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# Simple HPLC Determination of the Concentrations of Epiroprim in the Serum and Brains of Mice

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## Abstract

Epiroprim, an analogue of trimethoprim, has been shown to potentiate the efficacy of dapsone in experimental parasitic infections. A simple and accurate HPLC method has been developed to estimate epiroprim in serum and brain.

Blood and brains from mice were sampled 0, 30, 75, 120 and 240 min after 50 or 100 mg $kg^{-1}$ oral gavage. The drug and added internal standard metoprine in serum and brain supernatant were isolated by solid-phase extraction (Supelclean LC-SCX). The HPLC system consisted of a 150 × 4·6 mm Hypersil 5 μm ODS column. The mobile phases contained various proportions of acetonitrile, methanol and phosphate buffer (0·1 M). Peaks were detected by UV absorbance at 210 nm. Serum concentrations (mean ± s.e.m.) of epiroprim were highest at 30 min for both 50 and 100 mg $kg^{-1}$ doses, 173 ± 20 and 207 ± 25 ng $mL^{-1}$, respectively, falling to 8 ± 5 and 18 ± 6 ng $mL^{-1}$, respectively, at 240 min. Epiroprim concentrations in the brain correlated well with those in the serum, with levels of 223 ± 69 and 265 ± 21 ng $g^{-1}$ falling to 10 ± 10 and 31 ± 11 ng $g^{-1}$, respectively.

Epiroprim is rapidly absorbed and distributed to the brain.

Epiroprim, or 2,4-diamino-5[3,5-diethoxy-4-(pyrrol-1-yl)benzyl]pyrimidine (Ro 11-8958) is an analogue of trimethoprim (Fig. 1) with stronger inhibitory effects on bacterial dihydrofolate reductase (Then et al 1982). Its pharmacokinetic profile in dog is favourable in comparison with that of trimethoprim. Its longer elimination half-life ($t_{1/2\beta}$) and larger apparent volume of distribution at steady state ($V_{SS}$) could result in a reduction in dosing frequency and yet facilitate better access of the drug to infected tissues (Then et al 1982).

Epiroprim has recently been shown to be synergistic when used in combination with dapsone for the treatment of experimental pneumocystosis in immunosuppressed rats (Walzer et al 1993). The same combination used in acute toxoplasmosis in mice resulted in 100% survival whereas with epiroprim or dapsone alone the survival rates were 0% and 10%, respectively. Similarly, the combination was found to be more effective in reducing *Toxoplasma gondii* cysts in the brains of infected mice (Chang et al 1994).

Concentrations of epiroprim in serum have been determined by microbiological assay (Chang et al 1994). The objective of this study was to develop a simple and accurate high-performance liquid chromatographic (HPLC) method for the determination of epiroprim and to report serum and brain concentrations in mice after a single oral dose of 50 or 100 mg $kg^{-1}$.

## Materials and Methods

### *Materials*

Epiroprim (formerly Ro 11-8958) was kindly provided by F. Hoffmann-La Roche, Switzerland. Metoprine was supplied by Wellcome Research Laboratories, Beckenham, Kent, UK. All other reagents were of chromatographic grade from E. Merck, Darmstadt, Germany.

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### *Methods*

Experiments were performed on Swiss albino mice (ARC Swiss), 25 ± 1 g. Epiroprim at a low dose of 50 mg $kg^{-1}$ or a high dose of 100 mg $kg^{-1}$ was administered orally by gavage to each mouse. Five mice in each group were killed by cervical dislocation 0, 30, 60, 75, 120 and 240 min after dosing. Blood was sampled by cardiac puncture. The whole brain was removed, rinsed with buffered saline, dab-dried with Microwipes (Scott Paper Co, PA, USA) and weighed. Serum samples and whole brains were kept in glass tubes and stored at −20°C until assayed.

