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A quantitative high-pressure liquid chromatographic procedure for the determination of plasma and tissue levels of 2, 4-diamino-5-(3, 4-dichlorophenyl)-6-methylpyrimidine

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A QUANTITATIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF PLASMA AND TISSUE LEVELS OF 2,4-DIAMINO-5-(3,4-DICHLOROPHENYL)-6-METHYLPYRIMIDINE

by
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THESIS

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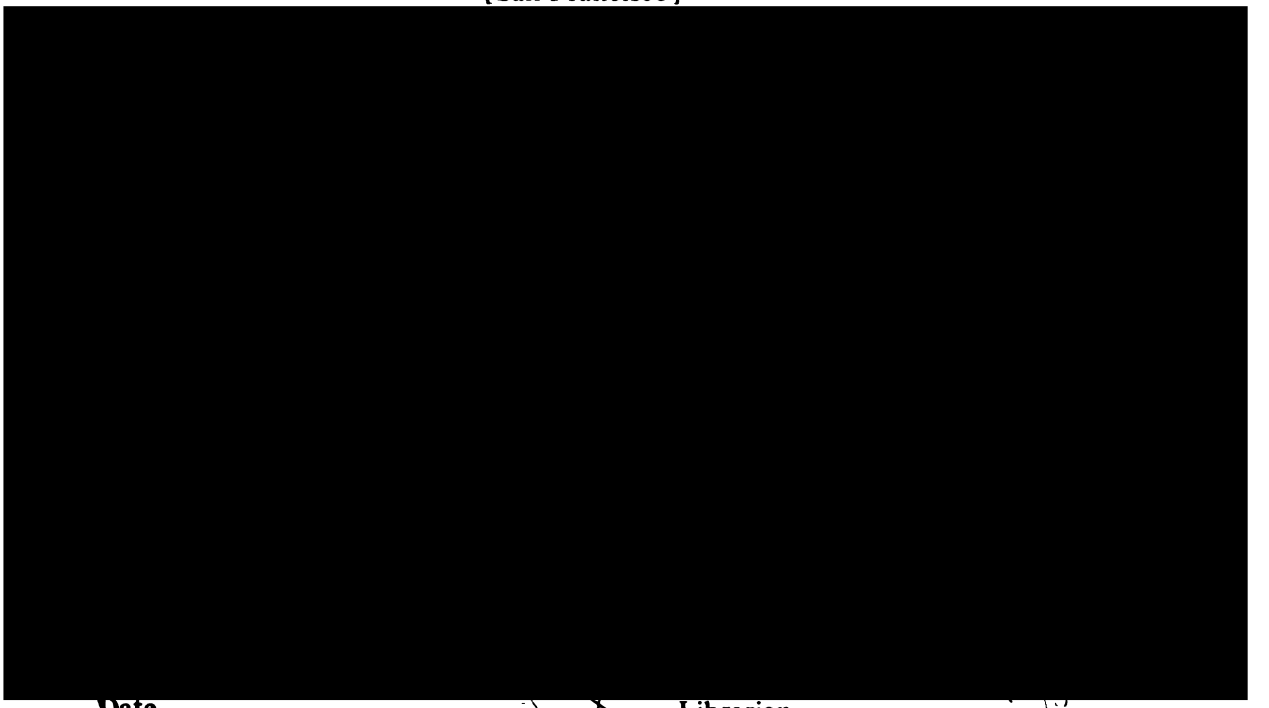

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I) INTRODUCTION

Tumors of the central nervous system (CNS) are responsible for nearly 2% of all cancer deaths, and available statistics indicate that more than 10,000 people in the United States will develop a brain tumor in 1977. In the first two decades of life, tumors of the CNS rank number one among solid tumors and are exceeded only by childhood leukemia as a cancer cause of death in children < 15 years of age (1).

Since surgery and radiotherapy cannot adequately control malignant brain tumors, multi-drug and drug radiation protocols are needed. Although chemotherapy holds enormous promise for more effective management of all brain tumors, nevertheless, there are few drugs available with demonstrable antitumor activity and the essential transport properties for blood-brain passage. Those agents shown to have the greatest success have been 1) lipophilic and able to cross normal brain capillaries with ease, and 2) cell-cycle phase non-specific in their mode of action (2,3,4). It is anticipated that drug therapy combinations with cell-cycle phase non-specific and phase-specific drugs would be advantageous if the drugs had dissimilar or controllable toxicities. Currently, none of the antimetabolites such as methotrexate or Baker's antifol (triazinate) are used effectively in the treatment of solid CNS tumors (3).

Two compounds synthesized by Burroughs Wellcome Laboratories, metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylprimidine; DDMP), and etoprine, the corresponding 6-ethyl analog (DDEP) are lipid-soluble compounds which cross the blood-brain barrier and penetrate brain tumors induced in rats by ethylnitrosourea (5). In addition, metoprine has

shown activity against a virally induced rat brain tumor (6). Therefore, investigation of the effectiveness of metoprine in the treatment of CNS tumors appears worthwhile.

The purpose of this paper is to present a new method of analysis for metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine) in plasma and tissues which will be useful for pharmacokinetic studies in animals and patients.

II) LITERATURE REVIEW

Diaminopyrimidines have been used medically 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 (7,8). Like methotrexate and Baker's antifol (triazinate), 2,4-diaminopyrimidines exert their cytotoxic action by strongly binding to and inhibiting the enzyme dihydrofolate reductase (9,10,11,12). Cancer therapists became interested in this class of compounds following the observation that pyrimethamine, given in rather high doses, achieved remission of menigeal leukemia in an adult patient suffering from acute myeloblastic leukemia (13). This increased interest in pyrimethamines led to the development of analytical methods to quantitate several 2,4-diaminopyrimidines; this was followed by pharmacokinetic studies and clinical trials of these compounds.

Schmidt, et al., measured pyrimethamine in urine and plasma by extracting it into an organic solvent and measuring the extract by direct spectrophotometry at 270 nm (14). This method lacked specificity as well as sensitivity. Golinsky, et al., reported a method for measuring levels of pyrimethamine and several related 2,4-diaminopyrimidines by selective desorption from Sephadex G-25 and G-10 columns. The concentrations were calculated from ultraviolet (UV) spectrophotometric measurements made at 270 nm. This method, although it had the required specificity, lacked sensitivity and was very time consuming (15).

