Biochemical Pharmacology, Vol. 36, No. 20, pp. 3365–3369, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

THE DISPOSITION OF PRIMAQUINE IN THE ISOLATED PERFUSED RAT LIVER

STEREOSELECTIVE FORMATION OF THE CARBOXYLIC ACID METABOLITE

DEBORAH D. NICHOLL,* GEOFFREY EDWARDS,*†‡ STEPHEN A. WARD,*†
MICHAEL L'E. ORME* and ALASDAIR M. BRECKENRIDGE*

* Department of Pharmacology & Therapeutics, University of Liverpool, New Medical Building, Ashton Street, P.O. Box 147, Liverpool, L69 3BX, † Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, U.K.

(*Received* 5 *February* 1987; *accepted* 7 *May* 1987)

Abstract—The disposition of (+) and (−) primaquine (PQ) was studied in the isolated perfused rat liver (IPRL) preparation following a bolus dose (2.0 mg diphosphate salt; N = 6) of each enantiomer. Perfusate plasma concentrations of PQ and the carboxylic acid metabolite (PQm) were determined using previously reported methods. To enable the simultaneous measurement of PQ and PQm in bile a selective and reproducible HPLC assay was developed. Clearance of (−)PQ (8.8 ± 2.9 ml min^{-1}) was significantly greater than that of (+)PQ (5.5 ± 1.5 ml min^{-1}) and the apparent volumes of distribution of (−)PQ (606 ± 182 ml) and (+)PQ (930 ± 171 ml) were significantly different. Stereoselectivity in the hepatic elimination efficiency was manifest as a significant reduction in half-life ((−)PQ 54 ± 29 min; (+)PQ 123 ± 33 min) and smaller area under the curve to infinity ((−)PQ 254 ± 96 $\mu g\ ml^{-1}.min$, (+)PQ 387 ± 108 $\mu g\ ml^{-1}.min$) for (−)PQ when compared with (+)PQ. A significantly greater peak concentration of PQm was achieved following administration of (−)PQ (0.61 ± 0.26 $\mu g\ ml^{-1}.min$) than (+)PQ (0.19 ± 0.09 $\mu g\ ml^{-1}$). There was no difference between the sum of the areas under the curve to 4 hr for (+) and (−)PQ and the corresponding carboxylic acid metabolite (322 ± 64 $\mu g\ ml^{-1}$ and 317 ± 75 $\mu g\ ml\ min^{-1}$ respectively). There was no difference in the biliary clearance of (+) and (−)PQ (0.08 ± 0.02 ml min^{-1} and 0.14 ± 0.10 ml min^{-1} respectively) or the corresponding carboxylic acid metabolites (0.24 ± 0.13 ml min^{-1} and 0.29 ± 0.09 ml min^{-1}). These results strongly suggest stereoselective formation of the carboxylic acid metabolite of primaquine. The significant increase in the volume of distribution of (+)PQ suggests the enantiomer has either an increased affinity for binding sites within the liver and/or erythrocytes or a decreased affinity for circulating perfusate albumin.

Primaquine is the only available drug for the radical cure of malaria due to *Plasmodium vivax* and *Plasmodium ovale* [1]. Primaquine contains an asymmetric carbon atom in the alkyl side chain and therefore exists as (+) and (−) enantiomers, although it is administered as the racemate. The clinical pharmacology of this drug was, until recently, poorly understood despite extensive use for over 40 years. Studies carried out over the past 5 years have characterized the pharmacokinetics of racemic primaquine in man [2, 3] and a number of animal models [4, 5]. The drug has been shown to undergo rapid and extensive metabolism [6–9]. A carboxylic acid formed by the oxidative deamination of primaquine has been identified as the major plasma metabolite in man [2], monkeys [4] and rats [8]. As yet the antimalarial and toxicological properties of this compound are unknown. Although the isomers of primaquine are equipotent antimalarial agents, significant differences exist with respect to toxicity. Sub-acute toxicity studies in monkeys have shown the (−) isomer to be three times as toxic as the (+) isomer whereas acute toxicity studies in mice have shown the (+) isomer to be four times as toxic as the (−) isomer [10]. Recent work in the isolated perfused rat liver (IPRL) preparation has shown the pharmacokinetics of primaquine to be dose dependent [11] with a large proportion of the drug excreted as primaquine and metabolites in the bile [5]. In addition to its dose dependent pharmacokinetics, a stereoselectivity in the rate of elimination of the isomers was observed at a dose of 2.5 mg primaquine base. The hepatic clearance of (−) primaquine was three times that of (+) primaquine. This difference in clearance was manifest as a threefold increase in half-life of (+) primaquine compared with (−) primaquine [11]. The mechanism responsible for this stereoselectivity has not been established.

