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Short Communication

Assay of artelinic acid in serum by high-performance liquid chromatography

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ABSTRACT

A simple and rapid high-performance liquid chromatographic method with UV detection for the determination in serum of the antimalarial artelinic acid is described. Artelinic acid is a water-soluble semi-synthetic derivative of artemisinin (or *Qinghaosu*), which is the parent compound of a completely new class of antimalarials with great potential value [1]. Calibration curves of artelinic acid in serum from 0.5 to 50 μg/ml were found to be linear, and the extraction recovery was *ca.* 70% from rabbit serum and 45% from bovine and human serum. The detection limit of the method was 50 ng/ml when 250 μl of serum were processed. Spiked samples (rabbit serum) proved to be stable for at least 43 days on storage at −20°C. The method has been used for the analysis of *ca.* 400 samples in a pharmacokinetic study. About 50 samples can be processed in half a day.

INTRODUCTION

Artelinic acid is a water-soluble semi-synthetic derivative of artemisinin (Fig. 1). Artemisinin, or *Qinghaosu*, is isolated from the herb *Artemisia annua*. It is the parent compound of a completely new class of antimalarials with great potential value. Being poorly soluble in water and oil, artemisinin is usually administered orally as a tablet or capsule, or intramuscularly as a suspension in oil or an aqueous vehicle. After oral administration the bioavailability in humans is low, *ca.* 30%, probably owing to a substantial first-pass effect [2]. Several semi-synthetic water-soluble derivatives of artemisinin (artesunic acid, artelinic acid; Fig 1), have been developed enabling parenteral administration, important in the treat-

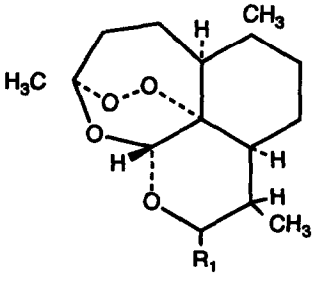

Fig. 1. Molecular structure of artemisinin and its water-soluble derivatives. R_1 = O–CH_2–(C_6H_4)–COOH, artelinic acid; R_1 = O, artemisinin; R_1 = O–(C=O)–$(CH_2)_2$–COOH, artesunic acid.

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ment of cerebral and complicated malaria [3,4]. Unlike artesunic acid, a hemi-succinic ester derivative, artelinic acid is an ether derivative, which is much more stable in aqueous solution [3].

This paper describes a simple high-performance liquid chromatographic (HPLC) method for the determination of artelinic acid in serum. Only one previous report of the assay of artelinic assay has appeared [5]. The methods and results of that study could not be reproduced, so the aim of this study was to develop a simple, rapid and reproducible assay for artelinic acid in serum for routine analysis. The method described here has been used in the pharmacokinetic evaluation of artelinic acid, administered intravenously, intramuscularly, orally and rectally, in rabbits [6], and has been proven to be accurate, reliable and simple to perform.

EXPERIMENTAL

Materials

Sodium artelinate ($C_{25}H_{29}O_7Na \cdot 1.5\ H_2O$) was provided by ACF (Maarssen, Netherlands); acetonitrile (HPLC grade) was obtained from Westburg (Leusden, Netherlands); triethylamine (TEA), diethylamine (DEA), orthophosphoric acid and acetone from Merck (Darmstadt, Germany); and diethyl ether from J.T. Baker (Deventer, Netherlands). Other reagents were of analytical grade.

Chromatography

The HPLC system consisted of an Applied BioSystems SF400 pump; a Waters WISP 710B autosampler; an Applied BioSystems SF783A variable-wavelength UV–VIS detector, operating at 240 nm and 0.05–0.1 a.u.f.s.; and a Hewlett-Packard 3390A integrator. The separation was achieved at ambient temperature on a LiChrosorb RP18 column, 10 cm × 3 mm I.D., particle size 5 μm (Chrompack, Middelburg, Netherlands), which was eluted with a mobile phase consisting of bidistilled water–acetonitrile–TEA (50:50:3, v/v/v) at a flow-rate of 0.4 ml/min. The eluent was adjusted to pH 5.0 using concentrated orthophosphoric acid.

Sample extraction

A 250-μl aliquot of rabbit serum was shaken with 4.5 ml of diethyl ether for 30 s in a stoppered glass 10-ml reagent tube. The phases were separated by centrifugation at 1400 *g* for 10 min and the aqueous layer was frozen in an acetone–dry ice bath. The organic layer was decanted into a clean, dry conical glass tube and evaporated to dryness at 40°C by means of a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase. An aliquot of 80 μl was injected into the HPLC system.

Calibration

Adequate aliquots of sodium artelinate stock solution in bidistilled water (0.1 mg/ml, freshly prepared) were diluted with blank rabbit serum (Gibco Europe, Leusden, Netherlands) to produce standards in serum in the range 0.5–50 μg/ml. Standards in serum were treated further as unknown samples. Additionally, spiked serum samples were processed within each run as a means of assay quality control.

