Biochemical Pharmacology, Vol. 34, No. 12, pp. 2193–2197, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

THE DISPOSITION OF PYRIMETHAMINE IN THE ISOLATED PERFUSED RAT LIVER

M. D. COLEMAN, G. W. MIHALY, S. A. WARD, G. EDWARDS,* R. E. HOWELLS† and A. M. BRECKENRIDGE

Department of Pharmacology and Therapeutics, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K.; Departments of Tropical Medicine* and Parasitology,† Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, U.K.

(*Received* 15 *November* 1984; *accepted* 28 *January* 1985)

Abstract—We have investigated the disposition of pyrimethamine base in the isolated perfused rat liver (IPRL) preparation after the administration of pyrimethamine (0.5 mg, 5 μCi). In the first half hour of the study, pyrimethamine underwent marked hepatic uptake, thereafter perfusate plasma drug levels declined monoexponentially with a half life ($t_{\frac{1}{2}}$) of 3.0 ± 1.0 hr. Area under the perfusate plasma concentration/time curve $(AUC)_{0\rightarrow\infty}$ was 6.9 ± 1.9 μg/hr/ml. Pyrimethamine was found to be a low clearance compound (78.4 ± 25.3 ml/hr ≡ 8.6% of liver perfusate flow) with a large volume of distribution (267.5 ± 55.3 ml) in the IPRL. The combined $AUCs_{(0\rightarrow 5hr)}$ for pyrimethamine (AUC 4.8 ± 0.5 μg/hr/ml) and pyrimethamine 3-N-oxide ($AUC_{0\rightarrow 5hr}$ 0.9 ± 0.6 μg/hr/ml) accounted for 57% of the total $AUC_{0\rightarrow 5hr}$ of [^{14}C] radioactivity (10.0 ± 2.6 μg/hr/ml). This indicates the presence of metabolites of pyrimethamine as yet unidentified in the perfusate. Biliary excretion of [^{14}C] during the course of the IPRL preparations was extensive (29.0 ± 10.3%) though only a small proportion was due to pyrimethamine and the 3-*N*-oxide metabolite. The majority of radioactivity in the bile was attributable to highly polar, but unidentified metabolites of pyrimethamine.

At the conclusion of each experiment (5 hr), a significant proportion of [^{14}C] radioactivity was recovered from the livers (22.9 ± 5.3%). Subsequent HPLC analysis of the liver tissue indicated this to be unchanged pyrimethamine, with trace levels of the 3-*N*-oxide metabolite. Sub-cellular fractionation of the homogenized livers revealed the most pronounced localisation of pyrimethamine to be in the lipid rich 10,000 *g* pellet (13.0 ± 2.6%), the remainder being distributed equally between the 105,000 *g* pellet and supernatant. Neither pyrimethamine, [^{14}C] radioactivity, nor pyrimethamine 3-*N*-oxide were extensively taken up by red cells throughout the study. Therefore, the large volume of distribution (267.5 ± 55.3 ml) underlines the extent of pyrimethamine localisation in the liver.

Pyrimethamine, combined with a sulphonamide or sulphone is a first choice treatment in areas of chloroquine resistant *Plasmodium falciparum* malaria. Due to analytical limitations, little is known of the pharmacokinetics and metabolic fate of pyrimethamine. However, the recent development of a selective and sensitive HPLC assay in our department [1] has enabled a number of aspects of this drug's disposition to be investigated.

In our earlier report [2], pyrimethamine was shown to undergo substantial hepatic uptake and elimination; a high proportion of the administered dose being recovered from the faeces.

Therefore, in the following study, we have investigated the hepatic disposition of pyrimethamine in the isolated perfused rat liver preparation (IPRL). We have chosen the IPRL as an experimental model, as this preparation allows the hepatic component of drug elimination to be examined in a whole organ system, excluding the influence of the other routes of elimination present in the intact animal. This experimental model has been of value in studies of the hepatic disposition of a number of drugs, e.g. cimetidine, propranolol, and the antimalarial primaquine [3–5].

Address for correspondence: M. D. Coleman, Department of Pharmacology and Therapeutics, University of Liverpool, P.O. Box. 147, Liverpool, L69 3BX, U.K.

