of the drug even over a 24-h period. Plasma samples were, therefore, stored in presilanised glass test tubes.

The method is specific for arteether as there was no interference from desoxyarteether, to putative major metabolite of arteether. Although desoxyarteether was found to decompose in acid to give UV-detectable products, these products were readily removed by washing the dichloromethane extract of the reaction mixture with alkali as described above. Incorporation of the alkali washing step also prevents possible interference from acid antimalarial drugs

such as sulphadoxine. Since the common basic antimalarial drugs such as chloroquine, amodiaquine, primaquine, pyrimethamine and proguanil are also unextractable under the highly acidic conditions in which the decalone derivative is extracted from the reaction mixture, this method is free from interference from these antimalarial drugs.

The assay method was applied to plasma samples obtained from rats administered the drugs. A chromatogram of rat plasma taken after intramuscular administration of the drug is shown in Fig. 5A. For comparison, chromato-

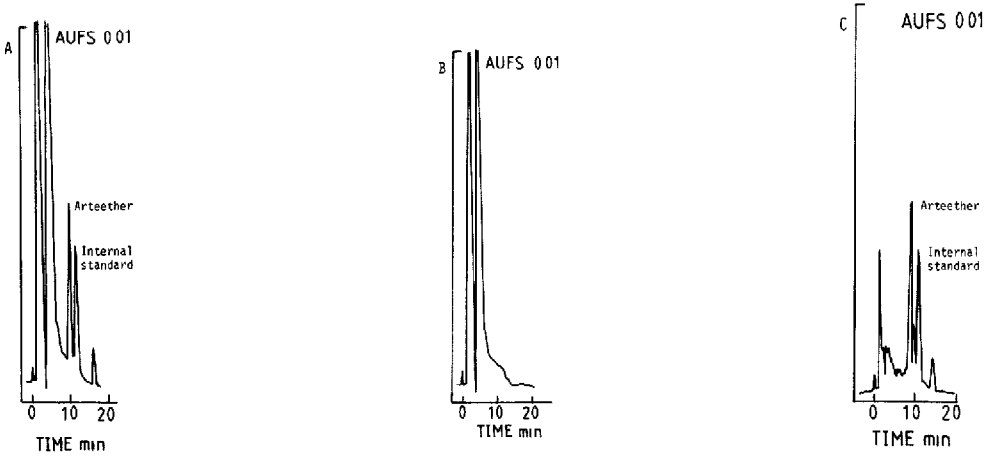

Fig. 5. Chromatograms of (A) rat plasma taken 20 min after intramuscular administration of arteether, (B) blank rat plasma and (C) standard aqueous solution of arteether (50 ng) and the internal standard progesterone (48 ng).

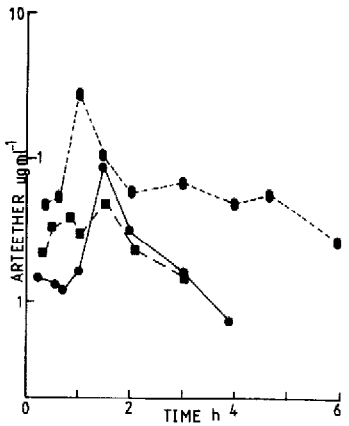

Fig. 6. Plasma versus time profile of arteether after intramuscular administration of the drug to rats.

grams of blank rat plasma and that of a standard aqueous solution of arteether are shown in Fig. 5B and C.

The plasma concentration versus time profile obtained from this animal study is shown in Fig. 6. Due to its insolubility in water, this animal study was beset by the problem of finding a suitable vehicle for the administration of the drug. Although sunflower oil was suitable as a solvent for the drug it may be not be the best in other respects. Establishment of a suitable vehicle and route for the administration of arteether is one of the problems in the development of the drug. The results of these preliminary animal study show that the method described herein will be useful in such investigations. The method may also be applicable to the analysis of artemether, the O-methyl analogue of arteether.

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REFERENCES

1 D.L. Klayman, Science, 228 (1985) 1049.
2 G.Q. Li, X.B. Guo, R. Jin, Z.C. Wang, H.X. Jian and Z.Y. Li, J. Trad. Chinese Med., 2 (1982) 45.
3 X. Luo, H.J.C. Yeh, A. Brossi, J.L. Flippen-Anderson and R. Gilardi, Helv. Chim. Acta, 67 (1984) 1515.
4 A.J. Lin, D.L. Klayman and W.K. Milhous, J. Med. Chem., 30 (1987) 2147.
5 A. Brossi, B. Venugopalan, L.D. Gerpe, H.J.C. Yeh, J.L. Flippen-Anderson, P. Buchs, X.D. Luo, W. Milhous and W. Peters, J. Med. Chem., 31 (1988) 645.
6 X.Y. Niu, L.Y. Ho, Z.H. Ren and Z.Y. Song, Eur J. Drug Metab. Pharmacokin., 10 (1985) 55
7 Y.D. Zhang, C.G. Wang, G Y Xu, Y.H. Zhuang and Y.L. Zeng, Acta Pharm. Sin., 17 (1982) 211.
8 Z.M. Zhou, J.C. Anders, H. Chung and A.D. Theoharides, J. Chromatogr., 414 (1987) 77.
9 A.J. Lin, D.L. Klayman, J.M. Hoch, J.V. Silverton and C.F. George, J. Org. Chem., 50 (1985) 4504.
10 A.J Lin, A.D. Theoharides and D.L. Klayman, Tetrahedron, 42 (1986) 2181.
11 S. Zhao and M.-Y. Zeng, Anal. Chem., 58 (1986) 289.
12 P.O. Edlund, D. Westerlund, J. Carlqvist, W. Bo-Liang and J. Yunhua, Acta Pharm. Suec., 21 (1984) 223.