Stability of artelinic acid in serum

Blank rabbit serum, spiked with artelinic acid at a concentration of 2 μg/ml was prepared as described above and stored at −20°C. After 0, 16 and 43 days, stored samples were assayed using the method described above.

Animal study

ICO White New Zealand rabbits, weighing 2.0–2.5 kg, received 0.2 ml of a 20% aqueous sodium artelinate solution intravenously in a marginal ear vein. Aliquots of 0.5–1.0 ml of blood were collected by puncture of the marginal vein of the opposite ear before, and 5, 10, 20, 30, 60, 90 and 120 min after administration. After clotting for at least 1 h at room temperature, blood samples were centrifuged for 20 min at 1400 *g* and the serum was transferred to 1.5-ml polypropylene cups (Greiner, Alphen a/d Rijn, Netherlands). Samples were processed the same day they were collected.

RESULTS

Chromatography

Fig. 2 shows chromatograms of a blank rabbit serum sample and a sample containing artelinic acid at a concentration of 2 μg/ml. The artelinic acid peak eluted at 9.5 min under the described conditions. Though a good retention time and good peak shape were achieved using several other chromatographic systems (reversed phase as well as normal phase systems), the conditions as described in Experimental were found to be the optimum for the separation of artelinic acid from endogenous serum components. It appeared necessary to set the run time to 25 min, owing to late-eluting peaks from endogenous serum components.

Sample preparation

The absolute recovery of artelinic acid from rabbit serum was 68.0 ± 1.37% (concentration 2.0 μg ml^{-1}, $n = 3$) using a simple liquid–liquid extraction with diethyl ether. During the development of the procedure it was observed that the source of the serum affected the recovery markedly. Bovine and human serum exhibited many more endogenous components than rabbit serum, resulting in interfering peaks in the chromatogram. Also the recovery from bovine and human serum was lower, *ca.* 45%.

Calibration

Regression analysis of a typical calibration curve yielded a straight line with a correlation coefficient of 0.9996 ($n = 6$, $p \ll 0.001$), with a slope of $5.107 \cdot 10^5$ μV s ml μg^{-1} and an intercept of $-2.69 \cdot 10^4$ μV s.

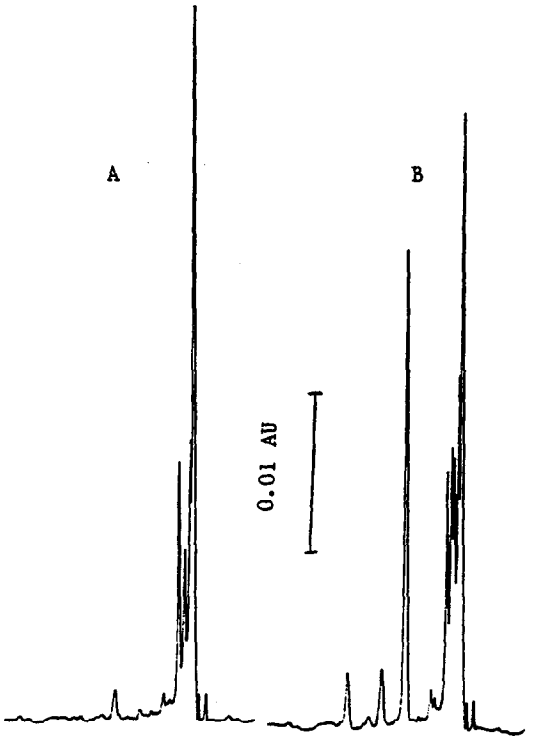

Fig. 2. Chromatograms of artelinic acid after i.v. administration of 20 mg/kg to a rabbit: (A) blank serum; (B) 1 h after administration (2 μg/ml).

Assay statistics and stability of artelinic acid in serum

Table I lists the results obtained from control samples processed during a period of 6 weeks.

Up to 43 days after storage of the spiked serum at −20°C, no degradation of artelinic acid was observed (Table II).

Animal study

Fig. 3 shows a representative concentration–time profile after intravenous administration of

TABLE I

ASSAY STATISTICS OF ARTELINIC ACID IN SERUM

Control sample concentration = 2.0 μg/ml (rabbit serum).

Recovery	68.0 ± 1.37% ($n = 3$)
Within-run variation	2.00 ± 0.033 μg/ml (mean ± S.D., $n = 3$)
Between-run variation:	2.01 ± 0.116 μg/ml (mean ± S.D., $n = 5$)

TABLE II

STABILITY OF ARTELINIC ACID IN SERUM AT −20°C

Spiked rabbit serum at a concentration of 2.0 μg/ml.

Days storage	Concentration (μg/ml)
0	2.00
16	1.98
43	2.05

TABLE III

PHARMACOKINETICS OF I.V. ADMINISTRATION OF SODIUM ARTENILATE TO A RABBIT

20 mg/kg aqueous solution.

