J. Pharm. Pharmacol. 1987, 39: 261–265
Received August 22, 1986

A preliminary pharmacokinetic study of the antimalarial drugs, proguanil and chlorproguanil

W. M. WATKINS*†‡, J. D. CHULAY¶, D. G. SIXSMITH*, H. C. SPENCER*§ AND R. E. HOWELLS‡

*Biomedical Sciences Research Centre, Kenya Medical Research Institute, Nairobi, †Wellcome Trust Research Laboratories, PO Box 43640 Nairobi, Kenya, ‡Liverpool School of Tropical Medicine, ¶Department of Immunology, Walter Reed Army Institute of Research, Washington DC, and §Division of Parasitic Diseases, Centers for Disease Control, Atlanta, GA 30333, USA

Pharmacokinetic parameters for cycloguanil and chlorcycloguanil, the active metabolites of proguanil (Paludrine] and chlorproguanil (Lapudrine) have been measured in a bioassay which assesses the in-vitro growth inhibition of a cycloguanil- and chlorcycloguanil-sensitive strain of *Plasmodium falciparum* produced by dilutions of plasma collected after oral administration of the pro-drugs. A single compartment model is applicable for cycloguanil with mean rate constants of elimination of 0·0624 h^{-1} and availability of 0·2398 h^{-1}. The elimination profile for chlorcycloguanil indicates partition of drug into more than one compartment. In 2 of 10 subjects dosed with proguanil and 1 of 11 subjects dosed with chlorproguanil, the active metabolite levels were significantly lower than the mean for the other subjects. Abnormally low cycloguanil or chlorcycloguanil plasma levels may be of importance in relation to effective prophylaxis against malaria.

The biguanide antimalarials proguanil and chlorproguanil, developed soon after the second world war, were removed from the World Health Organisation list of recommended prophylactics in 1982 because it appeared that resistance by *Plasmodium falciparum* to this class of drug was widespread (Bruce-Chwatt 1982; Peters 1982; Wernsdorfer & Kouznetsov 1980) although reports supporting their usefulness continued (Olsen 1983; McLarty et al 1984). In 1984 the biguanides were again officially recommended by WHO at a dosage of 200 mg proguanil daily or 20 mg chlorproguanil weekly (WER 1984) although, in the absence of recent controlled trials their efficacy remains uncertain (MMWR 1985).

The antimalarial activity of the biguanides is due to metabolism of the parent drug to a triazine metabolite which is a potent inhibitor of plasmodial dihydrofolate reductase (Schmidt et al 1952; Crowther & Levi 1953). The pro-drug proguanil and the minor metabolite or degradation product *p*-chlorophenylbiguanide (Carrington et al 1951) have virtually no action alone against the parasite in-vitro although they interact with the active metabolite, indicating a complex total biological effect (Watkins et al 1984a). The biguanides are free from serious side effects (White 1985) and are receiving renewed attention as a result of the continuing spread of chloroquine-resistant *P. falciparum*, including the emergence of such strains in East Africa (Sixsmith et al 1984; Watkins et al 1984b). Analytical techniques which have previously been used to measure the plasma concentrations of proguanil and its metabolites have either measured antifolate activity by means of a microbiological assay using *Streptococcus faecalis* as the test organism (Smith et al 1961) or the amount of material hydrolysable to *p*-chloroaniline (Maegraith et al 1946). These techniques measure both pro-drug and metabolites, and in neither case is the measurement necessarily related to biological activity against the malaria parasite. A high performance liquid chromatographic (HPLC) technique has been reported (Moody et al 1980), although this method was unable to detect cycloguanil in serum following an oral dose of proguanil 200 mg. In the present study, we describe a bioassay which utilizes a dihydrofolate reductase-sensitive strain of *P. falciparum* as the test organism, and expresses activity in terms of cycloguanil or chlorcycloguanil equivalents, from which pharmacokinetic parameters of antimalarial activity can be calculated.

† Correspondence.