Jones et al., measured pyrimethamine levels in urine utilizing the weak natural fluorescence of the compound. They chromatographed an

extract of urine on a thin-layer chromatography (TLC) plate and scanned it in a specially modified spectrophotofluorometer. The method was convenient and versatile, and the sensitivity of the fluorescence as a means of quantitation had potential. However, the lower limit for quantitative detection for pyrimethamine in urine was inadequate to support clinical studies (16,17). This led Simmons and DeAngelis to develop a spectrophotofluormetric method which could be used to measure very low levels of pyrimethamine in biological fluids. This method was based upon the induced fluorescence of these compounds when the silica gel TLC plates were sprayed with 2M aqueous ammonium hydrogen sulfate. The resulting fluorescence was then quantitated with a TLC scanner (18). This method was further improved by these investigators by utilizing absorbance to make it more sensitive, more versatile and faster over the induced fluorescence technique (19). At about the same time, Sigel and Grace developed a similar method for assaying trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) and its metabolites (20).

Although the method for quantitating the 2,4-diaminopyrimidines described by Simmons and DeAngelis was sensitive and versatile, the equipment needed to perform the analysis is elaborate and expensive. Hence, the reason for developing the high-pressure liquid chromatography (HPLC) method presented in this paper.

All types of chromatography are based on the phenomenon that each component in a mixture ordinarily interacts with its environment differently from all other components under the same conditions. Liquid chromatography (LC) in which solute molecules are separated by one or a combination of physio-chemical processes includes four distinct

families of procedures according to the mechanism of retention: 1) ion exchange, 2) exclusion (gel permeation), 3) liquid-solid (adsorption), and, 4) liquid-liquid (partition).

Ion exchange materials represent an important class of stationary phases used in LC. A reversible exchange of ions takes place between the stationary ion-exchange phase and the external liquid mobile phase when ion-exchange materials are employed in this type of chromatography (21).

The most widely used solid ion exchangers are the synthetic polymers and derivatives of cellulose. These synthetic resins contain polar groups, acidic or basic in nature, which are introduced either before or after the polymerization stage and consist of an insoluble polymeric matrix that is permeable. Numerous types of cation or anion exchange resins (depending on whether they have an affinity for cations or anions) are commerically available (21).

In gel permeation chromatography, the stationary phase is a porous solid and the solute molecules are retarded as a result of their permeation into the solvent-filled pores of the column packing. Larger molecules excluded from all or a portion of the pores by virtue of their physical size elute from the column before the small molecules, thereby, providing a separation based on molecular size in solution. Numerous packings are available for gel permeation chromatography and are classified by chemical type as being soft, semirigid, or rigid. Both gel performance and operating technique are related to this property (21).

Separations by liquid-solid chromatography are usually carried out on polar adsorbents such as silica, and alumina. The primary factor

in determining the relative adsorption of a sample molecule is its functional groups. Relative adsorption increases as the polarity and number of these functional groups increases, because the total interaction between the molecule and the polar adsorbent surface is thereby increased (21).

Liquid-liquid chromatography (LLC), sometimes called liquid-partition chromatography, has been a powerful technique for high resolution separations since its inception in 1941 by Martin and Synge (22). Separation by this method is based on differences in the solubility of individual solute molecules in two immiscible liquids that are in contact with one another. The degree to which the individual solutes distribute between the two solvents is expressed by the partition (distribution) coefficient K_d. This coefficient is defined as the ratio of the solubility of the solute in the receiving phase to the solubility in the original solvent. The greater the value of the partition coefficient, the more rapid and complete is the movement of solute from the original into the extracting solvent (23).

The mobile phase in liquid chromatography can be any combination of solvents that are compatible with the sample, column, and detector. The addition of small amounts of chemicals (other than the primary solvent) to the mobile phase solvent can impart special selectivity to the system. The column stationary phase is selected by considering the affinity for the components to be separated.

In normal-phase LLC, the sample and column packing material (stationary phase) are polar and the solvent (mobile phase) is non-polar. Consequently, the more polar compounds bind to the column and the least

polar compounds elute first (24).

In reverse-phase LLC, the sample and column packing material are non-polar while the solvent is polar. In this situation, the most polar compounds elute first (24). This technique has become quite popular for the separation of drugs in biological fluids.

In liquid chromatography, the detector used to monitor the concentration of the solute in the mobile phase as the separated sample components elute from the column is an important consideration in analytical liquid chromatography. The currently used detectors utilize a variety of detection principals and methods each suited for a limited range of applications. Of the four types of detectors currently used, two have the widest range of application and are most often used for quantitative analysis: 1) the refractive index (RI), and 2) ultraviolet absorption (UV) detectors. RI detectors monitor the differences between the analytical and reference mobile phases as elutes from the column and can detect solutes in the microgram range. It is useful for samples which do not absorb in the ultraviolet but it is very sensitive to changes in temperature and flow (21).

UV detectors measure light intensity ranging from 200 nm to 380 nm. These detectors are among the most sensitive and widely used in LC and are capable of detecting solutes in the nanogram range for samples which have moderate absorptivities. UV absorption is relatively insensitive to flow and temperature (21). Its simplicity of operation and the fact that the 2,4-diaminopyrimidines are ultraviolet absorbing compounds made it the most appropriate detector for the HPLC method described in this paper.

The fluorescent detectors provide specificity and high sensitivity

for qualitative and quantitative analysis of certain drugs and clinical compounds. Fluorescence, a property of electron rich compounds which absorb light at one wavelength and emit light of a longer wavelength and lower energy, is a characteristic of a relatively small number of compounds that fluoresce directly compared to the very large number that absorb energy. However, a large percentage of these absorbing compounds can be complexed or reacted chemically to transform them into fluorescent compounds. By selection of different complexing agents, fluorescence at different wavelengths may be produced making it possible to work at a wavelength significantly removed from interferences. Unlike UV absorbance, experimental conditions such as temperature, pH, and sample concentration have a great effect on the fluorescent emission and excitation spectrum (23,25).

In addition to the UV absorption, RI, and fluorescent detectors, LC with electrochemical detectors (LCEC) has three distinct advantages for applicable systems: 1) selectivity; 2) sensitivity; and 3) economy. Two detection techniques based on electrochemical phenomena are amperometry and coulometry, the former having the advantage of being more sensitive and less complex. Amperometry is based on measurement of the current flowing through an electrochemical cell when a constant electric potential is applied to the electrodes, whereas, coulometry is a technique used to measure the electrical charge between two electrodes in an electrochemical cell. Liquid chromatographic electrochemical chromatograms are obtained by plotting current as a function of time. It is possible to routinely detect picomole amounts of electroactive compounds separated by HPLC. This type of detector is well suited for ion exchange and reverse phase material since they are compounds with polar solvents

containing dissolved ions (23,26). LCEC is an important new chromatographic tool for trace analysis of organic compounds in complex biological media.

