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QUANTITATIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF PLASMA AND TISSUE LEVELS OF 2,4-DIAMINO-5-(3,4-DICHLOROPHENYL)-6-METHYLPYRIMIDINE (METOPRINE) AND ITS APPLICATION TO THE MEASUREMENT OF BRAIN CAPILLARY PERMEABILITY COEFFICIENTS

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SUMMARY

A high-pressure liquid chromatographic assay for metoprine levels in aqueous media, serum, and tissue extracts has been developed that can measure concentrations of the drug in the 25–100 ng range with accuracy, reproducibility, and ease. The half-time for metoprine disappearance from rat plasma and capillary permeability coefficients for metoprine in rat brain tissue determined with this method are in excellent agreement with values determined for related drugs using labeled compounds.

INTRODUCTION

Diaminopyrimidines have been used for the treatment of malaria for many years. Of the many 2,4-diaminopyrimidines synthesized and tested for antimalarial activity, pyrimethamine (Daraprim) is one of the most popular and effective[1,2]. Like methotrexate and Baker's antifol (triazinate), 2,4-diaminopyrimidines exert their cytotoxic action by strongly binding to and inhibiting the enzyme dihydrofolate reductase[3–6]. Cancer therapists became interested in this class of compounds after it was found that rather high doses of pyrimethamine achieved remission of meningeal leukemia in an adult patient suffering from acute myeloblastic leukemia[7].

Interest in the antitumor activity of diaminopyrimidines led to several methods for quantitating levels of these drugs in biological fluids[8–14]. While some methods were unsatisfactory for a number of reasons[8–11], several were versatile and sensitive[11–14]. However, the equipment needed to perform the latter assays is both elaborate and expensive. We report here a rapid, versatile and sensitive high-pressure liquid chromatographic (HPLC) assay for the antitumor drug 2,4-diamino-5-(3,4-

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dichlorophenyl)-6-methylpyrimidine (Metoprine, or DDMP) that employs apparatus commonly available in clinical laboratories.

MATERIALS AND METHODS

Chromatography apparatus

A Chromatronix Model 3500 (Spectra-Physics) high-pressure liquid chromatograph was equipped with a Model 220 UV detector (Spectra-Physics) set at 280 nm with optimal interference filters, a 30 cm × 4.0 mm μBondapak C_{18}–Corasil column (Waters Assoc., Milford, Mass., U.S.A.), a 1–20 μl loop injection port (Rheodyne), and a Varian Aerograph Model 20 10-in. strip chart recorder. The column was eluted with a degassed, Millipore filtered (47 mm, 0.45 nm) methanol–0.02 *M* phosphate buffer (pH 7.5) solution (65:35, v/v) at the rate of 1.4 ml/min at ambient temperature with an inlet pressure averaging 1600 p.s.i.

Drug standards

Metoprine (DDMP, B.W. 1970; Lot No. 58167), etoprine (2,4-diamino-5-[3,4-dichlorophenyl]-6-ethylpyrimidine, DDEP, B.W. 2760; Lot No. 64461), ^{14}C-pyrimethamine, and ^{14}C-etoprine were gifts from Dr. Charles Nichols (Burroughs Wellcome, Research Triangle Park, N.C., U.S.A.).

Each drug (1 mg/ml) was dissolved in methanol with gentle heating or sonification to give stock standard solutions, which were stable for at least one month at 25°. Working standards (0.1, 0.5 and 0.01 mg/ml) were prepared by diluting the stock solutions with methanol. Each drug in methanol was found to be analytically pure by chemical ionization mass spectroscopy.

Standard curves of metoprine and etoprine

Metoprine and etoprine were quantitatively diluted with methanol to give standard solutions with final concentrations of 25, 50, 100, 200, and 400 ng/10 μl. Ten μl of each concentration of metoprine and etoprine were injected into the chromatograph. The peak height of each standard was measured (mm) and plotted against the drug concentration. Duplicate determinations were performed on four separate days.