MATERIALS AND METHODS

Reagents. Pyrimethamine base, and pyrimethamine 3-*N*-oxide were supplied by Wellcome U.K. (Beckenham, Kent). Proguanil hydrochloride, the internal standard, was supplied by ICI Pharmaceuticals (Alderley Edge, Cheshire, U.K.). [^{14}C] Pyrimethamine base (specific activity 54 mCi/mmole) labelled in position 2 of the pyrimidine ring, was obtained from the Radiochemical Centre, Amersham International (Amersham Bucks, U.K.). N.C.S. tissue solubiliser and hydrogen peroxide (30% w/v) were supplied by B.D.H. Chemicals Ltd. (Poole, Dorset, U.K.). Emulsifying liquid scintillant (ES 299) was obtained from the Packard Instrument Company Ltd. (Caversham, Reading, U.K.). All other reagents were of HPLC or analytical grade (Fisons, Loughborough, U.K.).

Animals. Male Wistar rats (200–250 g) were housed in well-ventilated cages and kept at a room temperature of approximately 24°. They were allowed to feed *ad libitum* on pelleted food (Oxoid Breeding Diet, Oxoid, Ltd., London, U.K.) and tap water.

Isolated perfused rat livers (IPRL). Rats were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and their livers isolated using standard techniques and then perfused in a constant flow (15 ml/min) recirculating system at 37°, as previously

described [3]. The principal indices of liver viability were steady oxygen consumption (1.5–2 μmoles/g liver/min), sustained bile flow (0.2–0.6 ml/hr) constant perfusion pressure (6–8 cm H_2O) reproducible liver function tests and normal visual appearance.

Protocol. The elimination of pyrimethamine from the IPRL was studied over 5 hr following a 0.5 mg bolus dose of pyrimethamine containing 5 μCi of [^{14}C] labelled drug (N = 5). A pilot study had previously indicated that the pharmacokinetics of pyrimethamine were linear at this dose level. Pyrimethamine was added as a solution in dimethyl sulphoxide (25 μl) directly into the reservoir thereby simulating systemic dosage. Samples (1 ml) were removed from the perfusate reservoir pre dose, and at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min. After centrifugation (1100 *g*, 5 min) the perfusate plasma was removed and stored at −20° until assayed for pyrimethamine, its 3-*N*-oxide metabolite, and [^{14}C] radioactivity. Additional samples were taken at 60 and 180 min for measurement of whole perfusate levels of drug and metabolites. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. Bile was collected over 30-min intervals into pre-weighed vials and the bile volume determined by weight (assuming 1 ml bile = 1 g) before freezing at −20°. Perfusate blood gases were measured and liver function tests were performed before and after each experiment to ascertain liver viability. The subcellular localization of pyrimethamine, pyrimethamine 3-*N*-oxide and [^{14}C] radioactivity were determined for each liver at the end of each experiment (5 hr), as described below.

Preparation of liver tissue fractions. At the conclusion of each experiment, the livers were flushed with 0.9% saline, weighed, and then homogenised in three times the liver weight of ice-cold 0.067 M phosphate buffer (pH 7.5; containing 1.5% KCl) using a Teflon-in-glass homogeniser. The 25% homogenate was centrifuged at 10,000 *g* for 20 min at 4°. The resulting supernatant was decanted without disturbing the pellet and centrifuged at 105,000 *g* for 60 min at 4°. The microsomal pellet was then resuspended and stored in phosphate buffer (0.2 ml). Aliquots of whole liver homogenate, 10,000 *g* pellet, 10,000 *g* supernatant, the 105,000 *g* pellet and the 105,000 *g* supernatant were retained for analysis of pyrimethamine, pyrimethamine 3-*N*-oxide, and [^{14}C] radioactivity levels.

