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DETERMINATION OF ARTELINIC ACID IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the analysis of the new antimalarial drug artelinic acid in blood plasma The influence of mobile phase composition, pH and type of mobile phase modifier on the retention of artelinic acid on the reversed-phase column is reported Linear calibration curves were obtained in the range 0–500 ng/ml artelinic acid Intra-assay and inter-assay variability in the analysis of plasma samples spiked with the drug were $\leq 15\%$ Plasma samples of the drug were found to be unstable when stored at $-20\,^{\circ}C$, the concentration of the drug decreasing by over 50% within three days Plasma samples stored at $-70\,^{\circ}C$ remained stable for at least two weeks Initial pharmacokinetic studies in the rat showed that following intravenous administration, plasma concentrations of artelinic acid declined mono-exponentially The relatively short elimination half-life (17 ± 5 min) of artelinic acid is consistent with what is known for qinghaosu and its derivatives

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INTRODUCTION

Qinghaosu (QHS) is the antimalarial principle of *Artemisia annua*. This compound has assumed great importance in recent years during the search for alternative drugs effective against strains of *Plasmodium falciparum* exhibiting resistance to currently available antimalarials [1] There has been much interest in the synthesis and evaluation of derivatives of QHS as antimalarial agents because of their superiority to chloroquine (and other antimalarials presently in use) in the treatment of multi-drug-resistant *P falciparum* malaria, their superior chemotherapeutic index and their effectiveness in cases of cerebral malaria [2]

QHS (Fig 1a) is only poorly soluble in water and oils and its bioavailability is low following oral administration Attempts have, therefore, been made to produce semisynthetic derivatives of QHS with improved physico-chemical and/or pharmacological properties Because of their potential value in the treatment of cerebral malaria, water-soluble derivatives of QHS which can be administered parenterally are of considerable interest One such derivative is artesunic acid (ARTS, Fig 1b) which is prepared by reduction of QHS to dihydroqinghaosu (DQHS, Fig 1c) and subsequent esterification of DQHS with succinic acid to give ARTS which is the hemi-succinic ester of DQHS The sodium salt of ARTS (sodium artesunate) is water-soluble and has been found very useful in the treatment of cerebral malaria [2] A shortcoming of sodium

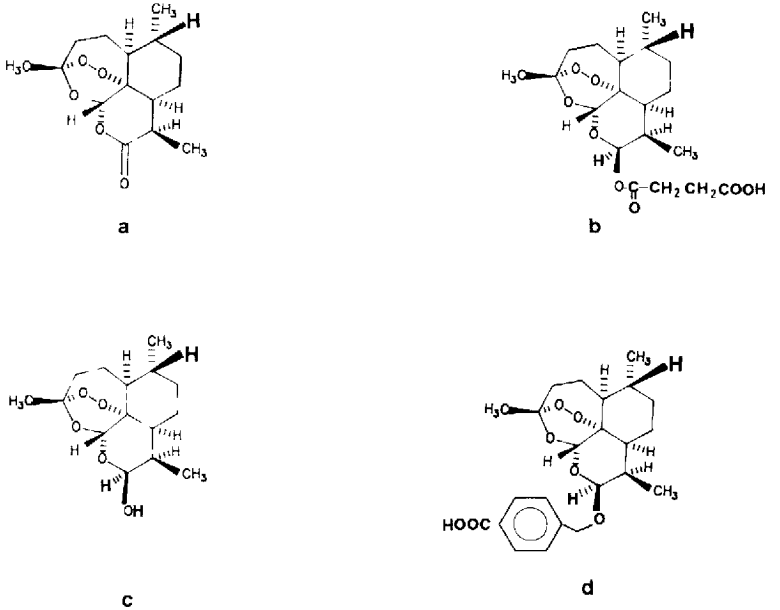

Fig 1 Structures of qinghaosu (a), artesunic acid (b), dihydroqinghaosu (c) and artelinic acid (d)

artesunate, however, is its poor stability in aqueous solution due to the lability of the ester linkage to hydrolysis.