The present study reports the use of the IPRL preparation to investigate further the stereoselective disposition of primaquine with particular reference to the formation and elimination of the carboxylic acid metabolite. To facilitate this, an analytical method for the simultaneous determination of PQ and PQm in bile has been developed.

‡ To whom correspondence should be addressed at: Department of Pharmacology & Therapeutics, University of Liverpool, New Medical Building, Ashton Street, P.O. Box 147, Liverpool L69 3BX.

MATERIALS AND METHODS

Drugs and chemicals

Primaquine diphosphate was obtained from the Aldrich Chemical Company Ltd. (Gillingham, Dorset, U.K.); (+) and (−) primaquine diphosphate were gifts from the Walter Reed Army Medical Research Centre (Washington, DC). The carboxylic acid metabolite of primaquine was a gift from Professor J. D. McChesney, Department of Pharmacognosy, University of Mississippi, U.S.A. All solvents were of HPLC grade, obtained from Fisons (Loughborough, U.K.), all other reagents were of analytical grade supplied by British Drug Houses (Poole, Dorset, U.K.).

The optical purity of the (+) and (−) isomers of primaquine was determined by measurement of the optical rotation of the individual isomers in aqueous solution using a polarimeter. The measured optical rotations were comparable with previously reported values [12], i.e. (+) PQ $[\alpha]_D^{22}$ + 28.7° and (−) PQ $[\alpha]_D^{22}$ − 27.8° [13].

Isolated perfused rat liver preparation

The livers of male Wistar rats (200–250 g) were isolated using standard techniques [14] following anesthesia with sodium pentobarbitone (60 mg ml^{-1}, 60 mg kg^{-1} i.p.). The common bile duct was cannulated (polythene tubing 0.10 × 0.61 mm) and connective tissue surrounding the liver cleared before cannulation of the hepatic portal vein (Argyle Medicut, 14G; Sherwood Indust.) which was then flushed with heparinised saline (50 u ml^{-1}). The inferior vena cava was cannulated (with polythene tubing 0.02 × 0.42 mm) through the left atrium after having been tied off above the renal vein. The liver was then removed and placed on a glass platform inside a humidified, thermostatically maintained environment (37°), where it was perfused in a constant flow (15 ml min^{-1}) recirculating system. The perfusate comprised washed human red blood corpuscles (10% v/v), bovine serum albumin (1% w/v) and glucose (0.1% w/v) in Krebs–Henseleit buffer. The principal indices of liver viability were steady oxygen consumption (1.5–2.7 μmoles g liver^{-1} min^{-1}), sustained bile flow (0.2–0.7 ml hr^{-1}), constant perfusion pressure 50–80 mm H_2O) reproducible liver function tests (perfusate Na^+, K^+, AAT, GGT concentrations), and normal physical appearance.

Experimental protocol

Studies using the IPRL have shown that primaquine exhibits dose-dependent pharmacokinetics [5]. For the purpose of this study it was necessary to identify a dose of (+) and (−) primaquine such that (a) low clearance behaviour was exhibited, when any difference in total clearance would reflect the difference in the affinity of drug metabolizing enzymes for the two isomers rather than organ perfusion rate; (b) the metabolizing capacity was not saturated, demonstrated by log-linear post-distributive decline in perfusate primaquine concentrations, and (c) formation of the carboxylic acid metabolite occurred to a measurable extent. A preliminary study identified a dose of 2.0 mg primaquine as satisfying these criteria.

The elimination of primaquine from the IPRL was studied over 4 hr following a bolus dose of 2.0 mg of (a) (+) primaquine disphosphate (N = 6) or (b) (−) primaquine disphosphate (N = 6), administered as an aqueous solution (200 μl) into the perfusate reservoir.