Standard curves in pooled rat plasma were prepared in a similar manner: 0.1 ml of each standard solution was mixed in 1.5 ml polypropylene centrifuge tubes with 0.1 ml of methanol–0.02 *M* phosphate buffer (pH 7.5) (40:60, v/v) solution, a reagent that precipitates endogenous material in plasma. The tubes were vortexed and spun at 8000 *g* for 5 min in a Brinkman 3200 centrifuge. A 10-μl aliquot of tle clear supernatant was injected into the chromatograph. Plasma samples were run in duplicate. The DDEP internal standard was checked each day.

The following formula was used to calculate the DDMP concentration in μg/g of plasma or tissue wet weight:

$$\text{DDMP, } \mu\text{g/g} = \frac{\text{mm peak ht. DDMP}}{\text{mm peak ht. DDEP}} \cdot \frac{R \text{ IS}}{\text{wt. in g}} \qquad (1)$$

where mm = millimeters; R = slope of DDEP/slope of DDMP from plasma; I.S. = weight of DDEP internal standard, μg.

In vivo rat brain capillary permeability studies

Adult Fischer rats weighing between 190–230 g were lightly anesthetized with ether and a PE 10 catheter was introduced into the saphenous vein via a cutdown. Metoprine and ^{14}C-etoprine (7.5–10 mg/kg) were dissolved with heating in 0.01 *N* lactic acid, mixed with 3 drops of tritiated water (12.5 mCi/ml) and pumped into the saphenous vein with a Harvard Infusion Syringe Pump (Harvard Apparatus) calibrated to deliver 0.146 ml in 5 sec. Five seconds after the infusion began, the animals were decapitated and the head was immediately frozen in liquid nitrogen. Blood samples were obtained from the neck stump. After removal from the liquid nitrogen, the head was cut with a Stryker bone saw, the brain was dissected free, and brain tissue slabs were cut. All samples were weighed in closed tared weighing bottles. Samples for scintillation counting were solubilized with NSC (Amersham-Searle, Arlington Heights, Ill., U.S.A.) and counted in a toluene base fluor. Samples for metoprine determination were extracted and measured as described above. The permeability coefficient (P) was calculated by the formula and constants given by Levin *et al.*[15].

Brain tissue extraction

Rat brain cortex and subcortex weighing 90–120 mg were homogenized with 0.270 ml of precipitating reagent containing 70 μl of the I.S. using 2.5 ml PTFE-pestle glass tissue grinders. The homogenate was decanted into 1.5 ml polypropylene centrifuge tubes, and the tissue grinders were rinsed with an additional 0.230 ml of precipitating reagent. The pooled solutions were spun for 10 min at 8000 *g*. A 10-μl aliquot of the clear supernatant was injected into the chromatograph, and the concentration of metoprine in μg/g of tissue was calculated using eqn. 1.

In vivo metoprine plasma disappearance curve

Two fasted adult Fischer rats weighing 330 and 310 g were given a therapeutic dose of metoprine (10 mg/kg) suspended in a mixture of Tween 80 and propylene glycol, which was administered by a stomach tube. Blood samples were taken at various times over a 24-h period by heart puncture from lightly anesthesized rats. Plasma samples of 100 μl were extracted and quantitated by the HPLC technique described above.

RESULTS AND DISCUSSION

Fig. 1 is a representative chromatogram of pyrimethamine, metoprine, and etoprine eluted with the methanol–phosphate buffer solvent system. Figs. 2 and 3 show typical chromatograms of extracts of rat plasma and brain tissue containing metoprine and etoprine. Good column resolution and efficiency were obtained as shown by the narrow base line width and band spread. The simple and rapid extraction procedure described here has advantages over the extraction procedure of De-Angelis *et al.*[13], because it eliminates interfering peaks from endogenous compounds in rat or human plasma, or in rat brain tissue. In addition, compounds such as heparin, propylene glycol, pentobarbital, dilatin and mysoline do not interfere with this procedure.

