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Comparison of bioassay and high performance liquid chromatographic assay of artesunate and dihydroartemisinin in plasma

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Abstract

The study was a comparison of bioassay and HPLC analysis of artesunate (ARTS) and dihydroartemisinin (DHA) in plasma. ARTS and DHA in plasma samples from patients treated with ARTS were quantified by HPLC and expressed as DHA. DHA-equivalents in the same plasma samples were measured using a standardised parasite culture technique. DHA concentrations estimated by both methods were highly correlated (bioassay $= 0.96 \times$ HPLC $+ 11.0$; $r^2 = 0.92$). At high concentrations ($> 12\,000$ nmol/l) bioassay sometimes overestimated DHA. Bioassay of active drug in plasma correlates well with specific chemical analysis by HPLC. ARTS and DHA appear to account for the total antimalarial activity in plasma after ARTS administration.
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1. Introduction

Bioassay is sometimes a convenient and sensitive method of analysis when chemical assays are either inaccessible or have not yet been developed. It also has the advantage that it can account for activity of drug metabolites that have not been identified or are not quantifiable by other methods.

The antimalarial drug artesunate (ARTS) is a hemisuccinate ester that is rapidly and completely hydrolysed to dihydroartemisinin (DHA) following the administration of ARTS by both parenteral and extravascular routes (Lee and Hufford, 1990; Batty et al., 1998; Davis et al., 2001; Ilett et al., 2002a). ARTS and DHA have been quantified in plasma by both bioassay (Bethell et al., 1997;

Na-Bangchang et al., 1997; Newton et al., 2000) and high performance liquid chromatographic (HPLC) (Batty et al., 1996a; Karbwang et al., 1997; Navaratnam et al., 1995) methods. Concordance between the bioassay and specific HPLC methods has not been investigated in a rigorous manner and, therefore, we set out to provide such data using plasma samples collected from patients with falciparum malaria who had been treated with intravenous and oral ARTS (Batty et al., 1998).

2. Patients and methods

2.1. Patients and blood sampling procedures

The plasma samples used for the method comparison were from a previously published pharmacokinetic study in nine Vietnamese volunteers who received either i.v. ARTS (120 mg), or oral ARTS (150 mg as 3×50 mg tablets) on two separate occasions, 8 h apart (Batty et al., 1998).

2.2. High performance liquid chromatographic analysis

Plasma samples were assayed for ARTS and DHA during November, 1997 using a validated HPLC method (Batty et al., 1996a). To enable a direct comparison with bioassay, the total concentration of drug in each plasma sample was expressed in terms of DHA concentration using the formula:

$$DHA \ (nM)$$
$$= DHA \ (nM \ by \ HPLC)$$
$$+ \left[\frac{ARTS \ (nM \ by \ HPLC) \times 284.36}{384.43} \right]$$

where 284.36 and 384.43 are the molecular weights of DHA and artesunic acid, respectively.

2.3. Bioassay

Parasites (chloroquine resistant K1 isolate from Thailand) were maintained in continuous culture (Trager and Jensen, 1976) using RPMI 1640 LPLF medium (Gibco, Grand Island, NY) supplemented with glucose (2 g/l), hypoxanthine (0.05 g/l), HEPES (5.97 g/l), sodium bicarbonate (2.1 g/l) and pooled human sera (10%). Parasite stages were synchronised using sorbitol (Lambros and Vanderberg, 1979) and were used for the assay procedure when more than 95% were rings and multiplication index was 5-fold or higher.

A modification of the 3H-hypoxanthine incorporation technique (Desjardins et al., 1979) was used to determine antimalarial activity of human plasma samples containing ARTS and/or DHA. Briefly, dilutions (1:4096 to 1:16 384) of drug-treated plasma (in drug free serum) were prepared in 96-well microplates. The susceptibility of parasites to ARTS (0.2–100 nmol/l) and DHA (0.2–100 nmol/l) also was assessed in parallel. All plates were inoculated with parasitised red blood cell suspensions in culture medium, incubated, and then harvested by standard procedures. The dilution of drug-plasma (ID_{50}) and the concentration of drug (IC_{50}) that inhibited 50% of parasites, relative to control cultures, developing from rings to schizonts were obtained by nonlinear regression analysis (logistic dose response equation $y = a + b/(1 + (x/c)^d)$, where x = plasma dilution, y = % parasite growth compared to control, a = minimum response, b = maximum response, $c = ID_{50}$ value and d = slope exponent) using TableCurve™ (Jandel Scientific, San Raphael, USA). The total antimalarial activity of plasma samples was expressed as DHA concentration equivalents ($ID_{50} \times IC_{50}$ for DHA). These analyses were carried out in April/May, 1999.