Samples (1.5 ml) were taken from the perfusate reservoir, pre-dose and at 5, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240 min post-dose, with the volume removed being replaced by fresh perfusate. After centrifugation (1000 *g* for 1 min) the perfusate plasma was removed and frozen at −20° until assayed for primaquine and the carboxylic acid metabolite of primaquine. The total volume of bile produced was collected into pre-weighed vials at hourly intervals, volume was measured and samples were frozen at −20° until assay.

Perfusate blood gases were measured and liver function tests performed before and after each experiment, to ascertain liver viability.

Analytical procedures

All assays were carried out on a high-performance liquid chromatograph (Spectra Physics, St Albans, U.K.). The system consisted of an SP8700 solvent delivery system, with an SP8750 organiser module equipped with a Rheodyne valve injection system. Chromatographic separation was carried out on a μBondapak "Rad-Pak" phenyl reversed-phase cartridge housed in a "Z-module" (Waters Assoc., Hartford, Cheshire, U.K.). Detection was by u.v. absorption at 254 nm.

(a) *Perfusate.* Primaquine and carboxylic acid metabolite levels were determined as previously described [12].

(b) *Bile.* To bile (25 μl) in a 1.5 ml capacity microfuge tube was added indomethacin as the internal standard (100 μg ml^{-1}, 25 μl) as used previously [2]. Endogenous components were precipitated by the addition of 25 μl each of $ZnSO_4$ (20%):methanol (50:50) and saturated $Ba(OH)_2$ solution. After vortex mixing (30 sec) and centrifugation (1000 *g*, 1 min) 25 μl of the supernatant was injected onto the chromatograph. The mobile phase consisted of methanol:water (50:50 v/v) containing triethylamine (1% v/v) buffered to pH 6.0 with orthophosphoric acid. Since the internal standard was strongly retained on the column the flow rate was programmed to increase after the elution of primaquine and the carboxylic acid metabolite (4.4 and 7.0 min respectively) from 3 ml min^{-1} to 6 ml min^{-1} and to return to the original flow rate following the elution of the internal standard (11.5 min). The minimum detectable concentrations of PQ and PQm using 25 μl of bile were 3 μg ml^{-1} and 2.5 μg ml^{-1} respectively, which produced peaks three times the baseline noise on the highest detector sensitivity used (× 0.005 AUFS). Calibration curves were linear ($r \geq 0.99$) in the range 0–18 μg ml^{-1}. The inter and intra assay variation of spiked samples for PQ ranged from 4.9 to 6.3% (N = 8) and 5.2 to 6.0% (N = 8) for PQm.

Pharmacokinetic calculations and statistical analysis

The terminal phase elimination rate constant (β) was determined by least squares regression analysis

of the post distributive, perfusate primaquine concentration–time data. The terminal phase half life ($t_{\frac{1}{2}}$) was obtained from the relationship $0.693/\beta$. The area under the perfusate concentration–time curve for primaquine and the carboxylic acid metabolite from $t = 0$ to 4 hr (AUC_{0-4}) was calculated by the trapezoidal rule [15]. In the case of primaquine, the area under the curve from 4 hr to infinity was calculated from the ratio C_4/β where C_4 was the perfusate primaquine concentration at 4 hr. The area under the curve from 0 to inifinity (AUC) for primaquine was obtained from the sum of the two areas. Primaquine clearance from the perfusate was calculated from the expression Cl = dose/AUC and the apparent volume of distribution (V_d) for primaquine was calculated from $V_d = (Cl \times t_{\frac{1}{2}})/0.693$. Biliary clearance, ($Cl_{bile}$) of primaquine and the carboxylic acid metabolite was calculated as Ae_{0-4}/AUC_{0-4} where Ae is the amount excreted into the bile. The AUC_{0-4} hr for primaquine and the corresponding carboxylic acid metabolite were summed to give AUC_{total}, i.e. $(AUC_{0-4(PQ)} + AUC_{0-4(PQm)}) = AUC_{total}$. Statistical comparisons between the two groups were made using Student's non-paired t-test.

Drug concentrations are quoted as base equivalents. Data are tabulated as mean ± SD (Table 1) and presented graphically as mean ± SE (Fig. 1). Statistical significance was accepted when $P \leq 0.05$.