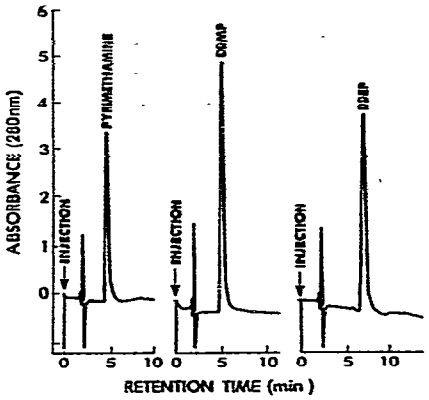
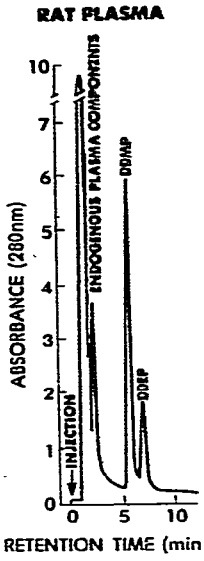


Fig. 1. High-pressure liquid chromatogram of pyrimethamine, metoprine (DDMP), and etoprine (DDEP) at 280 nm; attenuation, 0.01; chart speed, 0 17 in./min; flow-rate, 1.5 ml/min.



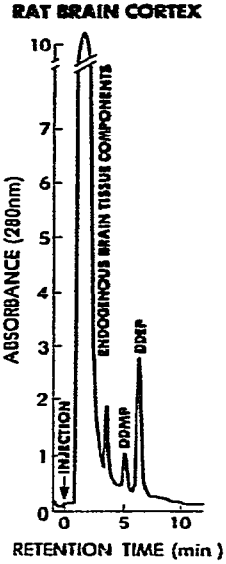
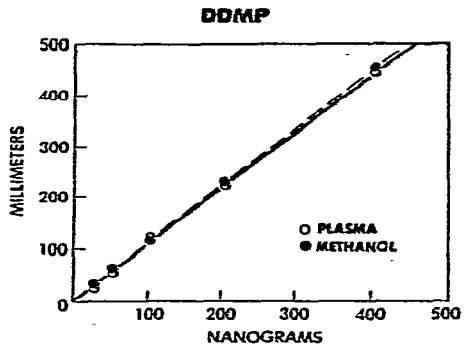


Fig. 2. High-pressure liquid chromatogram of metoprine (DDMP) and etoprine (DDEP) in rat plasma at 280 nm; attenuation, 0.01; chart speed, 0.17 in./min; flow-rate, 1.5 ml/min.

Fig. 3. High-pressure liquid chromatogram of metoprine (DDMP) and etoprine (DDEP) in rat brain tissue at 280 nm; attenuation, 0.01; chart speed, 0.17 in./min; flow-rate, 1.5 ml/min.

The calibration curves for metoprine and etoprine in methanol and plasma are shown in Figs. 4 and 5. The plots of concentration *vs.* peak height for each drug were linear between 10 and 400 ng.

Table I lists the analytical recovery ranges for metoprine and etoprine. The recovery procentage for the drugs over the entire linear range indicated an extraction efficiency of 83–111%. The accuracy of this method is shown in Fig. 6, which compares the amount of metoprine added to serum samples with the amount of metoprine determined by the HPLC method.



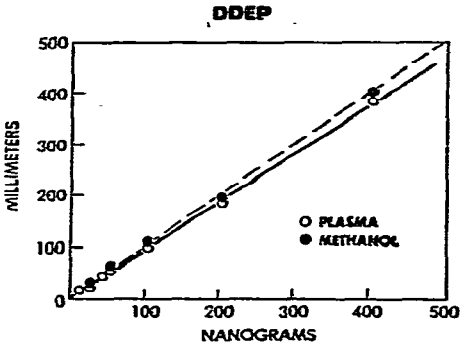


Fig. 4. Calibration curve of metoprine (DDMP) at 280 nm; attenuation, 0.01; plasma O—O; slope = 1.09303; $S.D._{slope}$ = 0.0173; $S.E._{est.}$ = 5.86; methanol ●—●; slope = 1.13085; $S.D._{slope}$ = 0.00862; $S.E._{est.}$ = 2.87.

Fig. 5. Calibration curve of etoprine (DDEP) at 280 nm; attenuation, 0.01; plasma O—O; slope = 0.93636; $S.D._{slope}$ = 0.0156; $S.E._{est.}$ = 5.36; methanol ●—●; slope = 1.00826; $S.D._{slope}$ = 0.0206; $S.E._{est.}$ = 7.81.