2.4. Statistical analysis

Differences between means were assessed using Student's t-test (two-sided). The comparison between bioassay and HPLC results was made by Passing and Bablock regression analysis, (Bablok et al., 1988) (Biostats® Software Ltd, Fremantle, WA), and the difference between the two methods was assessed as described by Bland and Altman (Bland and Altman, 1986).

3. Results

The molar IC_{50} bioassay values for ARTS (2.91 ± 0.96 nmol/l mean ± S.D.; $n = 4$) and DHA (2.42 ± 0.8 nmol/l) were not significantly different (t-test = 0.79, $P = 0.46$).

There was a significant correlation between bioassay and HPLC data (Fig. 1A; bioassay = $0.96 \times$ HPLC + 11.0; $r^2 = 0.92$). The 95% CI's for the slope and intercept were 0.93 to 0.99 and −20.5 to 44.3 nmol/l, respectively. Generally, the plot of bioassay–HPLC versus the mean of
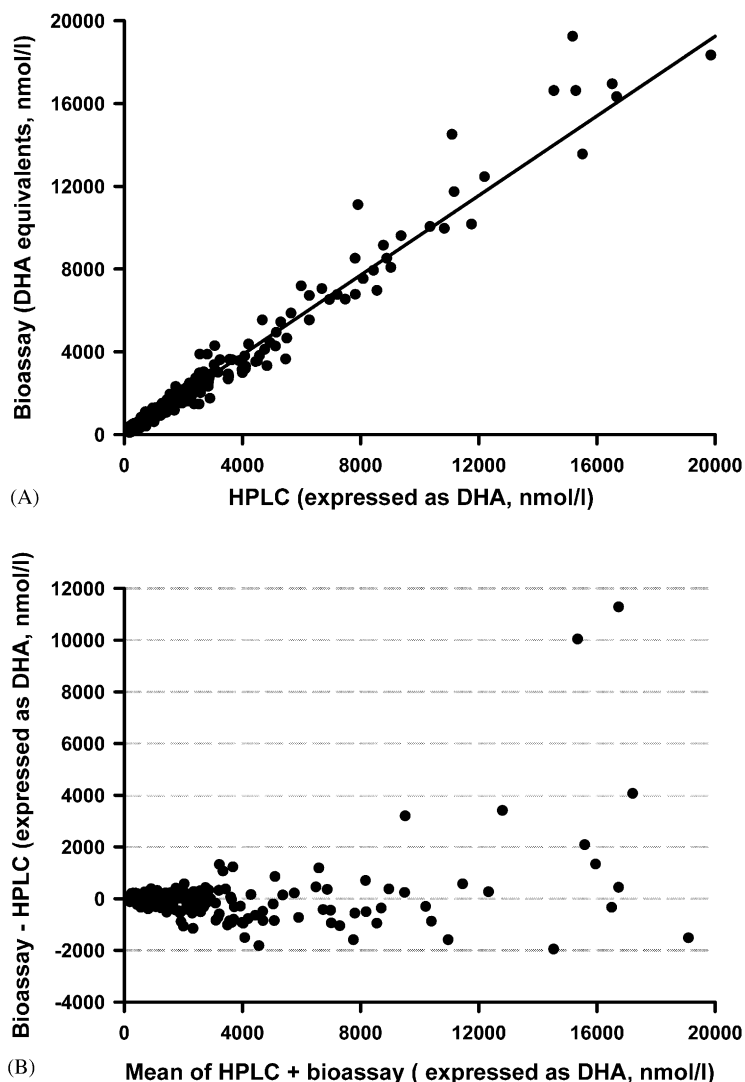