MATERIALS AND METHODS

Proguanil study

Ten healthy adult volunteers (five Kenyan, five European), with no history of antimalarial drug ingestion over the previous two months, each received a single dose of proguanil hydrochloride 2·5 mg kg^{-1}. The drug was given following overnight fasting and solid food was withheld for 4 h after dosing. Finger-prick blood samples were collected in heparinized microhaematocrit tubes, sealed at one end, centrifuged to separate cells from plasma, and

stored at 4 °C until assayed. Samples were taken immediately before dosage and at 1, 2, 3, 4, 5, 6, 10, 15 and 24 h after dosage. Haemograms (haemoglobin, white cell count, red cell count, packed cell volume, mean cell volume), renal function tests (serum Na, K and creatinine, blood urea nitrogen and uric acid) and liver function tests (alkaline phosphatase, serum alanine aminotransferase and total bilirubin) were determined for all subjects. All values were within normal ranges.

Chlorproguanil study

Eleven healthy adult volunteers (six Kenyan, five European) with no history of antimalarial drug ingestion over the previous two months each received a single dose of chlorproguanil hydrochloride 1·44 mg kg^{-1}. Blood samples were collected as above at 2, 4, 6, 8, 10, 24, 48, 72 and 96 h after dosage. Haemograms, renal function and liver function tests were normal for all subjects.

Assay method

In brief, plasma drug concentrations were determined by comparing the amount of test plasma required to produce 50% inhibition of *P. falciparum* growth in-vitro with the concentration of cycloguanil or chlorcycloguanil required to produce the same degree of inhibition in simultaneous control cultures using the same organism. Although the bioassay measured the total effect of pro-drug and metabolites on the parasite, results were expressed as nmol L^{-1} of cycloguanil or chlorcycloguanil equivalents, since these compounds are the active moieties.

P. falciparum strain M24, originally isolated from Malindi, Kenya, was used as the test organism, and was cultured by minor modifications to the methods of Trager & Jensen (1976) and Haynes et al (1976). RPMI medium 1640 containing no added *p*-aminobenzoic acid or folic acid (GIBCO Laboratories, Grand Island, NY, USA) was supplemented with 10% heat-inactivated normal human serum, 25 mM HEPES buffer and 25 mM $NaHCO_3$. Cultures were grown in a gas environment of 3% CO_2, 5% O_2 and 92% N_2. The M24 strain has been maintained in laboratory culture for over three years, during which time the concentration of cycloguanil causing 50% inhibition of growth (ID50) in this medium, as measured by the radioisotopic method of Desjardins et al (1979) has remained constant within the range 0·89 ± 0·61 nM (mean ± s.d.).

For the assays, culture medium (25 μL) was added to wells of a 96-well, flat bottom microculture plate by means of a repeating dispenser (Hamilton Co., Reno, NA, USA). Test plasma was removed from the haematocrit tubes by snapping them open above the cell sediment and approximating a micropipette tip to the section containing supernatant. Duplicate 25 μL plasma aliquots were added to the 25 μL culture medium in the second row of the plate. Serial two-fold dilutions were prepared using a 'Titertek' hand multidiluter (Flow Laboratories, Irvine, UK), giving seven duplicate plasma dilutions for each sample. A maintenance culture of the test organism was diluted with washed, 12–28 day-old O + ve RBC and fresh culture medium to a 1·5% haematocrit and 0·4% parasitaemia, and 200 μL aliquots were added to all test wells. The top row of 12 wells on each plate served as controls, 8 wells containing parasitized erythrocytes (PRBC control) and 4 wells containing non-parasitized erythrocytes (URBC control). For each subject studied, duplicate control columns were included to measure the ID50 of the test organism to cycloguanil or chlorcycloguanil alone and in the presence of pre-dose plasma. The plates were placed in a gas-tight box, flushed for 1 min with 3% CO_2, 5% O_2 gas mixture and incubated at 37 °C. After 48 h all the wells were labelled by the addition of 0·5 μCi [^{3}H]hypoxanthine in 25 μL medium. After a further 18 h incubation, the wells were harvested for the measurement of radioisotope incorporation (Desjardins et al 1979). We have previously reported that a 48 h drug exposure is more appropriate than 24 h in measuring the activity of dihydrofolate reductase inhibitors, which act late in the erythrocytic cycle (Sixsmith et al 1984).