Parameter	Value	Unit
c_0	55.2	mg l^{-1}
k_{el}	0.038	min^{-1}
$t_{1/2,el}$	18.2	min
V_d	0.31	l kg^{-1}
Cl	11.8	ml min^{-1} kg^{-1}

20 mg/kg sodium artelinate (aqueous solution) to a rabbit. The curve can be described by a one-compartment model with linear elimination kinetics. The pharmacokinetic parameters resulting from a specimen fit are listed in Table III.

DISCUSSION

Using the described chromatographic method artelinic acid is eluted 9.5 min after injection, well separated from endogenous serum components. The run time is relatively long (25 min) owing unidentified (serum) components eluting after artelinic acid. Though it was possible to achieve good chromatograms from pure standard solutions under several other chromatographic conditions, the described method gave the best selectivity.

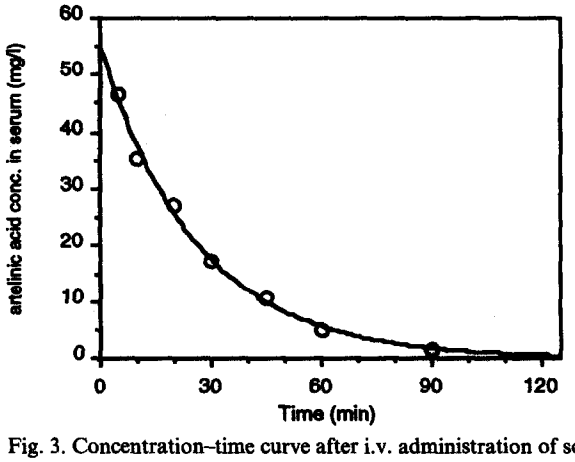

Fig. 3. Concentration–time curve after i.v. administration of sodium artelinate (20 mg/kg aqueous solution) to a rabbit.

During the development of the extraction procedure it was observed that the extracted fraction was constant for each serum source over long time periods, but a marked interspecies variation in extraction recovery occurred. It is not yet fully clear which factors cause this effect. One possibility is high-affinity binding to serum proteins or other serum components, because it was observed that the recovery was at a maximum from rabbit serum, which has the lowest content of proteins. Edwards *et al.* [7] reported recently a remarkable loss of arteether from whole blood on storage of spiked samples at 4 or −20°C, while developing an assay method for arteether. From their studies, the interaction appeared to involve the red cell membrane and the peroxide moiety of the sesquiterpene lactone. Although all our extraction studies were performed with serum or plasma, and no experiments with whole blood were performed in our laboratory, similar strong interactions with endogeous matrix components seem to occur with artelinic acid. Edlund *et al.* [8] reported the formation of a very strong complex of artesunate with haemoglobin, which inhibited the extraction of artesunate from plasma samples into the organic phase. Other effects, such as accumulation into the red cells and binding to, or partitioning into, the cell membrane have been described before for some other antimalarial drugs, *e.g.* primaquine and chloroquine [9,10]. Because these components can be recovered by simple extraction, the nature of their interactions is probably very different from those

involving artemisinin derivatives. Further investigations are needed to study the exact nature of these interactions and the importance of their effect on the interpretation of the pharmacokinetic data for artemisinin derivatives.

Apart from diethyl ether, several other organic solvents (hexane, *n*-chlorobutane, dichloromethane, ethyl acetate, hexane–butanol (95:5), dimethylformamide) were tested to increase the extraction recovery. Also sample treatment methods such as ion-pair extraction (with tetrabutylammonium phosphate), protein denaturation (with trichloroacetic acid, acetonitrile, acetone or the tungstate method [11]) or "salting out" did not yield better results than the simple diethyl ether extraction. The extraction method described by Idowu *et al.* [5], using acetonitrile as both extractant and protein precipitant, could not be reproduced. Besides, the liquid–liquid extractions described [5] were all used on acidified plasma samples. From other experiments in our laboratory [6], it is known that artelinic acid decomposes rapidly in aqueous acidic environments. Liquid–liquid extraction of a neutral serum sample with a solvent of medium polarity (diethyl ether) resulted in a simple, quick and reproducible sample preparation method, producing a reasonably clean extract with sufficient recovery. Calibration curves were linear in the concentration range 0–50 μg/ml. The limit of detection was 50 ng/ml, defined as the concentration of artelinic acid in serum under the given sample preparation method, using a signal-to-noise ratio of 3 as a lower limit.

The method has been used for the analysis of *ca.* 400 samples for a pharmacokinetic study [6]. About 50 samples can be processed in half a day.

The observed intravenous pharmacokinetics are in accordance with the results of Idowu *et al.* [5]. More detailed results and evaluations of the pharmacokinetic study are described elsewhere [6].

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