RESULTS

Perfusate disposition of primaquine

Clearance of (−) primaquine (8.8 ± 2.9 ml min^{-1}) was significantly greater than that of (+) primaquine (5.5 ± 1.5 ml min^{-1}). The volume of distribution for the (−) and (+) isomers (606 ± 182 ml and 930 ± 171 ml respectively) were significantly different. The stereoselectivity of hepatic elimination efficiency and volume of distribution were translated into both a significantly shorter half-life (54 ± 29 min) and smaller area under the curve to infinity (254 ± 96 μg ml^{-1}.min) for the (−) isomer when compared with the half-life (123 ± 33 min) and area under the curve to infinity (387 ± 108 μg ml^{-1}.min) of the (+) isomer.

The carboxylic acid metabolite of primaquine was produced rapidly after administration of both (+) and (−) primaquine. However, a significantly greater peak concentration was achieved following administration of (−) primaquine (0.61 ± 0.26 μg ml^{-1}) than following (+)primaquine (0.19 ± 0.09 μg ml^{-1}). After initially reaching a peak concentration the levels of carboxylic acid metabolite formed following administration of (−)primaquine declined at a rate similar to that of the parent drug whereas the concentration of metabolite formed from (+)primaquine persisted for the duration of the study. There was no significant difference between the sums of the AUC_{0-4} hr for (+) and (−)primaquine and the resultant carboxylic acid metabolite formed, (322 ± 64 μg ml^{-1}.min and 317 ± 75 μg ml^{-1}.min respectively).

Biliary excretion of primaquine and the carboxylic acid metabolite

Primaquine and the carboxylic acid metabolite were excreted into the bile. There was no significant difference in the absolute amount of the drug excreted unchanged in the bile as either (+) or (−) primaquine (1.1 ± 0.3% and 1.5 ± 0.8% of dose respectively). However, the amount of carboxylic acid metabolite recovered in bile was significantly greater after administration of (−)primaquine (1.2 ± 0.6% of dose) than (+)primaquine (0.3 ± 0.1% of dose). Despite the stereoselective difference in the total amount of drug excreted as the carboxylic acid in bile, when the data was normalized to take into account the differences in cir-

Table 1. Summary of pharmacokinetic parameters (perfusate plasma and bile) for primaquine (PQ) and its carboxylic acid (PQm) metabolite after 2.0 mg doses of (+) and (−)primaquine

Perfusate plasma	(+) PQ	(−) PQ
PQ		
Cl (ml min^{-1})	5.5 ± 1.5	8.8 ± 2.9*
V_d (ml)	930 ± 171	606 ± 182†
$t_{\frac{1}{2}}$ (min)	123 ± 33	54 ± 29‡
$AUC_{0-4\,(PQ)}$ (μg ml^{-1}.min)	287 ± 69	245 ± 81
$AUC_{0-\infty\,(PQ)}$ (μg ml^{-1}.min)	387 ± 108	254 ± 96*
PQm		
C_{max} (μg ml^{-1})	0.2 ± 0.1	0.6 ± 0.3‡
$AUC_{0-4\,(PQm)}$ (μg ml^{-1}.min)	35 ± 22	82 ± 31*
AUC_{0-4} (total) (μg ml^{-1}.min)	322 ± 64	317 ± 75

Bile	(+) PQ	(−) PQ	(+) PQm	(−) PQm
Ae (μg)	21.5 ± 6.6	30.1 ± 15.7	6.4 ± 1.9†	23.3 ± 12.7
Cl_{bile} (ml.min^{-1})	0.08 ± 0.02	0.14 ± 0.10	0.24 ± 0.13	0.29 ± 0.04

Perfusate plasma clearance Cl; volume of distribution V_d; elimination half-life ($t_{\frac{1}{2}}$); area under the curve (0–4 hr or 0–∞) AUC; maximum perfusate plasma concentration C_{max}; amount secreted into bile (0–4 hr) Ae; biliary clearance Cl bile.

* $P \leq 0.05$.

† $P \leq 0.01$.

‡ $P \leq 0.005$.