The seven duplicate test wells comprising the bioassay for each sample contained the subjects' plasma in concentrations ranging from 5·56 to 0·09%, in addition to the 10% serum in the culture medium. This additional plasma provides extra hypoxanthine and other purines which reduce the uptake of [^{3}H]hypoxanthine by parasites with the same growth rates (Chulay et al 1983). Since preliminary experiments showed that suppression of isotope uptake by parasites was proportional to the concentration of added plasma from 0·09 to 5·56%, the counts min^{-1} of all test wells were corrected for this effect by the inclusion of a duplicate control column of wells with pre-dose plasma only, at the same concentrations as test wells;

$$\text{corrected test counts min}^{-1} = \text{mean uncorrected test counts min}^{-1} \times \frac{\text{mean PRBC control cpm}}{\text{mean pre-dose plasma control counts min}^{-1} \text{ at the same concentration}}$$

The ID50 values for cycloguanil or chlorcycloguanil alone were computed by interpolation after logarithmic transformation of both the drug concentration and counts min^{-1} as described previously (Sixsmith et al 1984). The ID50 values for these drugs in pre-dose plasma were computed by the same method after applying the above correction for suppression of isotope uptake. The percentage (v/v) of each sample of test subject plasma required to inhibit growth of the test organism by 50% was calculated by the same method after correcting test counts min^{-1} values for isotope uptake suppression, and substituting plasma concentration for drug concentration. Drug equivalents for each plasma sample were given by:

$$\text{plasma drug equivalents (nmol)} = \frac{\text{ID50 (nmol)} \times 100}{\text{\% added plasma giving 50\% inhibition of growth}}$$

The sensitivity and reproducibility of the bioassay was assessed by the assay of normal plasma to which had been added known amounts of chlorcycloguanil.

RESULTS

The bioassay was able to measure the amount of chlorcycloguanil added to normal plasma with acceptable accuracy and reproducibility (Table 1).

Table 1. Sensitivity and reproducibility of the bioassay in measuring chlorcycloguanil added to normal plasma.

Added to plasma (nmol L^{-1})	Chlorcycloguanil found by bioassay (nmol L^{-1})	Mean ± s.d.
100	95·4, 83·2, 83·0, 107·8	92·3 ± 11·8
30	29·6, 29·6, 29·8, 29·2, 29·8	29·6 ± 0·24
10	15·1, 14·5, 14·5, 15·0, 14·9	14·8 ± 0·29

The ID50 of cycloguanil in the 10 bioassays performed was 1·05 ± 0·55 nmol L^{-1} (mean ± s.d.) for the drug alone and 1·19 ± 0·80 nmol L^{-1} (mean ± s.d.) in the presence of pre-dose plasma. These values are not significantly different ($P > 0·3$), indicating that the action of cycloguanil was not modified appreciably by pre-dose plasma constituents of any of the subjects studied. Similar results were obtained for chlorproguanil. There was an inverse relationship between isotope incorporation in the pre-dose plasma controls and the concentration of subject plasma in the culture system (Fig. 1). No significant difference ($P > 0·05$) was observed in this effect between black and white subjects, indicating that the immune status of individuals was either similar at the time or that antibodies present had no effect on the test system. None of the subjects had lived in an area endemic for malaria in the previous three years. Suppression by subject plasma of isotope uptake by parasites was greater than that predicted from theoretical considerations (Chulay et al 1983) but was controlled for in all individual bioassays.