### *Sample preparation*

As far as possible, glass rather than plastic apparatus was used. Serum (100 μL; sample or calibrator to which had been added 10 to 400 ng $mL^{-1}$ epiroprim) was mixed with metoprine (25 ng) as internal standard and water (1 mL). Whole brain (sample or calibrator to which had been added 10 to 200 ng epiroprim per whole brain) with metoprine (100 ng) in phosphate buffer (0·05 M, pH 4·5) was homogenized (Polytron Kinematic, Lucern, Switzerland) for 20 s in a silanized glass tube and centrifuged at 10 000 rev $min^{-1}$ (11 400 *g*) for 30 min. The supernatant was kept for extraction.

Extraction of epiroprim with added metoprine was performed using 1-mL (100-mg) solid-phase extraction (SPE) columns (Supelclean LC-SCX tubes for cation, organic base; Supelco, PA, USA). SPE tubes were conditioned with ammonia in acetonitrile (0·22 M; 1 mL), acetonitrile (1 mL) and water (2 × 1 mL). The serum mixture or brain supernatant was passed through the conditioned column which was then

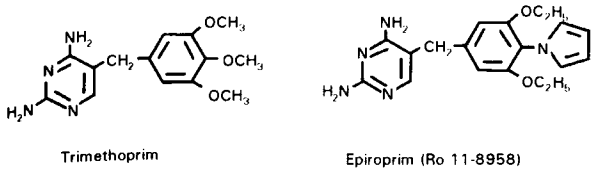


FIG. 1. The structures of trimethoprim and epiroprim (Ro 11-8958).

washed with ammonia in acetonitrile (0·22 M; 500 μL for serum and 3 × 500 μL for brain supernatant). The compounds were eluted from the column with ammonia in acetonitrile (0·22 M; 4 × 400 μL for serum and 5 × 500 μL for brain supernatant). The eluent collected was evaporated to dryness over $N_2$ and redissolved in mobile phase (100 μL). Volumes varying from 25-50 μL were injected into the HPLC system by means of a Rheodyne (Cotati, CA, USA) injector.

*HPLC*

HPLC was performed with a Waters 510 pump, a 150 × 4·6 mm 5 μm Hypersil ODS column (Alltech, Deerfield, IL, USA) with guard column, a 484 tunable absorbance detector set at 210 nm (Waters, Milford, MA, USA) and an HP 3396 series II integrator (Hewlett-Packard, Palo Alto, CA, USA). The mobile phase for serum samples was 35% acetonitrile in phosphate buffer (0·1 M, pH 3·5) flowing at 1 mL $min^{-1}$; that for brain samples was a 6:4 mixture of phosphate buffer (0·1 M, pH 5·5) and a mixture of acetonitrile and methanol (12:6 v/v); the flow rate was 1·2 mL $min^{-1}$.

Peak area ratios were used for determination of epiroprim concentrations, which were expressed as ng $mL^{-1}$ for serum. Although values obtained from the calibration curve for brain were ng per whole brain, concentrations were expressed as ng (g brain)$^{-1}$. Results are expressed as mean ± s.e.m.