Analytical procedures. Whole perfusate and perfusate plasma pyrimethamine and pyrimethamine 3-*N*-oxide levels were determined by a selective and sensitive HPLC method as previously described [1]. [^{14}C] Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer. Duplicate samples of perfusate plasma (10 μl) and bile (5 μl) were assayed directly for [^{14}C] radioactivity after the addition of scintillation fluid (5 ml). Samples of whole liver homogenate, various liver tissue fractions and whole perfusate, were incubated overnight at 60° with N.C.S. tissue solubilizer (500 μl). Discolouration was achieved by a further 2-hr incubation of the digest with hydrogen peroxide (500 μl) at 60°. To improve scintillation counting efficiency, elimination of chemiluminescence was achieved by the addition of glacial acetic acid (60 μl) and, after addition of scintillation fluid the samples were vortexed for 30 sec before being counted. Corresponding blank and spiked samples were processed in an identical fashion.

Analysis of biliary metabolites. In order to prepare a representative aliquot of the total bile production of each IPRL preparation, 40% of each half-hour bile sample was removed and combined. An aliquot (5 μl) of this pooled bile obtained from each IPRL preparation was used to determine the biliary level of [^{14}C] radioactivity. A further aliquot (100 μl) was removed from the pooled bile, and subjected to enzyme hydrolysis (pH 5.0, 37°, 6 hr) with H1 hydrolase preparation obtained from *H. pomatia* (Sigma, Poole, Dorset, U.K.) 100 units of enzyme activity per 100 μl of bile. This hydrolysing enzyme preparation contains both arylsulphohydrolase and β-glucuronidase activity. Control incubations contained 0.1 M sodium acetate buffer (pH 5.0). Prior to hydrolysis of the pooled bile samples both pyrimethamine and pyrimethamine 3-*N*-oxide were shown to be stable to hydrolysis by this enzyme preparation.

Pyrimethamine and pyrimethamine 3-*N*-oxide present in bile samples were separated by high performance liquid chromatography following direct injection onto the HPLC. Effluent fractions corresponding to the solvent front, (0–5.5 min) pyrimethamine 3-*N*-oxide (5.5–6.5 min) pyrimethamine (9.3–10.5 min) and 10.5–20 min were collected and assayed for [^{14}C] radioactivity after the addition of liquid scintillant (20 ml).

Pharmacokinetic calculations and statistical analysis. The terminal phase elimination rate constant (β) was determined by least squares regression analysis of the post distribution perfusate pyrimethamine-concentration–time data and the terminal phase half-life ($t_{\frac{1}{2}}$) from the ratio $0.693/\beta$. The area under the perfusate concentration/time curve for pyrimethamine, [^{14}C] radioactivity and the 3-*N*-oxide metabolite of pyrimethamine from time = 0 to time = 5 hr ($AUC_{0\rightarrow5}$) was calculated by the trapezoidal rule [6].

With pyrimethamine, the area under the curve from 5 hr to infinity was calculated from the ratio $C_{5/\beta}$ where C_5 was the perfusate pyrimethamine concentration at 5 hr. The area under the curve from zero to infinity (AUC) for pyrimethamine was obtained from the sum of the two areas. Pyrimethamine clearance from perfusate (Cl) was calculated from the equation:

$$Cl = \frac{\text{dose}}{\text{AUC}}$$

and the apparent volume of distribution (V_d) for pyrimethamine from

$$V_d = \frac{Cl \times t_{\frac{1}{2}}}{0.693}.$$

Whole perfusate (W) to perfusate plasma (P) concentration ratios (W/P) were determined from the quotient of perfusate and perfusate plasma drug levels. Statistical comparisons between two groups were made by using Student's paired *t*-test. Data are tabulated as mean ± S.D. and presented graphically

as mean ± S.E.M. Statistical significance was accepted when $P \leq 0.05$.

RESULTS

Perfusate disposition of pyrimethamine, pyrimethamine 3-N-oxide and [^{14}C] radioactivity

The mean log–pyrimethamine perfusate concentration time curve (Fig. 1) shows that pyrimethamine perfusate levels declined monoexponentially with a half life ($t_{\frac{1}{2}}$) of 3.0 ± 1.0 hr. $AUC_{0\rightarrow\infty}$ was 6.9 ± 1.9 μg/hr/ml. The clearance of pyrimethamine (78.4 ± 25.3 ml/hr) represented only 8.6% of liver perfusate flow, indicating pyrimethamine to be a low clearance compound in this experimental model. The apparent volume of distribution (267.5 ± 55.3 ml) significantly exceeded the actual volume of the circuit (i.e. 100 ml perfusate plus liver volume) and implies that hepatic uptake of pyrimethamine was considerable.

