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J. Pharm. Pharmacol. 1992, 44: 940–942
Communicated February 6, 1992

The binding of the antimalarial arteether to human plasma proteins in-vitro

S. WANWIMOLRUK*‡, G. EDWARDS*†, S. A. WARD†, A. M. BRECKENRIDGE*, *Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX, UK, and †Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK

Abstract—The binding of the novel antimalarial drug, arteether, to human plasma, pure albumin and α_1-acid glycoprotein has been investigated by ultrafiltration, using [^{14}C]arteether. The protein binding in plasma obtained from 11 healthy male subjects ranged from 73·4 to 81·8% bound, with a mean of 78·7 ± 2·1%. The binding of drug in plasma was mainly accounted for by binding to albumin and α_1-acid glycoprotein. Scatchard analysis of the binding data revealed that the binding affinity of arteether to α_1-acid glycoprotein is much greater (20-fold) than that to albumin. This suggests that α_1-acid glycoprotein is the more important binding protein in plasma. This may have clinical importance due to alterations in plasma protein binding in patients with malaria, as the concentration of α_1-acid glycoprotein is markedly increased during malarial infection.

Qinghaosu, also known as artemisinin, is an antimalarial drug isolated from the Chinese herb qinghao (*Artemisia annua* L.). The herb has been used in malaria therapy in China for over 2000 years. Since the discovery of artemisinin in 1972 by Chinese scientists, several semisynthetic derivatives have been prepared and tested for antimalarial activity. Among these derivatives, arteether and artemether, which are respectively ethyl and methyl ethers of dihydroartemesinin (a reduction product of artemisinin) were found to have greater antimalarial activity than artemisinin itself (Klayman 1985; Trigg 1989). Qinghaosu and its potent derivatives such as arteether and artemether, are effective against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, and are effective for the treatment of cerebral malaria (Trigg 1989). Malaria parasite resistance to chloroquine and mefloquine has been rapidly increasing in both degree and prevalence throughout the world (Wernsdorfer 1991). As a result, interest in these potent antimalarial derivatives of qinghaosu has increased. The World Health Organization and the Walter Reed Army Institute of Research have selected arteether for clinical development (Melendez et al 1991). This compound is currently in the preclinical phases of development.

As with many other drugs, pharmacokinetic information such as metabolism, clearance, drug distribution and protein binding, is essential for clinical use and dosage optimization. To our knowledge, clinical pharmacokinetic data of arteether are not available since this requires sensitive and reliable analytical methods. In this communication we report in-vitro protein binding of arteether in human plasma and characteristics of its binding to human serum albumin and α_1-acid glycoprotein.

‡Present address and correspondence: S. Wanwimolruk, School of Pharmacy, University of Otago, PO Box 913, Dunedin, New Zealand.

Materials and methods

Materials. Non-radioactive arteether was supplied by Sapec (Lugano, Switzerland). [^{14}C]Arteether (sp. act. 24·9 μCi mg^{-1}) was synthesized at Research Triangle Institute (Research Triangle Park, NC, USA) and was a gift from the Walter Reed Army Institute of Research (Washington DC, USA). The radiochemical purity of [^{14}C]arteether was 98% by TLC. Human serum albumin (HSA), essentially fatty acid-free; Fraction V HSA and human α_1-acid glycoprotein (AAG) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other chemicals were of analytical grade from conventional commercial sources. All the glassware used was silanized with dichlorodimethyl silane–toluene (5:95, v/v) before use.

Plasma samples. Human plasma or serum samples were obtained from 11 healthy male volunteers, aged 22–39 years (mean age = 30). These subjects had taken no medication for at least a week before blood sampling. The plasma for each subject was mixed with [^{14}C]arteether and non-radioactive drug to a final total drug concentration of 100 ng mL^{-1}. The plasma protein binding of arteether in each sample was then determined by ultrafiltration. Blood bank plasma was also used in a number of binding studies. The final total concentration of arteether in these plasma samples was 100 ng mL^{-1}, unless otherwise stated.

Protein solutions. To determine the extent of arteether binding to HSA and AAG, the individual protein was dissolved in 0·1 M isotonic phosphate buffer (pH 7·4). The protein solutions were then mixed with radiolabelled and non-radioactive arteether, and binding determined.

Methods. A commercially available ultrafiltration apparatus MPS-1 (Centrifree with YMT membrane, Amicon Corp., Danvers, MA, USA) was used. The device was loaded with 1 mL plasma or protein solutions and centrifuged at 1500 *g* for 15 min or until the filtrate amounted to about 10% (i.e. 100 μL) of the initial volume. Ultrafiltrate and the initial solution to which the drug had been added were analysed for radioactivity by liquid scintillation counting. All binding experiments were performed at room temperature (22°C), unless otherwise stated. An alternative ultrafiltration device, Millipore Ultrafree MC filter

Table 1. Binding of arteether to human serum albumin (HSA), α_1-acid glycoprotein (AAG) and plasma from various sources using a total drug concentration of 100 ng mL^{-1}.