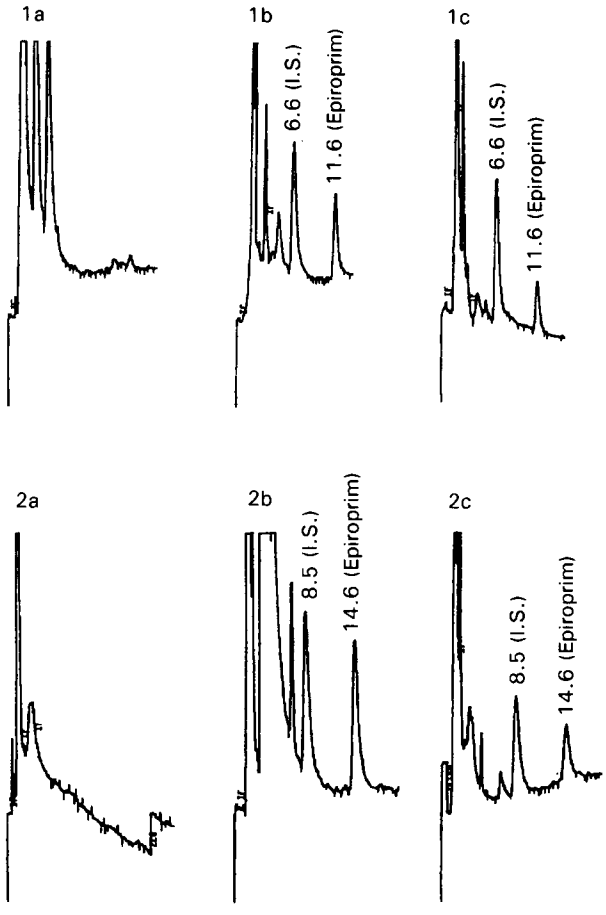


FIG. 2. Chromatograms obtained from drug-free serum sample (1a); drug-free serum sample with added epiroprim (100 ng $mL^{-1}$) and internal standard metoprine (1b); serum sample from mouse (1c); drug-free brain sample (2a); drug-free brain sample with added epiroprim (150 ng $brain^{-1}$) and internal standard (2b); brain sample from mouse (2c).

## Results and Discussion

Fig. 2 shows the chromatograms obtained from drug-free serum and brain, from samples of drug-free serum and brain to which epiroprim and the internal standard had been added, and serum and brain samples to which internal standard alone had been added. The use of glass tubes instead of plastic removed an interfering peak that eluted just after the internal standard.

Calibration curves were linear for 10 to 400 ng $mL^{-1}$ epiroprim in serum and for 10 to 200 ng epiroprim per brain. The linear regression equations were $y = 0{\cdot}004222x + 0{\cdot}00446$ ($r = 0{\cdot}9991$) for serum and $y = 0{\cdot}00654x - 0{\cdot}00513$ ($r = 0{\cdot}9998$) for brain. The inter-assay coefficient of variation obtained for the period of about 4 months was approximately 10% except for the lower concentration in brain, for which the coefficient was 13%. Accuracy of determinations was between 96 to 100% except for the lower concentration in brain, for which the accuracy was 88% (Table 1).

Fig. 3 shows the mean concentrations of epiroprim in the serum of mice after oral administration of 50 or 100 mg $kg^{-1}$. For both doses the highest serum concentrations were obtained after 30 min. With the 50 mg $kg^{-1}$ dose, the serum concentration (mean s.e.m.) at 30 min was 173 ± 20 ng $mL^{-1}$; that

Table 1. Inter-assay precision of determination of epiroprim.

| Serum Amount added (ng $mL^{-1}$) | Amount obtained (ng $mL^{-1}$) mean ± s.d. | Accuracy (%) | CV (%) | Brain Amount added (ng $mL^{-1}$) mean ± s.d. | Amount obtained (ng $mL^{-1}$) | Accuracy (%) | CV (%) |
|---|---|---|---|---|---|---|---|
| 25 | 24 ± 1·2 | 96 | 5 | 20 | 17·5 ± 2·2 | 88 | 13 |
| 100 | 99 ± 7·1 | 99 | 7 | 75 | 73·5 ± 6·6 | 98 | 8 |
| 200 | 199 ± 18·4 | 100 | 9 | 150 | 149 ± 15·4 | 99 | 10 |
| 400 | 401 ± 18·4 | 100 | 5 | | | | |

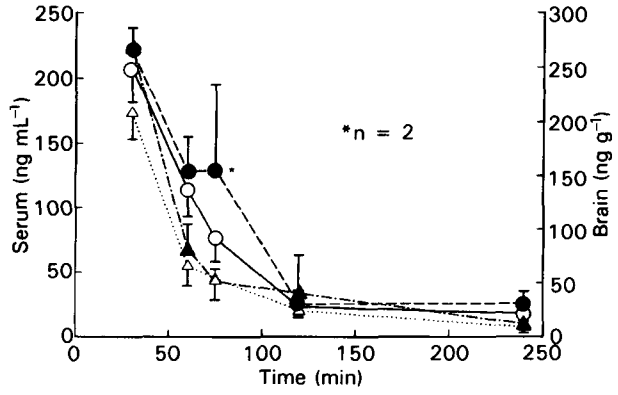


FIG. 3. Graphs of serum and brain epiroprim concentrations (mean ± s.e.m.; n = 2–5) over time. Serum (△) and brain (▲) concentrations after 50 mg $kg^{-1}$ single dose. Serum (○) and brain (●) concentrations after 100 mg $kg^{-1}$ single dose.