Initially, liquid chromatography was a slow separation technique performed in vertical columns by gravity flow. An increase in speed and resolution has been achieved by pumping the solution through the column at inlet pressures up to and frequently in excess of 1000 psi. Unique developments in structure and surfaces of packing material, the use of smaller diameter high-surface-area particles with a narrow range of particle size such as porasil and corasil, sensitive detectors, reproducible pumping systems, and theoretical insight have all been combined to make high-pressure liquid-liquid chromatography a versatile and practical means of separation. The μ bondapak C_{18}/Corasil column selected for the analysis to be described gives optimum retention and resolution for compounds having lipophilic character. In addition, this method offers the advantage of high resolution and rapid separation in an isocratic mode with a solvent system that favors partition mechanisms over adsorption.

III) MATERIALS AND METHODS

A. Principle

The 2,4-diaminopyrimidines, metoprine and etoprine, are separated by reversed-phased partition (liquid-liquid) chromatography employing a stationary organic phase and a mobile aqueous phase. The column eluents are measured spectrophotometrically using an ultraviolet absorption detector at 280 nm and the peak absorbance recorded on a strip chart recorder. The drug peaks are identified by their retention times and quantitated by the peak height ratio of the drug to the internal standard.

B. Chromatography Apparatus

A Chromatronix Model 3500 (Spectra-Physics) high-pressure liquid chromatograph was equipped with a Model 220 ultraviolet detector (Spectra-Physics) at 280 nm with optimal interference filters, a Varian Aerograph Model 20 ten-inch strip chart recorder, a 30 cm x 4.0 mm μ bondapak C_{18}/Corasil column (Waters Associates), and a 1-20 μl loop injection port (Rheodyne). The column was eluted with a degassed, filtered (Millipore filter, 47 mm, 0.45 μm) methanol/0.02M phosphate buffer, pH 7.5, mixture (65/35, v/v) at the rate of 1.5 ml/minute at ambient temperature with an inlet pressure averaging 1600 psi. The HPLC column was allowed to equilibrate with methanol/buffer for 30 minutes before analysis at the flow rate of 1.5 ml/minute.

C. Chemicals and Reagent Preparations

Methanol, AR grade (Mallinckrodt, St. Louis, Mo. 63160).

Ethyl ether, AR grade (Mallinckrodt).

Lactic acid, 0.01N, AR grade (J.T. Baker Chemical Co., Phillipsburg, N.J. 08865).

^{3}HOH, 12.5 mCi/ml (New England Nuclear Corporation, Boston, Mass. 02118).

Monobasic potassium phosphate and dibasic sodium phosphate AR grade (Mallinckrodt) together with double distilled water were used to prepare the 0.02M phosphate buffer, pH 7.5.

Pyrimethamine (Lot #65170), *Metoprine* (DDMP, B.W. 197U; Lot #58167), and *Etoprine* (DDEP, B.W. 276U; Lot #64461) were gifts from Dr. Charles Nichol (Burroughs Wellcome Co., Research Triangle Park, N.C. 27709).

A stock standard of each drug (1 mg/ml) was prepared by dissolving it in methanol with gentle heating or by sonification. These solutions were stable for at least one month at 25°C.

Working standards (0.1, 0.5, 0.01 mg/ml) were prepared by diluting the stock solutions with methanol.

Precipitating reagent: 0.02M phosphate buffer, pH 7.5, (filtered)/ methanol (40/60 v/v).

Solvent: methanol/0.02M phosphate buffer, pH 7.5 (65/35 v/v), filtered and degassed.

D. Procedures

1. Calibration Curve in Methanol

Known concentrations of metoprine and etoprine were made by diluting the stock solutions of each (1 mg/ml) in methanol in glass volumetric flasks using Hamilton repeating dispensers. 10 µl of each concentration of metoprine and etoprine was injected into the HPLC. The standards injected represented 25, 50, 100, 200, and 400 ng of drug in the final 10 µl volume. The peak height of each standard, detected at 280 nm with an attenuation of .01 and measured in millimeters (mm), was plotted against the drug concentration. Duplicate determinations were carried out at each concentration on four separate days.

D. Procedures (cont.)

2. Calibration Curve in Plasma

Known amounts of the stock standards (1 mg/ml in methanol) of metoprine and etoprine were added to glass volumetric flasks using Hamilton repeating dispensers and brought up to volume with pooled rat plasma. A 0.1 ml aliquot of each concentration was mixed with 0.1 ml of the precipitating reagent in 1.5 ml polypropylene centrifuge tubes, vortexed, and spun at 8,000 g for 5 minutes in a Brinkmann 3200 Centrifuge (Brinkmann Instruments, Inc.). A 10 µl aliquot of the clear supernate was injected into the HPLC. The peak height of each sample was plotted against its theoretical drug concentration. Duplicate determinations were carried out at each concentration on two separate days.

D. Procedures (cont.)

3. Plasma Extraction

Rat plasma (0.1 ml), either fresh or previously frozen, was combined with 30 µl of the working internal standard of DDEP and 70 µl of the precipitating reagent in 1.5 ml centrifuge tubes. The mixtures were vortexed and centrifuged as described. A 10 µl aliquot of the clear supernate was injected into the HPLC. Plasma samples were run in duplicate. As a check on the DDEP internal standard, 10 µl of known amounts of DDEP was injected into the HPLC and the actual amount of DDEP in the internal standard (IS) was determined for each day. In addition, the theoretical concentration of the IS was determined by measuring the actual peak height of the DDEP used to make the IS.

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

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

where,

mm = millimeters

R = slope of DDEP/slope of DDMP from the plasma calibration curves (figures 4 and 5)

IS = DDEP internal standard, µg

D. Procedures (cont.)

4. 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 IS using 2.5 ml teflon pestle glass tissue grinders. The homogenate was decanted into 1.5 ml polypropylene centrifuge tubes. The tissue grinders were rinsed with an additional 0.230 ml of precipitating reagent and this wash combined with the first sample. These pooled solutions were spun for 10 minutes at 8,000 g. A 10 µl aliquot of the clear supernate was injected into the HPLC. The concentration of metoprine in µg/gm of tissue was calculated using the peak height ratios of DDEP/DDMP and the amount of the IS in equation (1).