The combined $AUCs_{0\rightarrow 5hr}$ (Fig. 1) for pyrimethamine (4.8 ± 0.5 μg/hr/ml) and pyrimethamine 3-*N*-oxide (0.9 ± 0.6 μg/hr/ml) accounted for 57% of the total $AUC_{0\rightarrow 5hr}$ of [^{14}C] radioactivity (10.0 ± 2.6 μg/hr/ml). These findings indicate that substantial levels of unidentified metabolites of pyrimethamine were present in perfusate plasma.

The whole perfusate to perfusate plasma concentration ratios (W/P) were close to unity for pyrimethamine, its 3-*N*-oxide metabolite, and [^{14}C] radioactivity and there was no significant difference at 60 and 180 min in the value of this parameter for either pyrimethamine (t = 60 min, W/P = 1.0 ± 0.1; t = 180 min, W/P = 1.0 ± 0.1), the 3-*N*-oxide metabolite (t = 60 min, W/P = 1.1 ± 0.1; t = 180 min, W/P = 1.0 ± 0.4) or [^{14}C] radioactivity (t = 60 min, W/P = 0.84 ± 0.1; t = 180 min, W/P = 0.81 ± 0.1). This indicates that neither parent drug nor its metabolites accumulate extensively in red cells during the study.

A summary of the mass balance data is shown in Table 1. During the course of the study, 29.0 ± 10.3% of the [^{14}C] radioactivity administered was eliminated in bile. At the conclusion of each experiment, the majority of the radioactivity was located in the perfusate (46.4 ± 14.1%), while the remainder was recovered from the liver 22.9 ± 3.5%. Loss of radioactivity due to sampling was below 9%, and total recovery was virtually 100%.

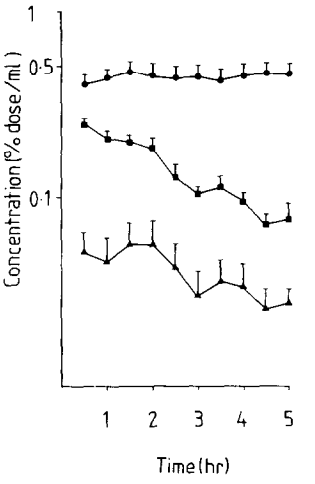

Fig. 1. Perfusate concentrations (% dose/ml) of pyrimethamine (■—■) ^{14}C radioactivity (●—●) and the 3-*N*-oxide metabolite of pyrimethamine (▲—▲) after systemic administration of pyrimethamine base (0.5 mg 5 μCi) to the IPRL.

Analysis of biliary metabolites

Although 29.0 ± 10.3% of the dose of [^{14}C] radioactivity was excreted in the bile over 5 hr, only 1.6% of the dose could be accounted for as pyrimethamine and the 3-*N*-oxide metabolite. However most of the remaining biliary radioactivity (24% of the dose) was recovered in the void volume, i.e. as more polar pyrimethamine derivatives. Less than 0.5% of the dose was eluted between 10.5 and 20 min. The remainder of the biliary radioactivity (4%) from all chromatographed samples being recovered from the HPLC column by flushing the HPLC system with methanol at the conclusion of each sample run.

Pyrimethamine and pyrimethamine 3-*N*-oxide levels were not significantly changed by subjecting bile to deconjugating enzymes, indicating that the excretion of pyrimethamine and pyrimethamine 3-*N*-oxide as conjugates were not important routes of elimination.

