Lipid-Soluble Inhibitors of Dihydrofolate Reductase III: Quantitative Thin-Layer and High-Performance Liquid Chromatographic Methods for Measuring Plasma Concentrations of the Antifolate, 2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido-[2,3-*d*]pyrimidine

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Abstract □ Specific methods using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) for the analysis of the potent antifolate, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido-[2,3-*d*]pyrimidine (I) in plasma were developed. The HPLC system employed paired-ion chromatography using a mobile phase of water–acetonitrile (65:35, v/v) in conjunction with a reversed-phase C-1 column and detection by UV absorbance measurement. The TLC system utilized a scanning densitometer operating in the reflectance mode with detection of I by fluorescence measurement. The lower limits of detection for the HPLC and TLC methods were ~0.005 μg/ml. The coefficients of variation for the measurement of drug concentrations over the range of 0.04–1.0 μg/ml of plasma were 5 and 6%, respectively.

Keyphrases □ Dihydrofolate reductase—quantitative thin-layer chromatographic and high-performance liquid chromatographic methods for measuring plasma concentrations of antifolate □ Antifolate—thin-layer chromatographic and high-performance liquid chromatographic methods for measuring concentrations of plasma, lipid-soluble inhibitors of dihydrofolate reductase □ High-performance liquid chromatography—method for measuring concentrations of an antifolate, lipid-soluble inhibitors of dihydrofolate reductase

2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido-[2,3-*d*]pyrimidine (I)[1] is a new lipid-soluble folate antagonist for the treatment of neoplastic diseases (1, 2). Methods using high-performance liquid chromatography (HPLC) and quantitative TLC for assaying this drug in plasma were developed and compared. For the measurement of I by HPLC, a reversed-phase C-1 column coupled with UV detection at 254 nm was employed; the TLC method fluorescence was measured after chromatography on conventional §silica gel plates. Chromatographic analyses were facilitated by the use of a microcomputer-based system for the acquisition and reduction of data (3).

BACKGROUND

In contrast to some other antifolate drugs, I exhibits activity comparable to methotrexate against mammalian dihydrofolate reductase, while minimally inhibiting the catabolism of histamine *via* histamine-*N*-methyltransferase (2, 4). The moderate lipophilicity [log *P* (octanol–water) = 1.73] of I facilitates cellular entry of the drug by diffusion, thus avoiding the dependence on the reduced folate facilitated transport system (2, 5). The short half-life of the drug (0.38 hr in the rat) and its response to calcium leucovorin rescue alleviate the problems of cumulative toxicity to normal cells (2, 6). Studies *in vivo* of the effect of I on solid W256 carcinoma in the rat resulted in reductions in mean tumor volumes of 70–90%, relative to tumorous control animals (1, 2).

The encouraging properties of I prompted development of specific analytical methods that could be used for measuring I in biological samples for chemotherapy, disposition, and toxicological studies. HPLC and TLC methods were evaluated and found to be comparable with respect to the analytical precision and net recovery of I.

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EXPERIMENTAL

Reagents—Acetonitrile[2], chloroform[2], methylene chloride[2], methanol[2], 2-propanol[2], ammonium hydroxide[3], sodium bicarbonate[3], and sodium hydroxide[3] were reagent grade. The ion-pairing reagent[4] was used as received. Distilled water was filtered through a 0.45-μm membrane filter[5] prior to use.

Apparatus—A liquid chromatograph[6] equipped with a valve-loop injector[7], strip-chart recorder[8], and a microcomputer-based data system was used with a commercially packed 25 cm × 4.6-mm i.d. column[9] containing 6-μm C-1 reversed-phase packing. A precolumn[10] filled with C-18 pellicular packing[11] was also used.

A motor-driven multispotter[12] was used to spot 20 × 20-cm nonfluorescing 0.25-mm layer thickness silica gel 60 TLC plates[13]. The plates were quantified using a scanning densitometer[14] interfaced with a microcomputer.

Standard Solutions—A stock solution of I was prepared in methanol that was diluted with either methanol or chloroform–methanol (90:10, v/v) to produce standard solutions for liquid and thin-layer chromatography, respectively.