TABLE I

RECOVERY RATES FOR DDMP AND DDEP IN RAT PLASMA

Drug	*n*	*Recovery ± S.D. (%)*	*Range (ng)*
DDMP	21	103 ± 14	25–400
DDEP	7	97 ± 14	10–400

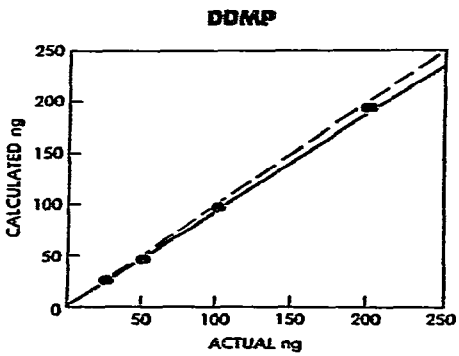


Fig. 6. Actual metoprine (DDMP) *vs.* calculated after extraction from plasma. ●—● = Calculated DDMP; O—O = actual DDMP.

The precision data for the drugs analyzed by this procedure are presented in Table II. Within-run and day-to-day precision was estimated ten times over a two-month period. The coefficient of variation (C.V.) was 4.7% for metoprine at a 200 ng level, and 6.3% for etoprine at a 100-ng level.

To demonstrate the sensitivity and applicability of this HPLC method, six 5-sec rat capillary permeability studies were done using the technique of Levin *et al.*[15]. Metoprine levels ranged from 14–84 μg/g of plasma, and 0.718 μg/g of brain

TABLE II

PRECISION DATA FOR DDMP AND DDEP IN METHANOL

Drug	*Actual*	*Amount calculated (ng) ± S.D.*	*C.V. (%)*
DDMP	200	216 ± 10.2	4.7
DDEP	100	93 ± 5.9	6.3

tissue. Capillary permeability coefficients (P) for brain tissue, obtained by the HPLC method (Table III) were compared to the tritiated water, ^{14}C-pyrimethamine and ^{14}C-etoprine P values. Metoprine has a permeability coefficient consistent with other lipophilic drugs of this size[15], as is evident from the rapid penetration of metoprine across the blood brain barrier in these 5-sec experiments. The permeability coefficients of metoprine were not statistically different (student's *t*-test on logs) from tritiated water, ^{14}C-etoprine, or ^{14}C-pyrimethamine. These compounds demonstrate infinite capillary permeability by the definition and limits of the measurement established by Levin *et al.*[15].

TABLE III

BRAIN CAPILLARY PERMEABILITY COEFFICIENTS

Substance	*Permeability coefficient (cm/sec)*	*% Log S.E.*	*n*
^{3}HOH	1.2×10^{-4}	5.1	6
^{14}C-Pyrimethamine	1.2×10^{-4}	2.4	7
Metoprine	1.5×10^{-4}	8.3	6
^{14}C-Etoprine	1.1×10^{-4}	6.8	2

The plasma disappearance curve is given in Fig. 7. The half-life of metoprine in plasma from 4–24 h was determined from the slope of the curve to be approximately 16.5 h. This value is in agreement with the 18-h half-life observed by Nichol *et al.*[16], using radioactivity measurements in rats.

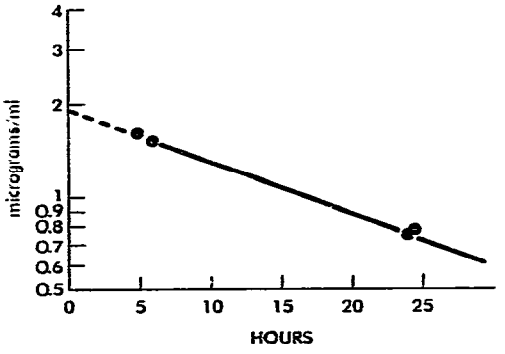


Fig. 7. Plasma metoprine levels after oral administration of 10 mg/kg. $T_{1/2}$ = 16½h; determined graphically.

This simple and accurate assay could be useful for pharmacological studies designed to correlate drug levels to toxicity. Such an approach with methotrexate has been invaluable in improving drug dose scheduling in combination with leucovorin rescue.

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