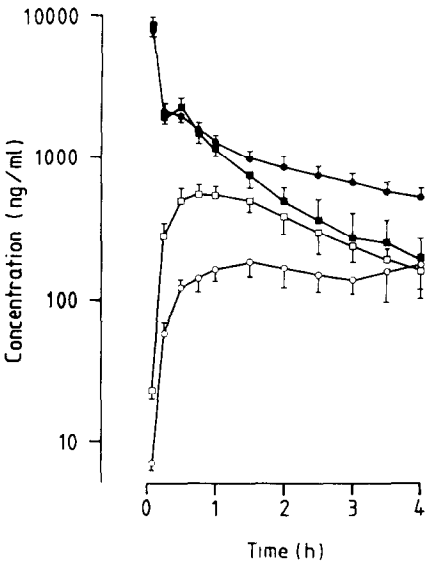

Fig. 1. Semilogarithmic plot of perfusate concentrations of (+) primaquine (—●—), (−) primaquine (—■—), and the corresponding carboxylic acids (—○—) and (—□—) following the administration of each enantiomer (2.0 mg diphosphate) to the isolated perfused rat liver system, (mean ± SE, N = 6).

culating perfusate metabolite concentrations, there was no difference in the biliary clearances of (+) or (−)primaquine (0.08 ± 0.02 ml min^{-1} and 0.14 ± 0.1 ml min^{-1} respectively) or carboxylic acid metabolite formed from (+) or (−)primaquine (0.24 ± 0.13 ml min^{-1} and 0.29 ± 0.09 ml min^{-1}).

DISCUSSION

Stereoselectivity in drug disposition and pharmacological activity have been observed for a number of agents including warfarin [16, 17], propranolol [18] and primaquine [11]. With respect to primaquine, although differences between enantiomers in plasma perfusate concentrations were described, metabolite levels were not reported, neither was there any discussion of biliary excretion of primaquine and its metabolites. The carboxylic acid metabolite of primaquine has been identified as the principal plasma metabolite in man. It is therefore necessary to identify any role it may have in the observed stereoselective disposition of primaquine. This present study has investigated further the stereoselective disposition of primaquine with particular reference to the formation and elimination of the carboxylic acid metabolite using the IPRL preparation. This model enables experimental conditions to be controlled rigorously while allowing the hepatic component of drug elimination to be examined in a whole organ system and without the influence of the other routes of elimination that are present in the intact animal.

The aim of this study demanded that a suitable technique be available to measure simultaneously concentrations of primaquine and the carboxylic acid metabolite in bile. Previously this had been accomplished by using separate extraction procedures followed by HPLC [2, 12]. Two distinct assay procedures were required as parent drug and metabolite possess different physico-chemical properties. A selective and reproducible HPLC assay has been developed which permits the simultaneous determination of the biliary concentration of both primaquine and the carboxylic acid metabolite without extraction thus reducing the time taken to assay samples.

The pharmacokinetics of primaquine in the IPRL preparation have been shown to be dose dependent. It was necessary, therefore, that the dose of primaquine administered to the IPRL in this study should satisfy several criteria. It was important that the rate of elimination of (+) and (−)primaquine was such that the half-life could be calculated accurately and that the carboxylic acid metabolite formation was measurable. In addition it was essential that primaquine behaved as a low clearance compound which is the case throughout the therapeutic range in man [19].

The significantly increased hepatic clearance and reduced elimination half-life of (−)primaquine compared to (+)primaquine is in agreement with the earlier study of Ward *et al.* [11]. In this study, however, no statistical difference in the volume of distribution was observed between the two stereoisomers. This previous study attributed the differences in elimination efficiency either to a reduced affinity of (+)primaquine for the enzymes responsible for its hepatic extraction or to different isoenzymes being responsible for the metabolism of each stereoisomer. We have now been able to demonstrate that biliary clearances of (+)primaquine and (−)primaquine are identical as are the corresponding clearance values for the carboxylic acid metabolites determined from the respective stereoisomers. There was also no difference in the sums of the AUC_{0-4} values for (+) and (−)primaquine and the corresponding carboxylic acid metabolites. These observations together with the observed significant differences in plasma clearance values for (+) and (−)primaquine strongly suggests that there is stereoselectivity with respect to the formation of the carboxylic acid metabolite of primaquine and that this most likely results from differences in substrate affinity for the enzyme active site. However, the possibility that the observed enantioselectivity is due to the participation of two isozymes with different selectivities cannot be excluded. The significant increase in volume of distribution of (+)primaquine suggests that this isomer shows an increased affinity for binding sites within the liver or within the erythrocytes or a decreased affinity for circulating perfusate plasma albumin. The pharmacological role of the carboxylic acid metabolite of primaquine has yet to be clearly defined. It is not believed to possess significant antimalarial activity but is the major plasma metabolite in man. However, the reduced plasma clearance of (+)primaquine to the carboxylic acid metabolite might be accompanied by an increased production of hydroxylated metabolites of primaquine which are thought to be precursors of those substances responsible for the acute intra-

vascular haemolysis which is often observed in response to primaquine [20].