Artelinic acid (ARTL, Fig 1d) is another semisynthetic water-soluble derivative of QHS in which the hydrophilic carboxylate group is on a benzyl moiety linked to DQHS by an ether linkage which should be resistant to hydrolysis The sodium salt of ARTL (sodium artelinate) has been reported to be effective against chloroquine-resistant *P falciparum* and possesses superior in vivo activity against *P berghei* compared to QHS Its solution in potassium carbonate has also been reported to be stable to hydrolysis [3] ARTL is, therefore, of potential value in the treatment of cerebral malaria Further pre-clinical evaluation of the compound requires an analytical method for measuring levels of the compound in blood plasma The following report describes the development of a simple high-performance liquid chromatographic (HPLC) method for determination of ARTL in plasma

EXPERIMENTAL

Chemicals

Sodium artelinate ($C_{23}H_{29}O_7Na \cdot 1.5H_2O$) was provided by the Walter Reed Army Institute of Research (Washington, DC, U.S A). Naphthalene, which was used as analytical standard, was obtained from BDH (Poole, U K) Acetonitrile (HPLC grade), triethylamine (TEA, analytical grade) and orthophosphoric acid were obtained from Fisons Laboratory Supplies (Loughborough, U K.). Tetrabutylammonium hydroxide (TBAH) and N,N-dimethyloctylamine (DMOA) were obtained from Aldrich (Gillingham, U K.)

Chromatography

The chromatographic system used consisted of a Spectra-Physics SP 8700 solvent delivery system, an SP 8750 organiser module containing a Rheodyne Model 7125 injector fitted with a 100-μl sample loop, a Beckman Ultrasphere-ODS C_{18} reversed-phase column (5 μm particle size; 15 cm$\times$4 6 cm) and an SP 8450 UV–VIS variable-wavelength detector operating at 240 nm During assay development, mobile phase composition was varied with respect to acetonitrile/water ratio, type and concentration of mobile phase modifier (i e TEA, TBAH or DMOA) and pH

Extraction

Two-phase extraction after acidification with hydrochloric acid or buffers

ARTL (0 5 μg ml^{-1}) in drug-free plasma was acidified by addition of hydrochloric acid (0 2 ml, 5 *M*) and extracted with dichloromethane (2 0 ml) by shaking on a vortex mixer for 1 min. The mixture was then centrifuged at 500 *g* for 10 min. The aqueous layer was removed and the organic phase dried with

sodium sulphate and evaporated to dryness under a stream of nitrogen at 40 °C The residue was redissolved in 50 μl of a solution of naphthalene in acetonitrile (2 0 μg ml^{-1}) and a 25-μl aliquot of the solution chromatographed The peak-height ratio of ARTL to naphthalene was measured. The extraction procedure was repeated using different concentrations of hydrochloric acid (1, 0 1 or 0 05 *M*) and also using either ethyl acetate or methyl *tert* -butyl ether in place of dichloromethane at these different concentrations of acid Extraction with either dichloromethane or ethyl acetate or methyl *tert* -butyl ether was repeated following acidification of the plasma solution of ARTL with a solution of sodium dihydrogen orthophosphate (30%, w/v), the pH of which had been adjusted previously to 4, 3 or 2, respectively

Ion-pair extraction using TBAH as ion-pairing agent A solution of ARTL in blood plasma (500 ng per 0 5 ml) was prepared as described above The solution was basified by addition of sodium hydroxide solution (0 5 ml, 5 *M*) After addition of TBAH (0.2 ml) and dichloromethane (2 0 ml) the mixture was shaken on a vortex mixer for 1 min The dichloromethane extract was dried, evaporated and the residue reconstituted in a solution of naphthalene in acetonitrile and chromatographed as described above

Extraction with acetonitrile Details of this approach to the extraction of ARTL from plasma are as described below for the calibration procedure.