D. Procedures (cont.)

5. *In Vivo* Rat Studies

a. Brain Capillary Permeability Studies

Adult Fischer rats weighing between 190-230 gm were lightly anesthetized with ether. A PE 10 catheter was introduced into the saphenous vein via a cutdown. Metoprine, 7.5-10 mg/kg, dissolved in either emulfor (polyethoxilated vegetable oil/50% ethanol, NIH) or heated 0.01N lactic acid, was mixed with 3 drops of tritiated water (^{3}HOH, 12.5 mCi/ml) and pumped into the saphenous vein with a Harvard Infusion Syringe Pump (Harvard Apparatus Co., Inc.). The pump was calibrated to deliver 1.75 ml/min. or 0.146 ml in 5 seconds. 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 the brain tissue slabs were cut with disposable razor blades. All samples were weighed in closed tared weighing bottles. Samples for scintillation counting were solubilized with NCS (Amersham-Searle) and counted in a toluene base fluor. Samples for metoprine determination were extracted and measured by the HPLC technique described above. The permeability coefficient (P) was calculated by the formula and constants given by Levin, et al. (27):

$$P\ (cm/sec) = \frac{0.28 \cdot (ICD) \cdot DS}{t\sqrt{BV}} \qquad (2)$$

where,

ICD = intercapillary distance, cm

DS = distribution space of drug or isotope in tissue

t = time, seconds

BV = blood volume of tissue

b. Plasma Disappearance Curve

Two fasted adult Fischer rats, weighing 330 and 310 gms, were given a dose of metoprine in the therapeutic range of 10 mg/kg. The drug, suspended in a mixture of Tween 80 and propylene glycol, was administered by a stomach tube. Blood samples were taken at various times over a 24 hour period by heart puncture from lightly etherized rats. 100 μl plasma samples were extracted for metoprine determinations and quantitated by the HPLC technique described above.

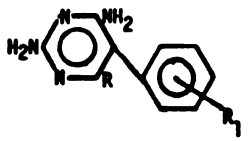
IV) RESULTS

In order to develop a successful separation of any group of compounds, knowledge of their physical characteristics and biological properties is important. Table 1 lists the structure, functional groups, and size of the 2,4-diaminopyrimidines. The dissociation constants and log P values listed in Table 1 indicate that these compounds are approximately 50% ionized within the physiological pH range, and based upon their octanol/water partition coefficients, are highly lipophilic (5).

The absorbance spectra of these compounds determined in freshly prepared 0.01N NaOH and 0.01N HCl gave a maximum absorbance at 285 nm.

Table 1

Physical Characteristics and Biological Properties of Three 2,4 Diaminopyrimidines



Compound	R	R_1	Name	Mol.[a] Wt.	Physical[a] Properties pK_a	log P	Binding to[b] Plasma Proteins	Retention[c] Time min.	λ Max.[d]
I	C_2H_5	4-Cl	Pyrimethamine (Daraprim)	248.71	7.34	2.69	87%	4.7	285
II	CH_3	3,4-Cl_2	Metoprine (DOMP)	269.13	7.15	2.82	88%	5	285
III	C_2H_5	3,4-Cl_2	Etoprine (DOEP)	284.16	7.20	3.19	97%	6	285

a Leo, Hansch, and Elkins. Chem. Rev. 71:525-554, 1971.

b See reference (5).

c From time of injection onto column (p. 11).

d Spectra determined in 0.01N NaOH.

The 2,4-diaminopyrimidines were completely soluble in DMSO and in methanol when gently heated. They were not very soluble in chloroform, hexane, ether, acetonitrile, methylene chloride, or dichloroethane.

After determining the solubility characteristics, the eluent for

the chromatographic separation of metoprine and etoprine was chosen by screening the following solvent systems: chloroform, methanol/chloroform, acetonitrile/chloroform, methanol/methylene chloride, and isopropanol/methylene chloride, using normal-phase LiChrosorb C_{18} and Porasil columns, and methanol/water, methanol/acetate buffer, and methanol/phosphate buffer using reversed-phase LiChrosorb C_{18} and μ Bondapak/C_{18} Corasil columns at various pH values. The methanol/phosphate buffer mixture was found to be the best and was evaluated further by varying the molar concentration (.002M - .05M) and pH (7.1 - 7.5).

Figure 1 (see page 21) is a representative chromatogram of pyrimethamine, metoprine, and etoprine eluted with the described methanol/phosphate buffer solvent system at a flow rate of 1.5 ml/min. The 2,4-diaminopyrimidines were detected at 280 nm with an attenuation of .01 and identified by their retention times. The retention times for pyrimethamine, metoprine and etoprine were 4.7, 5.0, and 6.0 mins., respectively. Good column resolution and column efficiency were obtained with this system as seen by the narrow baseline width and minimum band spread.

Each standard drug in methanol was found to be analytically pure by chemical ionization mass spectroscopy. Mass spectroscopy of the collected effluent corresponding to the resulting peak of each drug (metoprine and etoprine) was also done but background contaminants from the phosphate buffer made resolution by mass spectroscopy impossible.

Figures 2 and 3 (see pages 22 and 23) show typical chromatograms of extracts of rat plasma and brain tissue containing metoprine and etoprine using this HPLC method. The extraction procedure of DeAngelis, et al., (19) was tried but abandoned in favor of the simple and quick extraction procedure described in this paper. With this single step