for the 100-mg $kg^{-1}$ dose was 207 ± 25 ng $mL^{-1}$. By 240 min after administration, the serum concentrations had fallen to 8 ± 5 and 18 ± 6 ng $mL^{-1}$, respectively, for the two doses.

The concentrations of epiroprim in brain corresponding to those in serum are also shown in Fig. 3. The highest concentrations were again obtained from the 30-min sample, 223 ± 70 ng $g^{-1}$ for the 50-mg $kg^{-1}$ dose and 265 ± 21 ng $g^{-1}$ for the 100-mg $kg^{-1}$ dose. The concentrations at the last sampling time of 240 min were 10 ± 10 and 31 ± 11 ng $g^{-1}$, respectively, for the two doses used.

Epiroprim concentrations in the brain correlated well with those in the serum. The correlation coefficient for the 50 mg $kg^{-1}$ dose was 0·86 and that for the 100-mg $kg^{-1}$ dose was 0·93. The ratio of the concentrations in serum (ng $mL^{-1}$) and brain (ng $g^{-1}$) 30 min after administration of the 50-mg $kg^{-1}$ dose was 0·85 ± 0·16. The mean ratio for all samples for this dose was 0·93 ± 0·12. Similarly, the ratio for the 100-mg $kg^{-1}$ dose at 30 min was 0·79 ± 0·09 and that for all samples was 0·84 ± 0·11.

The method presented here is simple, sensitive, reproducible and accurate. The sensitivity of estimation of epiroprim in serum can be further increased by using a larger volume of serum or by reducing the integrator attenuation, or both; such conditions would enable quantitation of lower concentrations for full pharmacokinetic studies. Absolute recovery of epiroprim for serum samples, measured by comparison with standards analysed directly by HPLC, was approximately 90%. Absolute recovery from brain was approximately 55%. The different absolute recovery rates were compensated for by calibration using drug-free serum or drug-free whole brain to which epiroprim had been added.

Serum epiroprim concentrations analysed by microbiological assay after 100 mg $kg^{-1}$ oral gavage have been reported (Chang et al 1994). The concentrations found by Chang were much higher than those obtained in this study. Microbiological assays are usually less specific than HPLC analysis and are subject to greater variability. It is possible that epiroprim is metabolized to an active metabolite(s) which could then contribute to the higher concentrations obtained using microbiological assay. Indeed, when we chromatographed the serum sample with a more polar mobile phase, we observed another peak which had not been detected during calibration with drug-free serum.

Although maximum concentrations for both doses were obtained for the 30-min samples, these might not be the real maxima because sampling started at 30 min and the next sampling was at 60 min. The drug was still detectable in the serum and brain 4 h after oral administration of both doses. With the lower dose, however, concentrations in some serum and (especially) brain samples were undetectable.

A ratio of serum concentration to brain concentration of less than unity shows that the concentration in the brain is high. The slightly lower mean ratios for both doses at 30 min, compared with the respective overall mean ratios, seem to indicate that distribution of epiroprim to the brain is rapid, and that at 30 min concentrations are higher in the brain before redistribution reduces the concentrations, thereby subsequently resulting in higher serum/brain concentration ratios. Our data also support the notion that absorption from the gastrointestinal tract is rapid. The high concentrations in the brain could explain the efficacy of epiroprim in potentiating the effects of dapsone in both acute and chronic toxoplasma encephalitis induced experimentally in mice.

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