Sample	Arteether % unbound	Albumin ($g\ L^{-1}$)	Total protein ($g\ L^{-1}$)
Essentially fatty acid-free HSA (40 g L^{-1})	28·8 ± 1·1 (n = 5)	40	40
Fraction V HSA (40 g L^{-1})	32·6 ± 0·9 (n = 5)	40	40
AAG (0·75 g L^{-1})	39·0 ± 1·2 (n = 5)	—	0·75
Plasma samples from 11 healthy subjects	21·3 ± 2·4 (range: 18·2–26·6)	47·7 ± 2·3	73·2 ± 3·3
Blood bank plasma	20·0 ± 0·3 (n = 5)	39	72

Results are mean ± s.d.

Table 2. Binding constants for arteether (mean ± s.e.) in pure protein solutions, human serum albumin (HSA) and α_1-acid glycoprotein (AAG).

Protein solution	N_1	K_1 (M^{-1})	N_2	K_2 (M^{-1})
Essentially fatty acid-free HSA	0·95 ± 0·03	1·2 (± 0·1) × 10^4	—	—
AAG	2·1 ± 0·4	2·3 (± 0·2) × 10^5	4·3 ± 0·3	0·5 ± 0·0005

Protein concentrations used were 50 μM for HSA and 10 μM for AAG. All binding measurements were determined in duplicate by ultrafiltration, over the range 3–200 μM.

unit (Millipore Ltd., Chester, UK) with PLGC membrane was also used to determine the binding of arteether. Plasma (0·3 mL) was loaded onto the device and centrifuged (2700 *g*) for 20 min. The procedures were similar to those for MPS-1 (Amicon), except a bench-top centrifuge was used. The free (unbound) fraction of arteether was calculated as the ratio of the disintegrations min^{-1} in the ultrafiltrate to that in the protein solution. Protein binding determinations were performed in duplicate. Plasma albumin concentrations were measured by the bromocresol green method. Total plasma protein concentrations were determined by the Biuret reagent.

Data analysis. Binding data for arteether to HSA and AAG was analysed by a curve-fitting procedure using the nonlinear least squares programme Grafit (Data Analysis and Graphic Program, Erithacus Software Ltd, 1990) operated on an IBM computer. The best fit for the binding to albumin was obtained with the one-binding site model (eqn 1):

$$r = \frac{N_1 K_1 [D]}{1 + K_1 [D]} \quad (1)$$

where r = concentration ratio of bound drug to albumin; N_1 = number of binding sites; K_1 = association constant for the binding sites; and [D] = unbound (free) drug concentration. Binding data of arteether to AAG was fitted best with the two-binding sites model (eqn 2):

$$r = \frac{N_1 K_1 [D]}{1 + K_1 [D]} + \frac{N_2 K_2 [D]}{1 + K_2 [D]} \quad (2)$$

where N_1 = number of high affinity binding sites; K_1 = association constant for the high affinity sites; N_2 = number of low affinity binding sites; and K_2 = association constant of the low affinity sites.

Results are reported as mean ± s.d., unless otherwise stated. When appropriate, statistical analysis was performed using Student's *t*-test, $P < 0·05$ was used as the level of significant difference.

Results and discussion

Concentrations of arteether in the ultrafiltrate, after subjecting the buffer containing [^{14}C]arteether to ultrafiltration through the ultrafiltration device (MPS-1), were not significantly different from those in the original buffer samples (n = 4). This indicates that binding of arteether to the ultrafiltrate membranes (YMT) was negligible. Recovery of arteether after filtration through the membrane was 97 and 98% at drug concentrations of 100 and 500 ng mL^{-1}, respectively. Protein leakage, detected by Albustix dipstick (Ames Division, Miles Laboratories, Slough, UK); sensitivity of 0·05 g L^{-1}, was also negligible.

Binding of arteether was similar in serum and heparinized serum. Plasma protein binding was not altered by storage of plasma at −20°C for up to 3 months. The binding of arteether in human plasma was constant over the drug concentration range of 50–1000 ng mL^{-1} and the mean ± s.d. percentage unbound at 50 ng mL^{-1} was 20·0 ± 1·2% (n = 4) and that at 1000 ng mL^{-1} was 20·6 ± 1·3% (n = 4). The therapeutic plasma concentration range of arteether in man has not been established. Mean maximum plasma drug concentrations (C_{max}) of artemisinin in man were reported to be 440 and 331 ng mL^{-1} after oral and intramuscular administration of 40 mg artemisinin, respectively (Titulaer et al 1990). Percentage free fraction of arteether in plasma at room temperature was significantly lower than that at 37°C (20·0 ± 0·3 vs 22·2 ± 0·3%, n = 5), indicating binding decreased as the temperature increased.