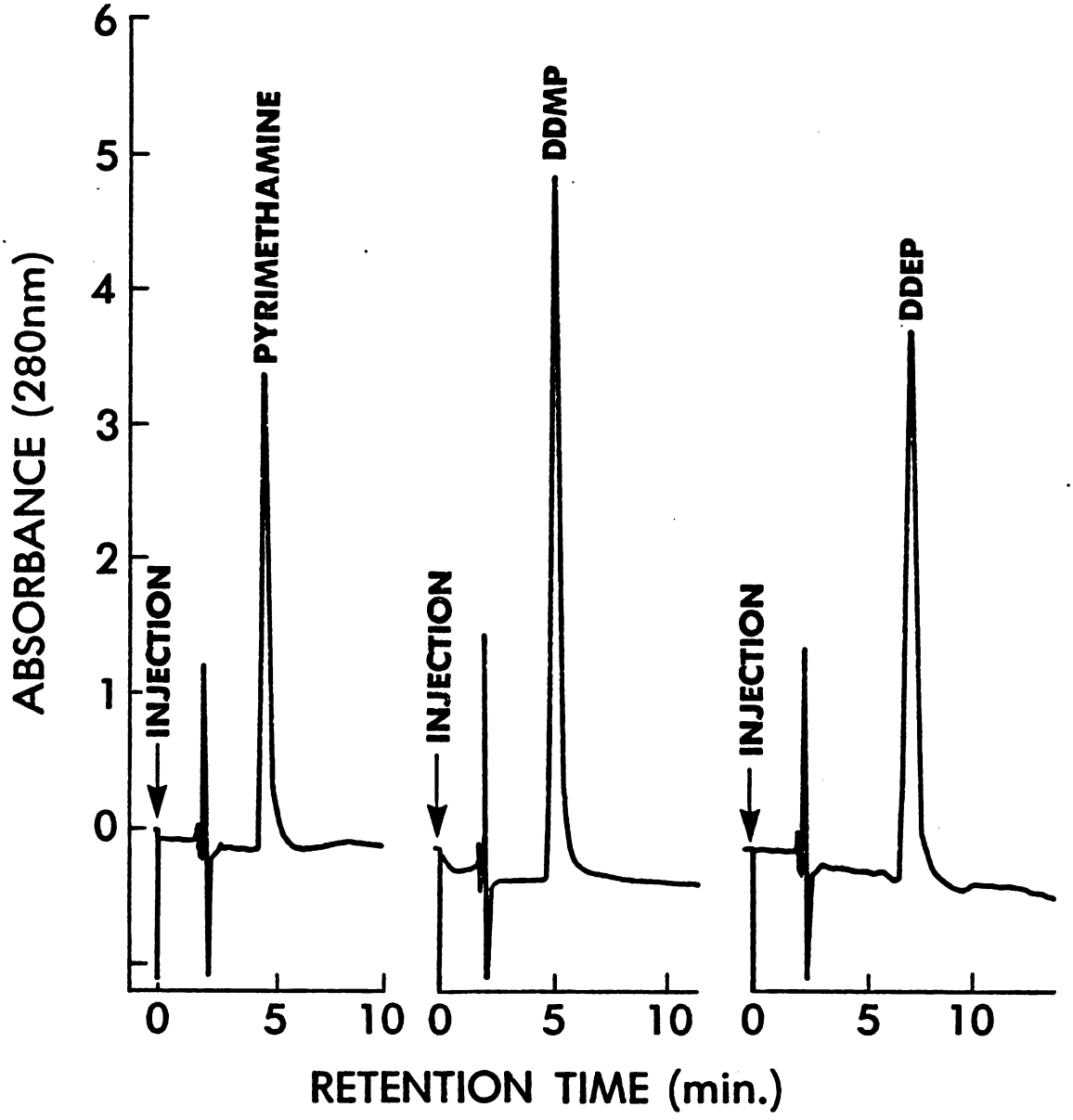


Figure 1. High-pressure liquid chromatogram of pyrimethamine, metoprine (DDMP), etoprine (DDEP) at 280 nm, attenuation .01, chart speed .17 inch/min., and a flow rate of 1.5 ml/min.

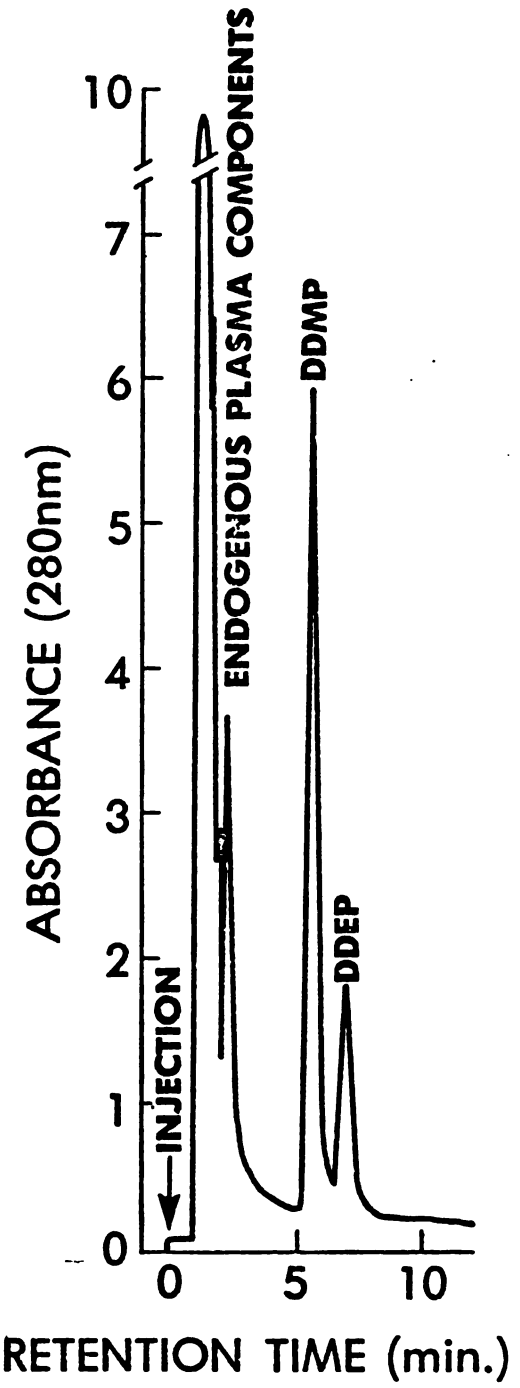


Figure 2. High-pressure liquid chromatogram of rat plasma at 280 nm, attenuation .01, chart speed .17 inch/min., and flow rate of 1.5 ml/min.

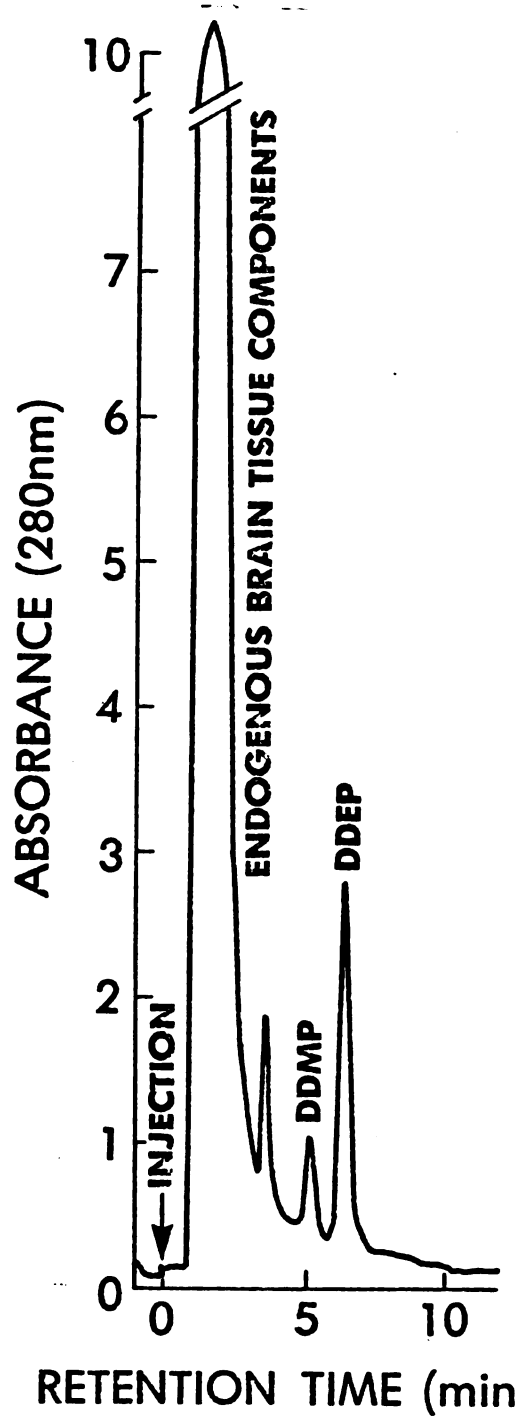


Figure 3. High-pressure liquid chromatogram of the rat brain tissue at 280 nm, attenuation .01, chart speed of .17 inch/min., and a flow rate of 1.5 ml/min.