(A)

(B)

Fig. 1. Comparison of HPLC and bioassay methods for analysis of DHA equivalents in plasma ($n = 249$) from Vietnamese volunteers who received ARTS i.v. (312 μmol) or orally (391 μmol) on separate occasions. Panel (A) shows a Passing and Bablock regression plot (bioassay = $0.96 \times$ HPLC + 11.0; $r^2 = 0.92$) and panel (B) a plot of the difference between the bioassay and HPLC data sets vs. the mean of the two methods.

bioassay plus HPLC (Fig. 1B) showed that differences between the methods were distributed evenly on either side of zero. However, bioassay sometimes significantly overestimated DHA at high concentrations (> 12 000 nmol/l).

4. Discussion

In a previous comparison of bioassay and HPLC methods of analysis for chloroquine, we used the relative IC_{50} potencies of chloroquine and its active mono-desethyl metabolite to convert the HPLC metabolite concentrations to equivalents of the parent drug (Kotecka and Rieckmann, 1993). In the present study, we were able to take a simpler approach to the method comparison. ARTS concentration in each plasma sample (nmol/l) was expressed in terms of its molar DHA content (nmol/l) and added to the measured DHA concentration to give a total DHA content in nmol/l. The data could then be compared directly to those for bioassay (also in nmol/l). The ability to take this approach, rather than that previously utilised, arises because of the similar the molar IC_{50} bioassay potencies for ARTS and DHA. It is not surprising that the IC_{50} values for ARTS and DHA are similar, given their close structural similarity and the fact that the neutral pH and 37 °C (conditions similar to those for the bioassay, ARTS undergoes a first-order hydrolysis to DHA with a $t_{1/2}$ of around 11 h (Batty et al., 1996b).

Our data support the contention that ARTS and DHA are the only pharmacologically active antimalarials present in significant quantities in plasma following ARTS administration (Zhao et al., 1988; Lee and Hufford, 1990; Batty et al., 1998). Moreover, they are in agreement with a recent finding that the major metabolic product of DHA in humans is an α-DHA-β-glucuronide (Ilett et al., 2002b) that lacks antimalarial activity (Ramu and Baker, 1995). The reason for the higher estimates of DHA equivalents for the bioassay compared with HPLC in a few plasma samples with high ARTS concentrations is unclear. However, these samples were those obtained after i.v. administration of ARTS and its our view that the large serial dilutions required to bring drug concentration down to the dynamic range of the bioassay may be involved. Between-day variability in the relative potencies (IC_{50}) for ARTS and DHA in the bioassay also may have contributed. Since the bioassay analyses were carried out some 17–18 months after HPLC analyses, degradation of ARTS or DHA does not provide the explanation for the higher DHA equivalents in some samples. Stability problems are also unlikely as our respective laboratories also have shown that these analyses (in frozen plasma) are stable over at least 12 months (data not shown).

Two studies have provided limited pharmacokinetics based on data derived from bioassay and HPLC. In one study where DHA was administered to eight patients with uncomplicated falciparum malaria (Na-Bangchang et al., 1997), the plasma DHA concentration profile as determined by HPLC paralleled the maximum inhibitory dilution of equivalent plasma samples. The second study used ARTS administration to patients with uncomplicated falciparum malaria (Newton et al., 2000), and in a subset of three patients given oral ARTS, the $AUC_{0,24h}$ values for bioassay were 72, 95 and 102%, respectively of the mean $AUC_{0,24h}$ for DHA measured by HPLC assay. These findings accord with those of the present study. Overall, we conclude that bioassay of ARTS/ DHA in plasma has an excellent correlation with specific HPLC analysis.

In the rural tropics, surveillance for treatment failure is an important aspect of malaria control. Parasite factors can be identified using in vitro sensitivity assays but sub-therapeutic drug concentrations, through factors such as poor drug quality, sub-optimal formulation and a lack of adherence to dosing schedules, may also be important contributors. Our data indicate that bioassay can provide reliable data in this respect. Since malaria parasite culture can be carried out in situations where only basic laboratory facilities exist, it can be used to identify causes of inadequate response to treatment and thus make a valuable contribution to malaria control programmes.

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