Calibration

A stock solution of ARTL (100 μg/ml) was prepared by dissolution of the appropriate amount of sodium artelinate in water This was diluted ten times to give a working aqueous solution of 10 μg/ml

Aliquots of the working solution (10–50 μl; 100–500 ng ARTL) were placed in 10-ml capacity culture tubes and drug-free plasma (0 5 ml) was added The plasma solutions were acidified by addition of 30% (w/v) solution of sodium dihydrogen orthophosphate (0.2 ml) previously adjusted to pH 4 The mixture was then shaken briefly on a vortex mixer Acetonitrile (2.0 ml) was added and after shaking on the vortex mixer for 1 min the mixture was centrifuged at 500 *g* for 10 min

Using a Pasteur pipette, the acetronitrile layer was transferred to a tapered glass centrifuge tube. To assist in obtaining optimal transfer of the extracted compound the tube was washed with acetonitrile (0.2 ml) and the washing transferred to the tapered centrifuge tube with a Pasteur pipette. The acetonitrile extract was evaporated to dryness at 40 °C under a stream of nitrogen The residue was redissolved in 50 μl of a 2 μg/ml solution of naphthalene in acetonitrile with shaking on a vortex mixer for about 1 min Aliquots of 20–40 μl were chromatographed The peak-height ratio of ARTL to naphthalene was plotted against the corresponding mass of ARTL.

All glass ware used for the extraction were pre-treated with dimethyldichlorosilane in toluene (5%, v/v) to prevent adsorption of ARTL to the glass

Assay precision and stability of artelinic acid in blood plasma

Over four days, a series of solutions of ARTL containing 150 ng/ml (or 200 ng/ml) drug in plasma were prepared and treated as unknown and the concentrations determined after running appropriate standard curves on each day

To evaluate the stability of ARTL solutions in plasma at different storage temperatures, solutions of sodium artelinate equivalent to 2 μg/ml ARTL in plasma were prepared in previously silanised glass culture tubes and stored at either −20°C or −70°C Aliquots (0 1 ml, 200 ng ARTL) of these solutions were analysed as unknowns over a two-week period using standard curves prepared on the same day

Animal studies

A solution of sodium artelinate equivalent to 12 5 mg/ml ARTL was prepared by dissolving 20 0 mg of the hydrated sodium artelinate in 1 5 ml of distilled water.

Four male Wistar rats were anaesthetised with pentobarbitone sodium (60 mg/kg) and the trachea, femoral vein and carotid artery were exposed and cannulated Heparin (200 U in saline) was then administered intravenously. The animals then received, intravenously, sodium artelinate (equivalent to 10 mg/kg ARTL) Blood (~0 5 ml) was removed from the carotid artery at 0, 5, 10, 20, 30, 45, 60, 90, 120 and 180 min into Microcap tubes (L I P Equipment and Services, Shipley, U K) An equivalent volume of isotonic saline was administered to replace blood lost in sampling The blood samples were centrifuged immediately (500 *g*, 15 min) and the plasma was transferred to 10-ml capacity glass culture test tubes and analysed as described for standard solutions

Mass spectrometry

During chromatography of extracts of plasma samples obtained from rats dosed with ARTL a peak appeared at a retention time of about 18 min (see Fig 3B) Since this peak does not appear in chromatograms of standard plasma solutions of ARTL or predose rat plasma samples it was suspected to be due to a metabolite or ARTL

In an attempt to identify the metabolite the eluate fractions corresponding to the peak with a retention time of 18 min were collected during chromatography of plasma samples from rats dosed with ARTL The collected fractions were extracted with dichloromethane The dichloromethane extract was dried over anhydrous sodium sulphate After evaporating at 40°C under a stream of nitrogen, the residue was redissolved in methanol (100 μl) and the solution examined by mass spectrometry in the fast ion bombardment (FIB) mode using a VG Tritech TS 250 mass spectrometer interfaced to a VG 250-11 data system

RESULTS AND DISCUSSION

Chromatography of artelinic acid

The influence of the acetonitrile content of the mobile phase, type of mobile phase modifier and pH of the mobile phase on the retention time of ARTL on the reversed-phase column used were investigated The results are shown in Table I In each case the proportion of mobile phase modifier was 3% (v/v) and the pH of the mobile phase was adjusted using orthophosphoric acid