Liver fractionation studies

HPLC analysis of whole liver homogenates revealed that the [^{14}C] radioactivity found in the livers consisted almost exclusively of parent drug

Table 1. Mass balance: % of pyrimethamine, pyrimethamine 3-*N*-oxide as well as [^{14}C] radioactivity at 5 hr post-administration of pyrimethamine base (0.5 mg, 5 μCi) to the IPRL

	Recovery of dose (%)				
Compound	Liver	Bile	Perfusate	Loss due to sample removal	Total recovery
Pyrimethamine HPLC identified	22.6 ± 6.9	*0.9 ± 0.3 †0.5 ± 0.2	8.3 ± 4.8	1.1 ± 0.7	32.6 ± 10.5
Pyrimethamine 3-*N*-oxide	3.1 ± 1.1	*0.7 ± 0.1 †0.6 ± 0.2	1.8 ± 1.4	0.3 ± 0.1	5.6 ± 1.2
Total [^{14}C] activity	22.9 ± 5.5	29.0 ± 10.3	46.4 ± 14.1	8.6 ± 1.3	99.5 ± 7.5

* Represents free compound.
† Represents free plus deconjugated compound.
N = 5 data expressed as mean ± S.D.

Table 2. The percentage of ^{14}C radioactivity, pyrimethamine and pyrimethamine 3-*N*-oxide present in whole liver and fractions at 5 hr post-administration of pyrimethamine base (0.5 mg; 5 μCi) to the isolated perfused rat liver

Tissue	Recovery of radioactivity (%)		
	^{14}C Radioactivity	Pyrimethamine	Pyrimethamine 3-*N*-oxide
Whole liver	22.9 ± 5.5	22.6 ± 6.9	3.1 ± 1.1
10,000 *g* supernatant	8.0 ± 2.9	5.6 ± 1.4	1.3 ± 0.5
10,000 *g* pellet	12.3 ± 4.2*	13.0 ± 2.6*	2.4 ± 1.0
105,000 *g* supernatant	5.2 ± 1.9	3.7 ± 2.0	1.0 ± 0.5
105,000 *g* pellet	3.2 ± 1.1	2.2 ± 0.9	0.3 ± 0.3

* Represents a significantly greater proportion of drug recovered from the 10,000 *g* pellet w.r.t. the 10,000 *g* supernatent.

N = 5, data expressed as mean ± S.D.

with only trace levels of the 3-*N*-oxide metabolite (Table 2). The greatest proportion of [^{14}C] radioactivity and pyrimethamine was localised in the 10,000 *g* pellet. Analysis of the 10,000 *g* supernatant as well as the derived microsomal fractions (both having been obtained from the 10,000 *g* post mitochondrial supernatant) revealed no significant difference in the distribution of both pyrimethamine and [^{14}C] radioactivity between these fractions. Pyrimethamine 3-*N*-oxide was recovered from the various liver fractions, but it was uniformly distributed and present only in trace proportions.

DISCUSSION

In a recent detailed study of the disposition of pyrimethamine in the mouse [2], substantial hepatic localisation of unidentified [^{14}C] radioactivity occurred after administration of radiolabelled pyrimethamine. This observation, as well as the discovery of high levels of [^{14}C] radioactivity in faeces, suggests that pyrimethamine undergoes considerable hepatic uptake and elimination *in vivo*. Therefore, we have investigated in detail the pharmacokinetics, metabolism, biliary excretion and hepatic subcellular localisation of pyrimethamine in the isolated perfused rat liver (IPRL) preparation. Within 30 min of administration of the dose, over 50% of the [^{14}C] radioactivity and 75% of the unchanged drug was no longer present in the perfusate, and had been localised in the liver (Fig. 1). Since pyrimethamine was not extensively taken up by red cells during the course of the study, the relatively high apparent volume of distribution of pyrimethamine (267.5 ± 55.3 ml) also indicates considerable hepatic uptake of this drug, which is consistent with the known high lipid solubility of pyrimethamine (octanol/water partition coefficient 106:1 at pH 7, Wellcome—personal communication). Therefore it would be expected that the [^{14}C] radioactivity located in the liver at 5 hr, would be almost entirely parent drug; indeed this proved to be the case. Furthermore, sub-cellular fractionation of the homogenised livers at 5 hr indicated that the majority of the pyrimethamine was recovered from the 10,000 *g* pellet. This is the most lipid rich fraction in homogenised liver which is known to contain cell nuclei, mitochondria and cell membrane debris [7]. The remainder of the drug and [^{14}C] radioactivity was distributed uniformly between the microsomal and soluble fractions of the 10,000 *g* supernatant where metabolism of lipid soluble compounds is known to occur [7]. It is likely, therefore, that the hepatic localisation of [^{14}C] radioactivity reported previously [2] in the mouse was mostly due to parent drug.