Reference solutions of I for HPLC analyses were prepared at concentrations of 0.25, 1.0, and 4.0 ng/μl; those for TLC analyses were prepared at concentrations of 1.0 and 5.0 ng/μl. No apparent degradation of stock

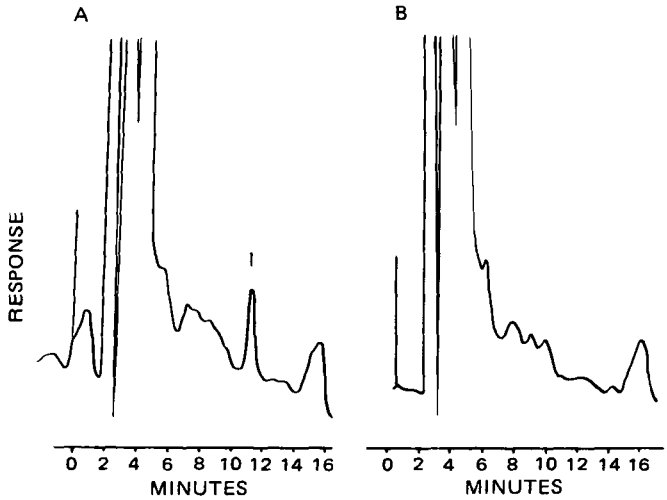

Figure 1—*Typical HPLC chromatograms of extracts from plasma (1 ml) (A) containing 0.040 μg of I and from blank plasma (1 ml) (B).*

[2] "Distilled in Glass," Burdick & Jackson, Muskegon, Mich.
[3] Analytical Reagent, Mallinckrodt, Inc. Paris, Ky.
[4] PIC B-8, Waters Associates, Milford, Mass.
[5] Millipore, Bedford, Mass.
[6] Series ALC 200, Waters Associates, Milford, Mass.
[7] Model U6K, Waters Associates, Milford, Mass.
[8] Omniscribe, Houston Instruments, Austin, Tex.
[9] ZORBAX TMS, E. I. Dupont de Nemours & Co., Wilmington, Del.
[10] Guard Column, Waters Associates, Milford, Mass.
[11] Permaphase ODS, E. I. Dupont de Nemours & Co., Wilmington, Del.
[12] AIS, Libertyville, Ill.
[13] No. 5763, E. Merck Laboratories, Elmsford, N.Y.
[14] Model SD3000, Schoeffel Instrument Corp., Westbrook, N.J.

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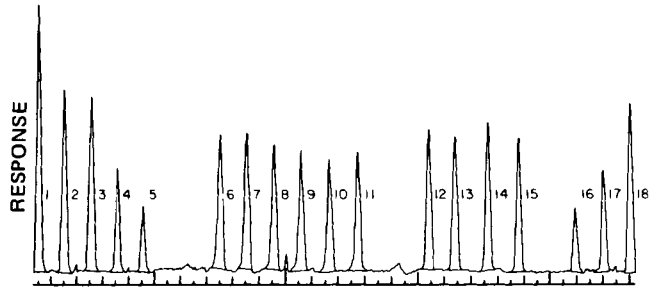

Figure 2—*Typical TLC chromatogram of extracts from plasma (1 ml) and I. Peaks corresponding to known amounts of I are as follows: 1, 0.080 μg; 2, 0.050 μg; 3, 0.050 μg; 4, 0.030 μg; 5, 0.020 μg; 16, 0.020 μg; 17, 0.030 μg; 18, 0.050 μg. Peaks 6–11 are the extracts from replicate plasma (1 ml) samples, each containing 0.040 μg of I. Peaks 12–15 are 20% of the extracts from replicate plasma (1 ml) samples, each containing 0.20 μg of I.*

or standard solutions was observed over a period of 6 months, when stored at −15°.