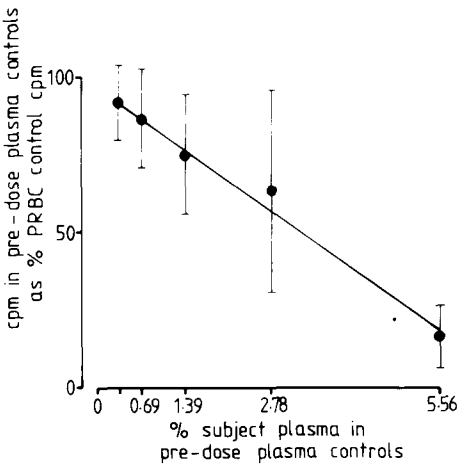

FIG. 1. The effect of concentration of subject plasma in the test system on the incorporation of [^{3}H]hypoxanthine in pre-dose plasma controls.

In the proguanil-dosed group, peak plasma cycloguanil concentrations occurred 5 h after administration. The rate constant of elimination, Ke, was determined from the linear portion of the log concentration vs time graph using standard methods, and did not differ significantly between black (0·0565 ± 0·014 h^{-1}) and white (0·0670 ± 0·025 h^{-1}) subjects ($P > 0·2$). Combining the results from all subjects gave Ke = 0·0624 ± 0·020 h^{-1}. The availability rate constant, Ka, which in the case of proguanil incorporates the constants of absorption and metabolism, was determined by the standard method of curve stripping (Portmann 1970), giving a mean value of 0·2398 ± 0·20 h^{-1}. The results are consistent with a one compartment model with first order availability and elimination (Fig. 2). Two individuals (one black, AN and one white, NP) exhibited plasma levels which were significantly lower than the mean for the other eight subjects ($P < 0·01$) 4 h after dosage and at all subsequent sampling times.

In the chlorproguanil-dosed group, chlorcycloguanil levels in plasma reached a maximum of 50·12 nmol L^{-1} 10 h after administration. There was no significant difference in peak plasma levels between black and white subjects ($P > 0·05$). Unlike cycloguanil, the elimination profile of chlorcycloguanil suggests that the drug distributes into more than one compartment. Chlorcycloguanil was elimi-

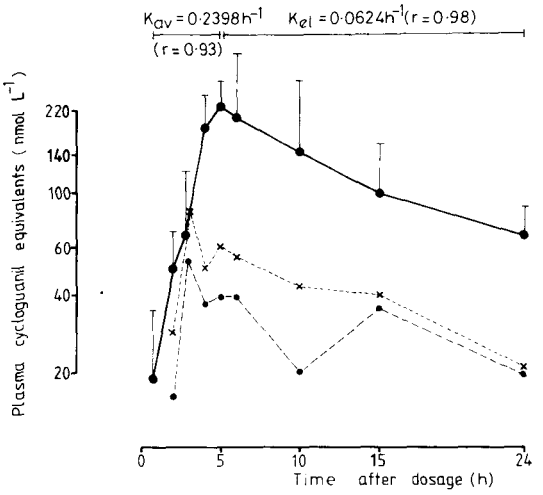

FIG. 2. Plasma concentrations of cycloguanil following a single oral dose of proguanil hydrochloride 2·5 mg kg^{-1}. Shown are the geometric means and standard deviations for 4 Kenyan and 4 European adults (—●—), and individual values for subjects AN (— • —) and NP (—×—).

nated from a central compartment of approximately 53 L kg^{-1} with a rate constant a = 0·063 h^{-1}, followed by a phase of slower elimination (b = 0·0080 h^{-1}) (Fig. 3). The intercept on the ordinate of the back-extrapolated exponential slope was 20·42 nmol. As was found in the proguanil study, one of the eleven subjects dosed with chlorproguanil exhibited significantly lower levels of chlorcycloguanil ($P < 0·02$) which were measurable only in the 6, 8 and 10 h plasma samples.