Although there was close agreement between [^{14}C] radioactivity and pyrimethamine localisation in the liver, there was a marked disparity between [^{14}C] radioactivity and pyrimethamine levels in both perfusate plasma and bile at 5 hr (Fig. 1, Table 2). In perfusate plasma, the contribution of the combined AUC's$_{(0\text{–}5\,hr)}$ of pyrimethamine and its 3-*N*-oxide metabolite accounted for 57% of the $AUC_{0\rightarrow 5hr}$ of [^{14}C] radioactivity. Therefore the remaining 43% consisted of unidentified metabolites of pyrimethamine present in the perfusate. Analysis of bile revealed that 29.0 ± 10.3% of the dose was excreted over 5 hr, however only negligible proportions could be accounted for as unchanged drug or the 3-*N*-oxide metabolite.

In bile, the majority of [^{14}C] eliminated was accounted for as highly polar but unidentified pyrimethamine metabolites. As hepatic [^{14}C] radioactivity was almost exclusively parent drug, with only trace levels of 3-*N*-oxide, it may be that the unidentified perfusate plasma metabolites of pyrimethamine are also of a polar nature. Consequently, the chemical components of this polar material both in bile and perfusate may be alkyl or aryl hydroxylated derivatives of pyrimethamine and its N-oxides as described in an earlier report [8].

Interestingly, although pyrimethamine is a potent intraerythrocytic schizonticide, we found that neither pyrimethamine nor its metabolites selectively accumulated in red cells in this experimental model.

In conclusion our studies indicate that pyrimethamine is a low clearance compound of large volume of distribution which undergoes considerable uptake into the lipid rich fractions of rat liver. However, metabolism of the drug was extensive, there being considerable excretion of pyrimethamine metabolites into bile.

Acknowledgements—MDC is supported by the Medical Research Council, GWM by the National Health and Medical Research Council of Australia, SAW by the Mersey Regional Health Authority and GE by the Wolfson Foundation. This work also received financial support from the

UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. The authors are also grateful to Miss Susan Oliphant for typing the manuscript.

REFERENCES

1. M. D. Coleman, G. Edwards, G. W. Mihaly, R. E. Howells and A. M. Breckenridge, *J. Chromatogr.* **308**, 363 (1984).
2. M. D. Coleman, G. W. Mihaly, G. Edwards, S. A. Ward, R. E. Howells and A. M. Breckenridge, *J. Pharm. Pharmac.* **37**, 170 (1985).
3. G. W. Mihaly, R. A. Smallwood, J. D. Anderson, D. B. Jones, L. K. Webster and F. J. Vajda, *Hepatology* **2**, 828 (1982).
4. D. B. Jones, G. W. Mihaly, R. A. Smallwood, L. K. Webster, D. J. Morgan and N. P. Madsen, *Hepatology* **4**, 461 (1984).
5. S. A. Ward, G. W. Mihaly, D. D. Nichol, G. Edwards and A. M. Breckenridge, *Drug Metab. Dispos.* (in press).
6. M. Gibaldi and D. Perrier, in *Pharmacokinetics* Vol. 15: *Drugs in the Pharmacoceutical Sciences* (Ed. J. Swarbrick), p. 445. Marcel Dekker, New York (1982).
7. B. N. La Du, H. G. Mandel and E. Leong Way (Eds.), *Fundamentals of Drug Metabolism and Drug Disposition*, pp. 402–3. Williams Wilkins Company, Baltimore, U.S.A. (1972).
8. J. P. Hubbell, H. L. Henning, M. E. Grace, C. A. Nichol and C. W. Sigel, in *Biological Oxidation of Nitrogen* (Ed. J. W. Gorrod), p. 177. Elsevier/North Holland Biomedical Press, Amsterdam (1978).