Sample Preparation—To a 12-ml culture tube[15], fitted with a polytef[16]-lined screw cap, was added 1.0 ml of plasma. The pH of the plasma was adjusted with 1.0 ml of pH 10 bicarbonate buffer and vortex mixed[17] for 5 sec. The basified plasma was extracted by addition of 4.0 ml of methylene chloride and rotary mixing[18] for 10 min. The organic phase was transferred and retained. A second 4.0-ml portion of methylene chloride was used to extract the aqueous phase, as above, and the organic extracts were combined and concentrated at ~40° to 1.0 ml using a gentle stream of dry nitrogen. Extraneous material was separated from I by passing the concentrated extract through a disposable silica gel cartridge[19] that was preeluted sequentially with 25 ml of methanol and 15 ml of methylene chloride. Ten milliliters of methylene chloride was passed through the silica cartridge and discarded. A 6.5-ml eluate of methanol was collected and evaporated at ~55° with nitrogen. The residue was reconstituted, using methanol for HPLC analysis or chloroform–methanol (90:10, v/v) for TLC analysis, to a final volume dependent on the expected concentration of I in the plasma. For both analyses, if low levels (5–200 ng) of I were anticipated, sample tubes were washed with two 40-μl portions of the appropriate solvent, which were drawn into a 100-μl syringe; for higher levels, residues were dissolved in 250–1000 μl of solvent and a suitable volume (10–100 μl) was drawn into a syringe.

Analytical Procedure for HPLC—The mobile phase, filtered distilled water containing 0.005 *M* 1-octane sulfonic acid–acetonitrile (65:35, v/v), was degassed by agitation under reduced pressure. The C-1 column was operated isocratically with an eluent flow rate of 1.5 ml/min and a column inlet pressure of 2500 psi. The retention time for I was ~11 min. Volumes of reference solution (20–100 μl) were injected to bracket expected amounts of I in the samples. The column effluent was monitored by UV absorbance measurement at 254 nm. Chromatograms were obtained in an analog form using a recorder with a chart speed of 0.5 cm/min and in a digital form using a microcomputer[20] with a data sampling rate of 1 point/sec. The use of a microcomputer in this laboratory as an adjunct to the acquisition and reduction of chromatographic data has been described previously (3).

Analytical Procedure for TLC—A motor-driven multispotter was used to spot gradually the extracts and reference solutions onto a nonfluorescing silica gel plate. Volumes of reference solution (10–100 μl), containing a sufficient quantity of I to bracket expected amounts in the sample residues, were drawn into 100-μl syringes for deposit on the TLC plate. Warm air from a 460-watt hair dryer[21] was blown across the plate to hasten evaporation of the solvent and to minimize spot diffusion. Plates were developed to 10 cm with chloroform–2-propanol–concentrated ammonium hydroxide (25:20:01, v/v). The plate was air dried in a hood for 30 min, then the fluorescence of I was measured using a scanning densitometer operated in the single-beam reflectance mode at 340 nm excitation wavelength with a 400-nm cutoff interference filter. Plates that were spotted with quantities of I at the low end of the concentration range were stored overnight and scanned the next day after the intensity of the fluorescence of I had increased.

[15] KIMAX, Owens-Illinois, Inc., Toledo, Ohio.
[16] TEFLON, E. I. Dupont de Nemours & Co., Wilmington, Del.
[17] Vortex-Genie, Scientific Industries, Inc., Bohemia, N.Y.
[18] Multi-Purpose Rotator, Scientific Industries, Inc., Bohemia, N.Y.
[19] SEP-Pak, Waters Associates, Milford, Mass.
[20] Lilliputer, Digital Specialities, Carrboro, N.C.
[21] Model 202, Oster Corp., Milwaukee, Wis.

Table I—Recovery of I from Spiked Plasma

Compound I, μg/ml	HPLC			TLC		
	N	Recovery, %	*CV*	*N*	Recovery, %	*CV*
0.040	4	84	6	10	95	7
0.200	4	80	0	10	89	5
1.00	4	86	5	10	93	5
Composite	12	84	5	30	92	6

Identification and Quantification of I in Plasma—Programmable microcomputers were interfaced with the HPLC and TLC equipment to provide for ready assessment of chromatograms (3). The microcomputers monitored either the output of the HPLC absorbance detector or the output of the TLC scanning densitometer.

The chromatographic peak corresponding to I in samples was identified by comparison of retention time or R_f value with those for reference solutions containing I. Quantification was achieved by calculating the ratio of the peak area of I in the sample to the peak areas of varying amounts of external reference I as determined by least-squares regression fit. Although peak heights were also available, calculations based on peak areas were found to provide more reliable results (3).