The use of DMOA as mobile phase modifier resulted in satisfactory chromatography for ARTL with sharp peaks being obtained at convenient retention times ($\leqslant$15 min) However, baseline instability was a problem associated with the use of DMOA A similar problem was encountered when TBAH was used as modifier The problem of baseline instability was overcome by the use of TEA Therefore, the retention characteristics of ARTL in presence of TEA were studied in greater detail The results are shown in Table I Using TEA, a mobile phase of 65% acetonitrile and a pH of 6 was found to be optimum Under these conditions, it was possible to obtain satisfactory calibration curves for extracts of aqueous solutions of ARTL at concentrations between 18 and 350 ng/ml However, determination of ARTL from plasma extracts under these conditions was not feasible because of interference from endogenous compounds For plasma extracts of ARTL, the optimum chromatographic conditions were achieved by reduction of the pH to 5 and decreasing the proportion of acetonitrile 65 to 50% This displaced the ARTL peak from peaks due to endogenous components, the retention times of which were unaffected by the

TABLE I

VARIATION OF THE RETENTION VOLUME OF ARTELINIC ACID WITH MOBILE PHASE COMPOSITION, pH OF MOBILE PHASE AND USING DIFFERENT MOBILE PHASE MODIFIERS

Mobile phase composition (acetonitrile–water, v/v)	Mobile phase modifier[a]	pH of mobile phase	Retention volume of ARTL (ml)
60 40	DMOA	3	12
65 35	DMOA	5	4 5
65 35	DMOA	7 5	7 5
60 40	TBAH	7 5	8 0
40 60	TBAH	7 5	20 0
65 35	TEA	5 0	5 0
65 35	TEA	6 0	4 5
65 35	TEA	6 8	2 5
60 40	TEA	6 8	5 0
50 50	TEA	5 0	9 0

[a]DMOA = N,N-dimethyloctylamine, TBAH = tetrabutylammonium hydroxide, TEA = triethylamine

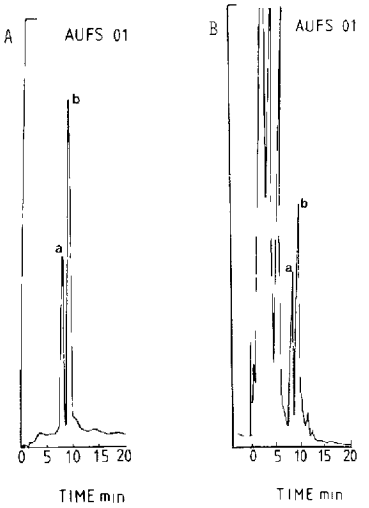

Fig 2 (A) Chromatogram of standard aqueous solution of naphthalene (50 ng) and artelinic acid (500 ng) (B) Chromatogram of an extract of a standard plasma solution of artelinic acid (500 ng) and naphthalene (50 ng) Peaks a = naphthalene, b = artelinic acid

change in pH It was found that the column required 2–2 5 h for equilibration. The mobile phase selected was acetonitrile–water (50 50, v/v) containing TEA (3%, v/v) buffered to pH 5 02 with orthophosphoric acid Flow-rate was 1 5 ml min^{-1}

Under the optimum chromatographic conditions for plasma extracts, the retention times for ARTL and naphthalene were 10 and 8 5 min, respectively Sample chromatograms of standard aqueous solution of ARTL and of extracts of plasma solutions, along with naphthalene, are shown in Fig 2