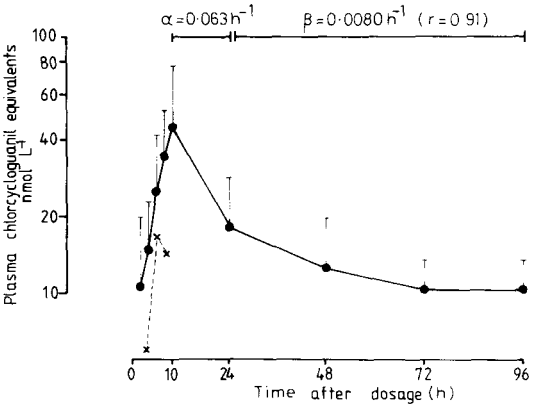

FIG. 3. Plasma concentrations of chlorcycloguanil following a single oral dose of chlorproguanil hydrochloride 1·44 mg kg^{-1}. Shown are the geometric means and standard deviations for 5 Kenyan and 5 European adults (—●—), and individual values for subject GC (—×—).

DISCUSSION

Our results indicate that the pharmacokinetics of proguanil and chlorproguanil are similar in Kenyans and Europeans. At a proguanil dose of 2·5 mg kg^{-1} (equivalent to 200 mg proguanil for an 80 kg individual), the geometric mean plasma concentration reached a peak of 162·2 nmol L^{-1} cycloguanil equivalents after 5 h and averaged 51·3 nmol L^{-1} after 24 h, the time when the next dose would normally be administered. The mean elimination rate constant for cycloguanil (Ke = 0·0624 h^{-1}) is equivalent to a half-life ($t_{\frac{1}{2}}$) of 11·1 h. This value is comparable with a mean clearance time (5 half-lives) of 64 h reported by Urbanski et al (1964) and the mean half life of 12·4 h which can be derived from the urinary excretion data for 4 subjects presented by Smith et al (1961). Repeated daily dosage with proguanil will lead to drug accumulation, as is indicated by the amount of drug remaining in plasma at 24 h (Fig. 2). Applying Dost's formulae for multiple dose kinetics (Ritschel 1982), repeated doses of 200 mg proguanil at 24 h intervals should result in a steady state maximum plasma concentration of 265 nmol L^{-1} cycloguanil equivalents, which is reached 4·37 h after dosage, and a minimum plasma concentration of 91 nmol L^{+1}. These values for the triazine metabolite are similar to those reported for proguanil and metabolites by Maegraith et al (1946) who found a maximal plasma concentration 4 h after oral dosage, and a low degree of drug accumulation after repeat doses, leading to steady state conditions after approximately three days.

The calculation of mean drug equivalent levels from individual results (Figs 2, 3) was associated with variance which resulted in part from imprecision in the method and also from the differences in plasma drug concentration between subjects at the same sampling time. However, the drug concentrations of 2/10 subjects in the proguanil study and 1/11 subjects in the chlorproguanil study were significantly lower than the mean values for the other subjects. These anomalies could not be related to the liver function test results, which were normal in all cases. Although the number of subjects studied is small, this observation may be of importance, since it suggests that a substantial proportion of individuals may exhibit abnormal absorption or metabolism of proguanil and/or chlorproguanil. The plasma concentrations of cycloguanil equivalents in subjects AN and NP ranged from 21 to 62 nmol L^{-1}, and 21 to 84 nmol L^{-1}, respectively. In a comparable in-vitro test, 14 nmol L^{-1} cycloguanil was required to achieve 50% growth inhibition of 16 resistant *P.*

falciparum isolates (Watkins et al unpublished observations). The comparatively low cycloguanil plasma concentrations achieved by subjects AN and NP may be associated with an increased risk of breakthrough of prophylaxis by the erythrocytic stages of resistant parasites. The abnormally low chlorcycloguanil levels of subject GC present a potentially greater risk of breakthrough. At the chlorproguanil dosage used, which is over four times that recommended by WHO for prophylaxis, minimum chlorcycloguanil levels 96 h after dosage were approximately 10 nmol L^{-1} and would be even lower at 168 h, the time of the next dose. Since resistant parasites have an ID50 to chlorcycloguanil in-vitro of 2 nmol L^{-1} (Watkins et al unpublished observations), and, from our results, weekly dosing with chlorproguanil would not lead to drug accumulation, any further reduction of chlorcycloguanil plasma levels would increase the risk of prophylaxis breakthrough by resistant parasites.