extraction, there were no interfering peaks from endogenous compounds in rat or human plasma or in rat brain tissue. In addition, none of the tested compounds such as heparin, propylene glycol, pentobarbital, dilantin, and mysoline interfered with this procedure.

The results of the calibration curves of metoprine and etoprine in methanol and plasma are graphically presented in Figures 4 and 5 (see pages 25 and 26). The concentration and peak height of each drug were linearly related from 10 ng to 400 ng. Also represented on the graph are the calculated slope of the line, the standard deviation of the slope, and the standard error of the estimate.

Table 2 lists the analytical recovery ranges for metoprine and etoprine, The % recovery for the drugs for the entire linear range indicated an extraction efficiency of 83-111%.

Table 2

Recovery Rates For DDMP and DDEP in Rat Plasma

Drug	n	% Recovery $\pm$ S.D.	Range (ng)
DDMP	21	103 $\pm$ 14%	25-400
DDEP	7	97 $\pm$ 14%	10-400

The data showing the accuracy of this method are represented in Figure 6 (see page 27). Each point on the graph represents a measured plasma sample containing known amounts of each drug. The actual metoprine concentration calculated by the peak height was plotted against the concentration of metoprine which was calculated by measuring the peak heights of the drugs relative to the internal standard.

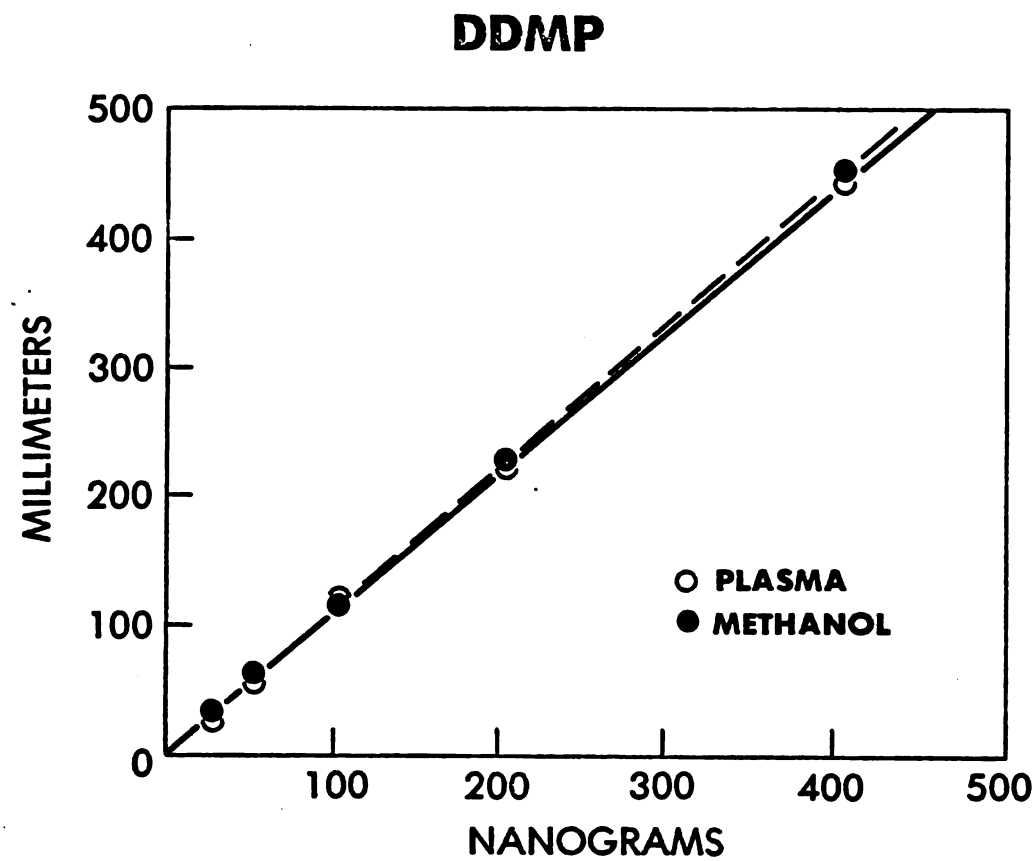


Figure 4. Calibration curve of metoprine (DDMP) at 280 nm, attenuation .01, Plasma o—o; Slope = 1.09303, S.D. Slope = .0173, S.E. EST = 5.86. Methanol ●--●; Slope = 1.13085, S.D. Slope = .00862, S.E. EST = 2.87.