Extraction

The extraction procedure described above for calibration was adopted following the observation that ARTL is readily decomposed by aqueous mineral acid Thus the recovery of the drug was either very poor or zero when plasma samples were acidified with different concentrations of hydrochloric acid prior to extraction with different organic solvents such as dichloromethane, ethyl acetate or methyl *tert* -butyl ether Extraction of ARTL under basic conditions (where it is stable) with TBAH as an ion-pairing agent was evaluated ARTL was readily extracted from basified aqueous solution with either dichloromethane or ethyl acetate, in the presence of TBAH However, due to interference from endogenous substances in plasma the method was found to be unsatisfactory for quantitative determination of ARTL and was abandoned in favour of the procedure described for calibration, with acetonitrile acting as both extractant and protein precipitant The optimum pH for the recovery of ARTL

was found to be 4. An acidifying solution of high ionic strength was chosen to enhance the extraction of ARTL through the salting out effect This approach also minimises the partitioning of water into the acetonitrile phase

Comparison of the slopes of calibration curves obtained as described above with slopes of calibration curves obtained from the direct analysis of standard solutions of ARTL in sodium dihydrogen orthophosphate (pH 4) shows that the recovery of ARTL was 44 5% In spite of the low recovery, a detection limit of 50 ng/ml was estimated for the procedure when, as indicted above, the detector was set at 240 nm which was found to be the λ_{max} ($\epsilon = 1\ 10 \cdot 10^4$) for ARTL

Calibration

Several compounds, including carboxyl group-containing compounds such as indomethacin and naproxen, and some sulphonamides were tested as possible internal standards in the analysis of ARTL Only naphthalene was found suitable as the other compounds had low retention times ($\leqslant 5$ min) similar to those of endogenous substances in plasma Naphthalene was, however, used only as an external rather than as an internal standard as its extraction from plasma was found to be irreproducible

Following the procedure described above, linear calibration curves were obtained ($r \geqslant 0\ 976$) in the range 0–500 ng of ARTL in plasma Both standard stock and working solutions used for calibration were prepared daily following the observation of the instability of aqueous solutions of sodium artelinate as discussed below

Assay precision and stability of artelinic acid in plasma

The precision and accuracy of the method were determined by replicate analysis of freshly prepared plasma samples. The results are shown in Table II for plasma samples spiked with 150 or 200 ng of ARTL and treated as unknown The intra- and inter-assay coefficients of variation were, respectively, 13 5% (150 ng/ml, $n=5$) and 14 5% (200 ng/ml, $n=16$) The assay values obtained (Table II) also show that the method is of acceptable accuracy despite the absence of a conventional internal standard

Although ARTL has been reported to be stable when dissolved in a solution of potassium carbonate [3], the compound was found to be unstable in distilled water. Early in the present investigation it was found that using the same stock solution which was stored at 5 °C to prepare calibration curves over a number of days resulted in a decrease in the slope of the calibration curves For example, a slope of 0 00308 was obtained for calibration curves constructed with a freshly prepared solution of sodium artelinate in water while a slope of 0 00196 was obtained for curves prepared using the same solution after three days of storage at 5 °C This is equivalent to a 63 6% decrease in the concentration of ARTL in the stock solution after three days of storage at 5 °C Further evidence

TABLE II

PRECISION AND ACCURACY OF THE ANALYTICAL METHOD FOR THE DETERMINATION OF ARTELINIC ACID IN PLASMA

Actual concentration (ng/ml)	Concentration determined (mean ± S D) (ng 1 ml)	
	Intra-assay	Inter-assay
150	140 6 ± 19 0 (n=5)	141 6 ± 20 4 (n=16)
	138 1 ± 22 0 (n=5)	
	147 8 ± 22 2 (n=3)	
	142 8 ± 8 5 (n=3)	
200	212 8 ± 53 1 (n=5)[a]	230 5 ± 48 0 (n=8)
	111 3 ± 23 6 (n=4)[b]	
	205 0 ± 26 5 (n=6)[c]	

[a]Freshly prepared plasma solution
[b]Plasma solution after storage at −20 °C for three days
[c]Plasma solution after storage at −70 °C for two weeks

of decomposition of the compound was the appearance of a microcrystalline deposit in the container

The decomposition of ARTL in the aqueous solution may be attributed to the slight acidity of the distilled water used in preparing the solution The pH of the stock solution of sodium artelinate was found to be 6 30. When a solution of the drug was prepared using distilled water which had been purged previously with helium for 20 min, the appearance of the microcrystalline deposit which is indicative of decomposition was not observed until after about two weeks of storage