RESULTS

High-Performance Liquid Chromatography—Typical chromatograms of the entire extracts from 1.0 ml of control (blank) plasma and 1.0 ml of plasma to which was added 0.040 μg of I are presented in Fig. 1. At a capacity factor (k') of 6, the linear calibration range for I was 0.010–0.320 μg. A correlation coefficient of 0.999 was obtained for a typical regression analysis, $y = 0.2134x - 0.0008$, where y is the peak area and x is the peak amount in micrograms. At a signal-to-noise ratio of 2.5, the HPLC limit of detection for I in plasma was 0.005 μg/ml. The mean recovery of I in plasma over the range of 0.04–1.0 μg/ml was 84% with a coefficient of variation (CV) of 5% ($n = 12$) (Table I).

Thin-Layer Chromatography—Using the chromatographic conditions indicated, I had an R_f of 0.35. A microcomputer-generated plot of peaks obtained for I by scanning an entire TLC plate is presented in Fig. 2. No peaks corresponding to the drug were found in control samples. A typical TLC calibration curve was linear over the range of 0.004–0.300 μg with a correlation coefficient of 0.997 for the line $y = 1.167x + 0.0128$, where y is the peak area and x is the peak amount in micrograms. The limit of detection for plasma extracts, at a signal-to-noise ratio of 2.5, was 0.004 μg/ml. The mean recovery of I over the range of 0.040–1.0 μg/ml of plasma was 92% with a CV of 6% ($n = 30$) (Table I).

DISCUSSION

Concentration-dependent variations in the extraction efficiency of BW 301U were observed over the measured range when a one-step methylene chloride extraction procedure was utilized; consequently, a two-step extraction procedure was investigated. Although this refinement did decrease the variability to an acceptable level, an excessive amount of endogenous material was extracted also. The coextractives resulted in considerable streaking from the origin to the solvent front of TLC silica gel plates, deforming the spots of compound I. Silica gel cartridges were used to remove these relatively nonpolar materials. Prior to use, the cartridges were eluted with methanol to reduce plate streaking and to remove a contaminant, R_f of ~0.85, that fluoresced when exposed to long wavelength (366 nm) UV radiation. Once the sample extract was loaded, the cartridge was eluted with methylene chloride to remove the sample components responsible for the streaking. Compound I, strongly retained on silica gel, was not eluted until methanol was passed through the cartridge.

Immediately after removal of the TLC plate from the developing chamber the fluorescence of I was most intense, but it diminished markedly to a minimum within 20 min. Plates could be analyzed at that time; however, for increased sensitivity, plates were retained for 24 hr before scanning to permit the fluorescence of I to increase to an intensity that was comparable to that observed initially. The change in fluorescence intensity did not require the presence of light. Following this initial interval, the enhanced fluorescence was stable for at least 1 month.

The substances responsible for the streaking of TLC silica gel plates initially did not appear to adversely affect HPLC chromatograms; however, the silica gel cartridges and a C-18 guard column were used to maximize column longevity, providing a useful column life in excess of several months.

The tendency of I to exhibit fluorescence in solution is potentially useful for analysis by HPLC. However, this fluorescence is readily quenched in aqueous solutions, thereby hindering its use in many reversed-phase liquid chromatographic systems. Normal-phase liquid chromatography does not appear promising due to the strong affinity of I for silica gel columns, resulting in asymmetric peak shapes and excessive capacity factors. Thus, paired-ion reversed-phase chromatography in conjunction with detection by UV absorbance is used for the analysis of I by HPLC. The parameters chosen provided good peak shape, a moderate retention time, and analytical sensitivity comparable to that obtained by fluorescence detection.

The selection of one procedure in preference to the other for the analysis of plasma samples from clinical studies is a matter of available equipment and efficiency. TLC is used preferentially to HPLC in this laboratory for the rapid analysis of limited numbers of samples (typically 1–20). Larger numbers of samples may be analyzed more efficiently by HPLC using an auto-injector to provide for unattended operation. The chromatographic equipment is interfaced with microcomputers to facilitate quantification, thereby achieving high levels of precision and accuracy.

REFERENCES

(1) E. M. Grivsky, S. Lee, C. W. Sigel, D. S. Duch, and C. A. Nichol, *J. Med. Chem.*, **23**, 327 (1980).

(2) D. S. Duch, C. W. Sigel, S. W. Bowers, M. P. Edelstein, J. C. Cavallito, R. G. Foss, and C. A. Nichol, in "Current Chemotherapy and Infectious Disease," Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Washington, D.C., 1980, p. 1597.

(3) R. G. Foss, C. W. Sigel, R. J. Harvey, and R. L. DeAngelis, *J. Liq. Chromatogr.*, **3**, 1843 (1980).