In summary, this study has demonstrated that the clearance of primaquine to its carboxylic acid metabolite is stereoselective. The implications of this observation are dependent upon the role of this and other metabolites in the therapeutic and toxicological effects of the drug. Schmidt and his colleagues [10] have suggested that the stereoisomers of primaquine possess equal antimalarial potency but that their acute and sub-acute toxicities are different, suggesting that different mechanisms are responsible for each effect. Recent reports suggest that this may not be the case [21]. Clearly this is an area where further investigation will be necessary.

Acknowledgements—The authors are grateful to Mrs Angela Brown for her expert technical assistance. We should also like to thank Miss Susan Oliphant for typing this paper.

This work received financial support from the UNDP/WHO/World Bank Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. L. J. Bruce-Chwatt, in *Essential Malariology*. William Heinemann, London (1980).
2. G. W. Mihaly, S. A. Ward, G. Edwards, M. L'E. Orme and A. M. Breckenridge, *Br. J. clin. Pharmac.* **17**, 441 (1984).
3. J. Greaves, D. A. P. Evans, H. M. Gilles, K. A. Fletcher, D. Bunnag and T. Harinasuta, *Br. J. clin. Pharmac.* **10**, 399 (1980).
4. J. K. Baker, J. A. Bedford, A. M. Clark and J. D. McChesney, *Pharm. Res.* **2**, 98 (1984).
5. S. A. Ward, G. W. Mihaly, D. D. Nicholl, G. Edwards and A. M. Breckenridge, *Drug Metab. Dispos.* **13**, 425 (1985).
6. A. Strother, I. M. Fraser, R. Allahyari and B. Tilton, *Bull. W.H.O.* **59**, 413 (1981).
7. A. Strother, R. Allahyari, J. Bucholz, I. M. Fraser and B. E. Tilton, *Drug Metab. Dispos.* **12**, 35 (1984).
8. J. K. Baker, J. D. McChesney, C. D. Hufford and A. M. Clark, *J. Chromatogr.* **230**, 69 (1982).
9. C. D. Hufford, A. M. Clark, I. N. Quinones, J. K. Baker and J. D. McChesney, *J. Pharm. Sci.* **72**, 92 (1983).
10. L. H. Schmidt, S. Alexander, L. Allen and J. Rasco, *Antimicrob. Agents Chemother.* **12**, 51 (1977).
11. S. A. Ward, G. W. Mihaly, D. D. Nicholl, A. M. Breckenridge and G. Edwards, *Biochem. Pharmac.* **36**, 2238 (1987).
12. S. A. Ward, G. Edwards, M. L'E. Orme and A. M. Breckenridge, *J. Chromatogr.* **305**, 2329 (1983).
13. F. I. Carroll, B. Berrang and C. P. Linn, *J. Med. Chem.* **21**, 326 (1978).
14. G. W. Mihaly, R. A. Smallwood, J. D. Anderson, D. B. Jones, L. K. Webster and F. J. Vajda, *Hepatology* **2**, 828 (1982).
15. M. Gibaldi and D. Perrier, in *Pharmacokinetics*, 2nd Edn. Marcell Dekker New York (1982).
16. A. M. Breckenridge, M. L'E. Orme, H. Wesseling, R. J. Lewis and R. Gibbons, *Clin. Pharmac. Ther.* **15**, 424 (1974).
17. R. A. O'Reilly, *Clin. Pharmac. Ther.* **16**, 348 (1974).
18. B. Silber, N. H. G. Holford and S. Riegelman, *J. Pharm. Sci.* **71**, 699 (1982).
19. G. W. Mihaly, S. A. Ward, G. Edwards, D. D. Nicholl, M. L'E. Orme and A. M. Breckenridge, *Br. J. clin. Pharmac.* **19**, 745 (1985).
20. E. Beutler, *Pharmac. Rev.* **21**, 73 (1969).
21. I. A. Clark and N. H. Hunt, *Infec. Immun.* **39**, 1 (1983).