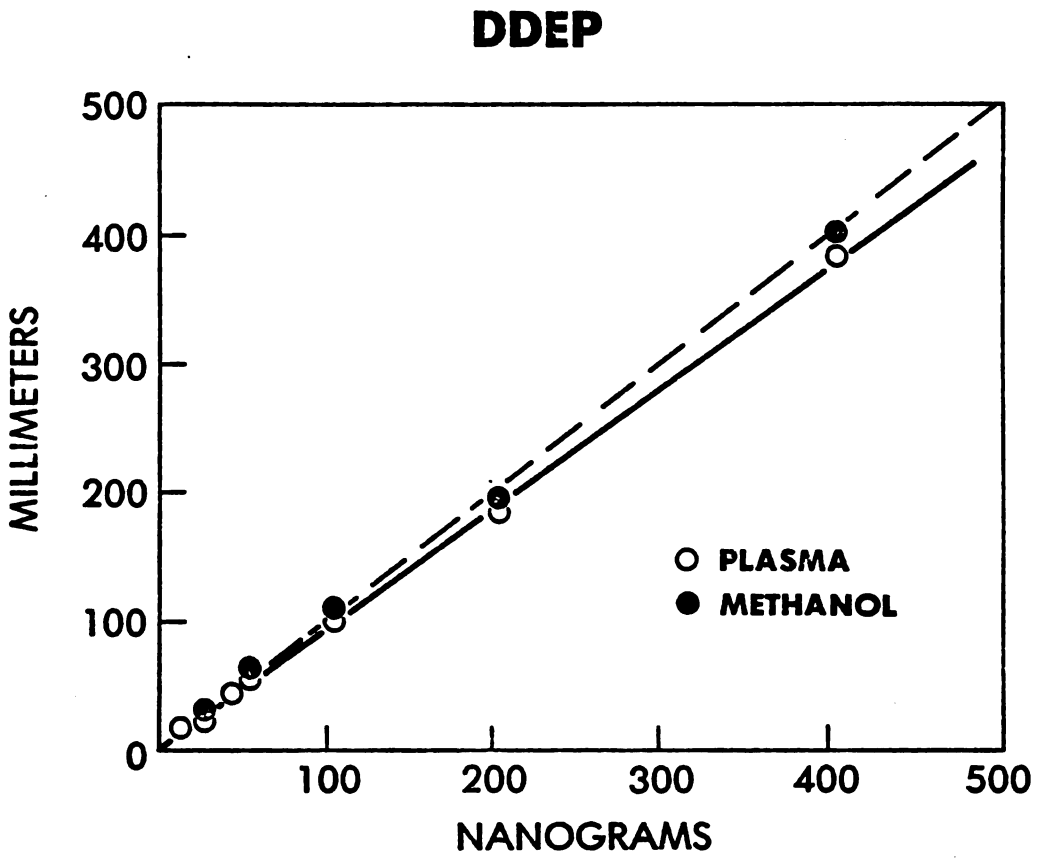


Figure 5. Calibration curve of etoprine (DDEP) at 280 nm, attenuation .01, Plasma o—o; Slope = .93636, S.D. Slope = .0156, S.E. EST = 5.36. Methanol ●----●; Slope = 1.00826, S.D. Slope = .0206, S.E. EST = 7.81.

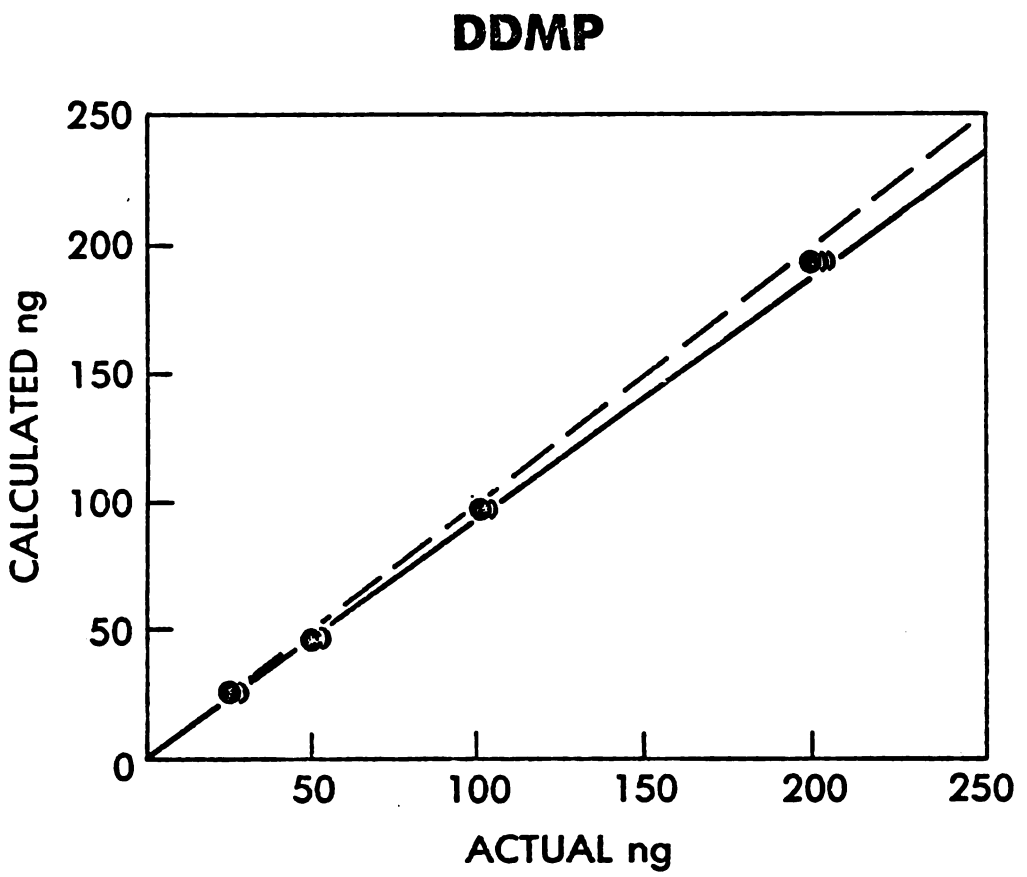


Figure 6. Actual metoprine (DDMP) vs calculated after extraction from plasma.

●--● = calculated DDMP

o—o = actual DDMP

The precision data for the drugs analyzed by this procedure are presented in Table 3. Within-run and day-to-day precision was estimated by 10 day analyses of metoprine and etoprine in methanol over a 2 month period. The coefficient of variation (CV) was 4.7% for metoprine at a 200 ng level, and 6.3% for etoprine at a 100 ng level.

Table 3

Precision of Assays For DDMP and DDEP in Methanol

Drug	Actual	Amount, ng Calculated ± S.D.	CV, %
DDMP	200	216 ± 10.2	4.7
DDEP	100	93 ± 5.9	6.3

To demonstrate the sensitivity and application of this HPLC method, six five-second rat capillary permeability studies were done using the technique of Levin et al., (27). Metoprine levels ranged from 14-84 μg/gm of plasma, and 0.7-18 μg/gm of brain. Capillary permeability coefficients (P) for brain tissue obtained by the HPLC method were compared to the tritiated water, ^{14}C-pyrimethamine, and ^{14}C-etoprine P values and are listed in Table 4. Metoprine has a permeability coefficient consistent with other lipophilic drugs of this size (27). This is evident from the rapid penetration of metoprine across the blood brain barrier in these five-second experiments. The permeability coefficients of metoprine were not statistically (student's t-test on logs) different from ^{3}HOH, ^{14}C-etoprine, or ^{14}C-pyrimethamine (28). These compounds demonstrate infinite capillary permeability by the definition and limits of the measurement established by Levin, et al., (27).