Binding of arteether was also evaluated with an alternative ultrafiltration device, Ultrafree MC (Millipore). This new device provides an advantage over the MPS-1 (Amicon) device in that it can be easily centrifuged using a bench-top centrifuge and uses a smaller plasma sample (0·2–0·4 mL). Our results showed that there was a negligible loss of arteether due to binding to the Ultrafree MC (Millipore) filter membrane. The mean value of arteether free fraction in plasma determined by the Ultrafree MC device was 22·6 ± 0·3%, similar to that determined by the MPS-1 (Amicon) device (22·4 ± 0·8%), confirming that the Ultrafree MC is an acceptable alternative technique for measuring plasma protein binding of arteether. This device may be particularly useful for use in paediatric patients with malaria, where volumes are limited.

The extent of arteether binding was examined in two HSA preparations (essentially fatty acid-free and Fraction V), AAG solution, and various samples of plasma. The results are shown in Table 1. The percentage free fractions of arteether in plasma obtained from 11 individuals varied from 18·2 to 26·6%

indicating that the drug was 73·4–81·8% bound in human plasma. The degree of plasma protein binding of arteether is of the same order as those reported for artemisinin and other derivatives. Li et al (1982) reported that artemisinin, dihydroartemisinin, artemether and sodium artesunate all bind to human serum proteins, with a degree of binding of 64, 43, 76 and 59%, respectively. The extent of plasma protein binding of arteether at 100 ng mL^{-1} is relatively low when compared with other antimalarial drugs such as quinine (Mihaly et al 1987; Mansor et al 1991; Silamut et al 1991) and pyrimethamine (Rudy & Poynor 1990). The degree of binding with HSA or AAG was similar to that observed with plasma. This suggests that arteether is bound to both HSA and AAG. The concentrations of albumin (40 g L^{-1}) and AAG (0·75 g L^{-1}) used are the normal physiological concentrations. The concentration of AAG employed in this study was much less than that of HSA, suggesting that AAG is the more important binding protein in human plasma for arteether binding. The mean percentage unbound fraction of arteether in Fraction V HSA (containing 1·4 mol fatty acid (mol HSA)$^{-1}$) was $32·6 \pm 0·9\%$ and this was significantly ($P < 0·05$) greater than that in the fatty acid-free HSA solution ($28·8 \pm 1·1\%$). This indicates that fatty acids may displace arteether from the binding sites on albumin.

The binding characteristics of arteether to pure HSA (50 μM) and AAG (10 μM) were evaluated by ultrafiltration, and the binding constants were determined (Table 2). Results of fits to data obtained for HSA revealed that the binding of drug to albumin was adequately described by the model with one binding site and the Scatchard plot for the binding of arteether to HSA was linear. HSA has approximately one binding site ($N = 0·95$) for arteether with an estimated association constant of $1·2 \times 10^4$ M^{-1} (i.e. dissociation constant of 82·1 μM). In contrast, the binding data for AAG was best fitted by the model with two binding sites (Table 2). The nonlinearity of the Scatchard plot indicated that the binding sites for arteether on AAG were heterogeneous. The data suggests there are two high affinity binding sites ($K_1 = 2·3 \times 10^5$ M^{-1}; $N_1 = 2·1$) and at least four further binding sites with a very low affinity for arteether ($K_2 = 0·5$ M^{-1}; $N_2 = 4·3$) on AAG. The affinity of the low affinity binding sites on AAG is extremely low which suggests that this binding may be a minor contribution from some impurities (e.g. globulins) in the AAG preparation used. The binding data obtained from pure protein solutions (Table 2) also revealed that arteether is bound more strongly to AAG than to albumin; the binding affinity for AAG is approximately 20-fold greater than that for albumin. This supports our suggestion that AAG is the more important binding protein in plasma for the binding of arteether. A similar observation was previously reported for the antimalarial drug, quinine (Mihaly et al 1987) and many other basic drugs, e.g. lignocaine, alprenolol and imipramine (Piafsky & Borga 1977; Routledge 1986).

Although the interindividual variation in arteether binding to plasma was low, the variation may be large in patients with malaria as the concentration of acute phase proteins (including AAG) has been shown to increase during malaria infection (Mansor et al 1991; Silamut et al 1991). This could have clinical importance due to alteration in plasma protein binding in patients with malaria.

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