(4) D. S. Duch, S. W. Bowers, and C. A. Nichol, *Mol. Pharmacol.*, **18**, 100 (1980).

(5) W. D. Sedwick, R. Birdwell, and J. Laszlo, in "Current Chemotherapy and Infectious Disease," Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Washington, D.C., 1980, p. 1593.

(6) D. S. Duch, M. P. Edelstein, S. W. Bowers, and C. A. Nichol, *Proc. Am. Assoc. Cancer Res.*, **22**, 264 (1981).

NMR Determination of Isosorbide Dinitrate and β-Adrenergic Blocking Agents in Tablets

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Abstract □ An NMR spectroscopic method for the determination of isosorbide dinitrate, alone or together with alprenolol or propranolol, is described. Spectra are determined in dimethyl sulfoxide-d_6 containing maleic acid or 1,4-dinitrobenzene as internal standards. Both synthetic mixtures and commercial formulations were assayed, and the results were compared using compendial procedures.

Keyphrases □ Isosorbide dinitrate—NMR determination, β-adrenergic blocking agents in tablets □ β-Adrenergic blocking agents—NMR determination of isosorbide dinitrate, tablets □ NMR spectroscopy—determination of isosorbide dinitrate and β-adrenergic blocking agents in tablets

Isosorbide dinitrate (I) is a member of the group of vasodilator drugs, having a nitrite or nitrate function, that are used particularly in the treatment of angina pectoris and ischemia of skeletal muscle (1). It is used alone or together with β-adrenergic blocking agents such as propranolol (II) (2) or alprenolol (III) (3).

Several methods for the analysis of I, II, and III have been described. The official compendia describe a polarographic titration (4) or a colorimetric reaction with *p*-phenolsulfonic acid (5) for isosorbide dinitrate and a spectrophotometric assay for alprenolol (6) and propranolol (7). However, their application for the quantitative determination of I in tablets is laborious and time consuming. The present report describes a new quantitative method for the determination of I alone or together with II or III by ^{1}H-NMR spectroscopy. Furthermore, it allows the simultaneous analysis of II or III.

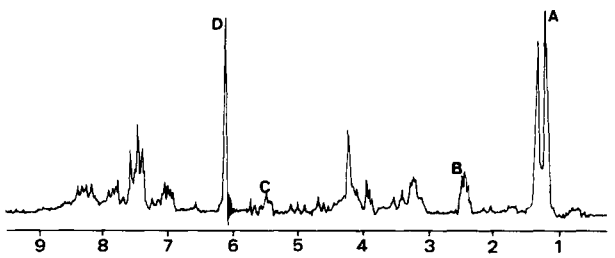

Figure 1—*NMR spectrum of a typical I and II mixture analysis in dimethyl sulfoxide-*d_6. *Key: (A) methyl protons of II; (B) solvent impurity; (C) protons on* C_2 *and* C_5 *of I; (D) single signal of IV.*

EXPERIMENTAL[1]

Materials—Standard isosorbide dinitrate (I), propranolol hydrochloride (II), and alprenolol hydrochloride (III) were purified by recrystallization from ethanol–water, *n*-propanol, and ethyl acetate, respectively.

Maleic acid (IV) was used as internal standard after recrystallization from water, mp 130–131°; 1,4-dinitrobenzene (V) (8), also used as internal standard, was purified by sublimation, mp 173–174°. Dimethyl sulfoxide-d_6 (VI) was used as the solvent and tetramethylsilane was the internal standard.

Samples—Tablets from two batches of each brand locally obtained were used.

Isosorbide Dinitrate Tablets—Twenty tablets were weighed and a sample equivalent to 50 mg of I was dissolved in 10 ml of 2% sodium bicarbonate solution and extracted from methylene chloride (3 × 7 ml). The solution was evaporated in a Craig tube, and 75 mg of IV was added. The mixture was dissolved in 1.0 ml of VI, and ~0.4 ml of the solution was transferred to an NMR tube where the spectrum was obtained (Table I).

Isosorbide Dinitrate and Propranolol Hydrochloride Tablets—The same procedure as just described was used with a sample equivalent

[1] A Perkin-Elmer R12 NMR spectrometer, 60 MHz was used. All spectra were scanned at a probe temperature of 35°.

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