Table 4

Brain Capillary Permeability Coefficients

Substance	Permeability Coefficient cm/sec	% Log S.E./animals
^{3}HOH	1.2×10^{-4}	5.1/6
^{14}C-Pyrimethamine[a]	1.2×10^{-4}	2.4/7
Metoprine	1.5×10^{-4}	8.3/6
^{14}C-Etoprine [a]	1.1×10^{-4}	6.8/2

[a]V.A. Levin and A. Byrd, unpublished observations, 1977.

The results of the plasma disappearance curve appear in Figure 7 (see page 30). The half-life of metoprine in plasma as observed from 4-24 hours and determined graphically was approximately 16½ hours. This value is in agreement with the 18 hour half-life observed by Nichol, et al., using radioactivity measurements in rats (5).

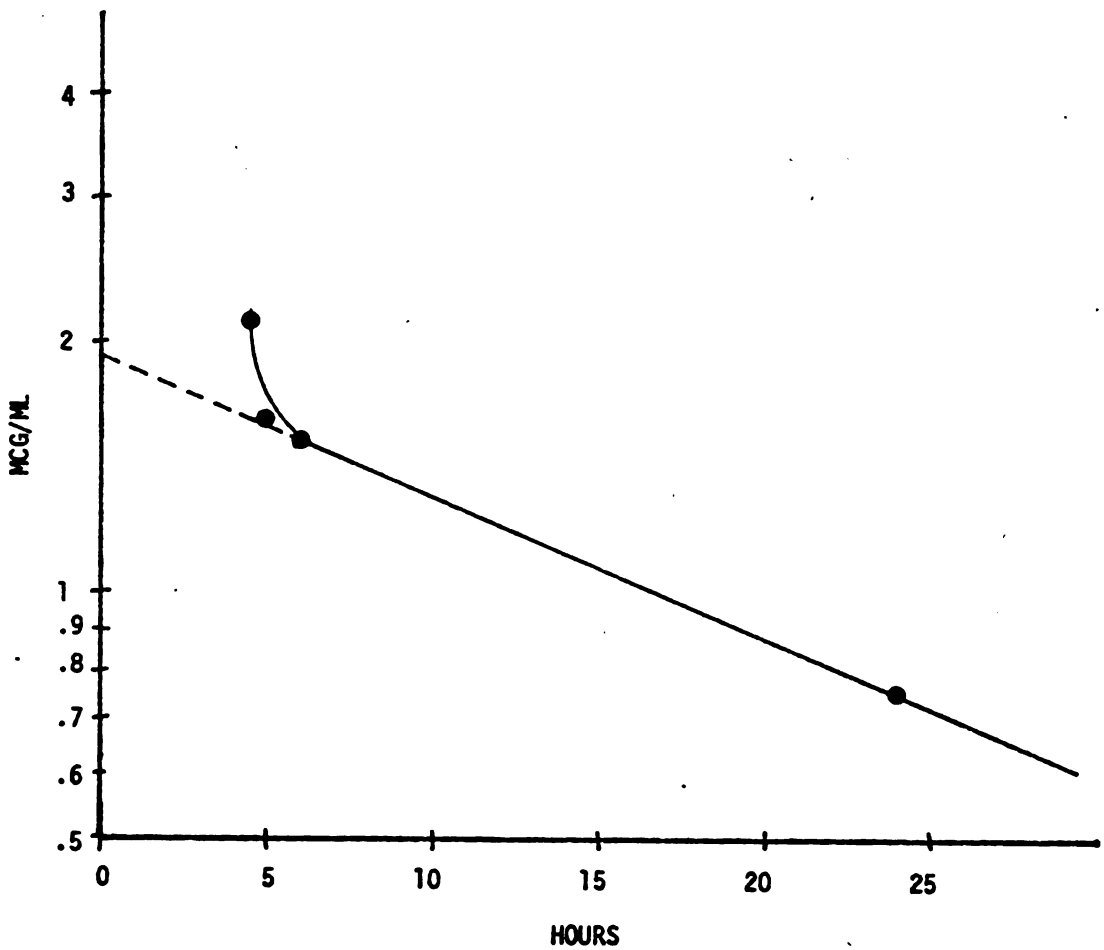


Figure 7. Plasma metoprine levels after oral administration of 10 mg/kg. $T_{1/2}$ = 16½ hours; determined graphically.

V) DISCUSSION

A sensitive and reliable method was developed for quantitating metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine) using etoprine (the corresponding 6-ethyl analog) as the internal standard. This method offers the distinct advantages of speed, reproducible high resolution separation, specificity, and ease of sample preparation over the traditionally employed thin-layer methods.

Metoprine, a lipophilic antifol of potential effectiveness in the treatment of brain tumors, was quantitated following a single step extraction procedure and separation by high-pressure liquid chromatography utilizing the ultraviolet absorption of the pyrimidine ring at 280 nm.

The drug was well separated from the normal blood constituents in less than 10 minutes on the HPLC. As little as 50 µl of plasma and 100 mg of tissue could be extracted using etoprine as the internal standard. The minimum detectable concentration was 10 ng in either methanol or plasma. Drug levels between 0.7-18 µg/gm of brain were accurately measured.

Application of this method demonstrated quantitatively detectable plasma and brain tissue pyrimidine levels in five-second brain capillary permeability studies. The capillary permeability coefficients were determined and found to be similar to the P values for tritiated water (^{3}HOH), ^{14}C-etoprine, and ^{14}C-pyrimethamine. This was interpreted as evidence that there is virtually no blood brain barrier to metoprine.

The half-life for plasma disappearance of metoprine between 4 and 24 hours after oral administration was approximately 16½ hours. Nichol, et al., observed a half-life of 18 hours with radioactive measurements

of metoprine in rats. The extensive binding of metoprine to plasma proteins (Table 1) undoubtedly contributes to its retention in plasma and its slow rate of elimination.

The development of this method could be useful for the pharmacological studies designed to correlate drug levels to toxicity. Such an approach with methotrexate has been found invaluable in improving drug dose scheduling in combination with leucovorin rescue (29).

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VII) ABBREVIATIONS

CNS = Central nervous system

DDMP = Metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine

DDEP = Etoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine

TLC = Thin layer chromatography

HPLC = High-pressure liquid chromatography

LC = Liquid chromatography

LLC = Liquid-liquid chromatography

RI = Refractive index

UV = Ultraviolet absorption

LCEC = Liquid chromatography with electrochemical detectors

mm = millimeters

IS = Internal standard

R = Slope of DDEP/slope of DDMP from the plasma calibration curves

SD = Standard deviation

SE = Standard error

CV = Coefficient of variation

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