In general, bioassays have poor reproducibility in comparison with more precise physicochemical methods, and consequently they require a more complex assay design. It is therefore likely that a physicochemical assay for cycloguanil, perhaps by HPLC, will prove more advantageous than the assay described here. However, our results have provided kinetic data on two potentially useful chemoprophylactic agents. Further, our results may explain why the HPLC technique described by Moody et al (1980) was unable to detect cycloguanil in serum following 200 mg oral proguanil, since the detection level for cycloguanil of 60 ng mL^{-1} (243 nmol L^{-1}) which they report exceeds the mean peak plasma concentration of 162 nmol L^{-1} cycloguanil measured by bioassay in our study.

Acknowledgements

We are grateful to Professor M. Mugambi, Director, Kenya Medical Research Institute, for permission to publish these results. We acknowledge with thanks the excellent technical assistance provided by the staff of the Biomedical Sciences Research Centre, KEMRI. Financial support was received from the Wellcome Trust, and from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. W. M. W. is also grateful to the Wellcome Trust for personal support.

REFERENCES

Bruce-Chwatt, L. J. (1982) Br. Med. J. 285: 674–676

Carrington, H. C., Crowther, A. F., Davey, D. G., Levi, A. A., Rose, F. L. (1951) Nature 168: 1080

Chulay, J. D., Haynes, J. D., Diggs, C. L. (1983) Exp. Parasitol. 55: 138–146

Crowther, A. F., Levi, A. A. (1953) Br. J. Pharmacol. 8: 93–97

Desjardins, R. E., Canfield, C. J., Haynes, J. D., Chulay, J. D. (1979) Antimicrob. Ag. Chemother. 16: 710–718

Haynes, J. D., Diggs, C. L., Hines, F. A., Desjardins, R. E. (1976) Nature 263: 767–769

Maegraith, B. G., Tottey, M. M., Adams, A. R. D., Horner Andrews, W. H., King, J. D. (1946) Ann. Trop. Med. Parasitol. 40: 493–506

McLarty, D. G., Webber, R. H., Jaatinen, M., Kihamia, C. H., Murry, M., Kumano, M., Aubert, B., Magnuson, L. W. (1984) Lancet ii: 656–658

Moody, R. R., Selkirk, A. B., Taylor, R. B. (1980) J. Chromatog. 182: 359–367

Morbidity and Mortality Weekly Report (1985), Centers for Disease Control, Atlanta, USA, vol. 34, no. 14, p. 189

Olsen, V. V. (1983) Lancet i: 649

Peters, W. (1982) Br. Med. Bull. 38: 187–192

Portmann, G. A. (1970) in: Swarbrick, J. (ed.) Current concepts in the Pharmaceutical Sciences, Biopharmaceutics, Lea and Febiger, Philadelphia, pp 1–56

Ritschel, W. A. (1982) Handbook of Basic Pharmacokinetics, 2nd Edn., p. 263

Schmidt, L. H., Loo, T. L., Fradkin, R., Hughes, H. B. (1952) Proc. Soc. Exp. Biol. Med. 80: 367–370

Sixsmith, D. G., Watkins, W. M., Chulay, J. D., Spencer, H. C. (1984) Am. J. Trop. Med. Hyg. 33: 772–776

Smith, C. C., Ihrig, J., Menne, R. (1961) Ibid. 10: 694–703

Trager, W., Jensen, J. B. (1976) Science 193: 673–675

Urbanski, P., Serafin, B., Clyde, D. F. et al (1964) Tetrahedron 14, 1 suppl: 463–468

Watkins, W. M., Sixsmith, D. G., Chulay, J. D. (1984a) Ann. Trop. Med. Parasitol. 78: 273–278

Watkins, W. M., Sixsmith, D. G., Spencer, H. C., Boriga, D. A., Kariuki D., Kipingor, T., Koech, D. K. (1984b) Lancet i: 357–359

Weekly Epidemiological Record (1984) 59: 234

Wernsdorfer W. H., Kouznetsov, R. L. (1980) Bull. W.H.O. 58: 341–352

White, N. J. (1985) Clin. Pharmacokinet. 10: 187–215