As shown in Table II, ARTL was also found to be unstable in plasma when a solution of the drug in plasma was stored at −20 °C After three days of storage the concentration of ARTL in the plasma solution had decreased by over 50% A solution of the drug in plasma stored at −70 °C was, however, found to remain stable for at least two weeks Similarly, plasma extracts of ARTL reconstituted in acetonitrile are stable when stored at either 5 °C or −20 °C or −70 °C

Animal studies

The assay method was applied to plasma samples obtained from rats given ARTL A chromatogram obtained from analysis of rat plasma taken after intravenous administration of the drug is shown in Fig 3B For comparison, a chromatogram of an extract of a pre-dose plasma sample is also shown in Fig 3A The peak appearing at the retention time of 18 min in Fig 3B was thought to be due to a metabolite of ARTL since the peak height–time profile of this peak was found to be parabolic as might be expected if the peak was due to a

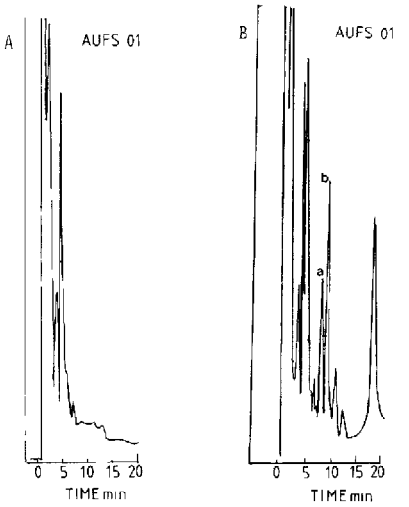

Fig 3 (A) Chromatogram of an extract of drug-free rat plasma (B) Chromatogram of an extract of rat plasma (2 4 μg ml^{-1} artelinic acid) taken 20 min after intravenous administration of artelinic acid as sodium artelinate Peaks a = naphthalene, b = artelinic acid

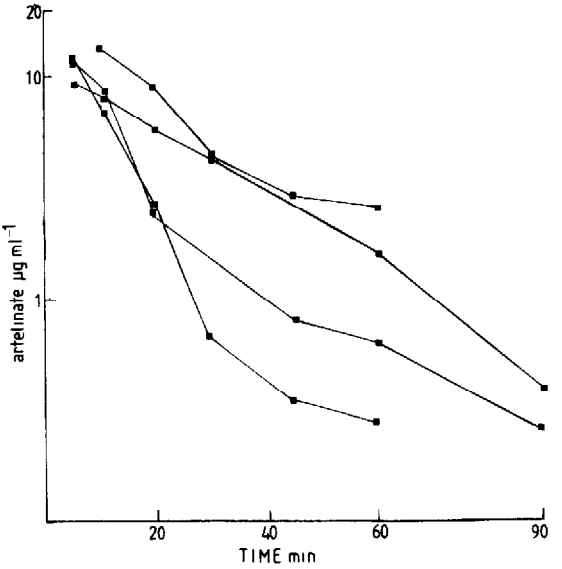

Fig 4 Plasma concentration-time profiles of artelinic acid in four rats given as sodium artelinate (10 mg kg^{-1})

metabolite of ARTL However, mass spectrometry of the eluate fraction corresponding to this peak gave no conclusive information on the structure of this putative metabolite.

Plasma concentration–time profiles of ARTL in four rats given ARTL (10 mg kg^{-1} intravenously) as sodium artelinate are shown in Fig 4. The plasma concentration–time data declined mono-exponentially This contrasts with the observation for QHS and other derivatives of QHS with plasma concentration–time profiles which decline bi-exponentially [4–6] The rapid disappearance of ARTL from the plasma ($t_{\frac{1}{2}} = 17 \pm 5$ min) ($n = 4$) following intravenous administration is, however, consistent with what has been observed for QHS and its other derivatives ARTL could not be detected in the rat plasma samples obtained at 120 and 180 min after intravenous administration

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