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Note

High-performance liquid chromatographic assay for trimetrexate in human plasma

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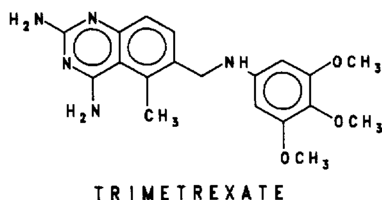
Trimetrexate, 5-methyl-6-[[(3,4,5-trimethoxyphenyl)amino]methyl]-2,4-quinazolinediamine, is a novel anticancer compound first synthesized by Elslager and Davoll [1]. It is a potent inhibitor of dihydrofolate reductase [2,3] and has shown promising activity against solid tumors [3] and *Pneumocystis carinii* pneumonia [4,5] in clinical and preclinical studies.

Existing assays for trimetrexate are based on high-performance liquid chromatographic (HPLC) [6–9], gas chromatographic–mass spectrometric (GC–MS) [10], dihydrofolate reductase inhibition [3,8,11] or competitive protein-binding [12] techniques. Previous HPLC assays were not considered for use in planned pharmacokinetic studies due to insufficient sensitivity (18.5 ng/ml) [6–9], long run times (60 min) [8], lack of an internal standard [6] or variable recovery [7]. Dihydrofolate reductase inhibition assays, while sensitive, are not specific because trimetrexate metabolites can inhibit dihydrofolate reductase in vitro [7,9]. Since competitive protein-binding measures displacement from dihydrofolate reductase, the same metabolites could also interfere with this assay. The GC–MS assay was considered too expensive for routine use. In order to support pharmacokinetic studies, our objective was to develop a trimetrexate assay that met the following requirements: sensitive enough to quantitate 2–5 ng/ml, run times of 15–20 min, internally standardized, reproducible, selective and relatively inexpensive. Structures of trimetrexate and the internal standard (I) are shown in Fig. 1.



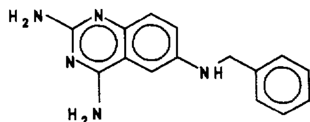


Fig. 1. Structures of trimetrexate and internal standard.

EXPERIMENTAL

Materials

Trimetrexate and internal standard (I), N6-(phenylmethyl)-2,4,6-quinazolinetriamine, were synthesized at Parke-Davis Pharmaceutical Research (Ann Arbor, MI, U.S.A.).Acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Methanol was obtained from Mallinkrodt (Paris, KY, U.S.A.). Triethylamine (99%, w/w) and N,N-dimethylacetamide were purchased from Aldrich (Milwaukee, WI, U.S.A.). All solvents, unless noted otherwise, were HPLC grade and used without further purification. Orthophosphoric acid and ammonium dihydrogenphosphate (AR grade) were obtained from Mallinkrodt. Heparinized human plasma was obtained from healthy volunteers by Plasma Alliance (Knoxville, TN, U.S.A.) and stored frozen. Plasma was clarified by centrifugation at 2250 *g* for 30 min. Bond-Elut C_2 cartridges, 100 mg, were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Potential metabolites 2,4-diamino-5-methyl-6-quinazolinecarboxaldehyde, 2,4-diamino-5-methyl-6-quinazolinecarbonitrile, 5-methyl-2,4,6-quinazolinetriamine, 6-(aminomethyl)-5-methyl-2,4-quinazolinediamine, 2,4-diamino-5-methyl-6-quinazolinecarboxylic acid, cisplatin, lomustine and morphine were obtained from Parke-Davis Pharmaceutical Research. Prednisolone, melphalan, cytosine arabinoside, 6-thioguanine, 5-fluorouracil, vinblastine, vincristine, doxorubicin, chlorambucil, trimethoprim, sulfamethoxazole and 5-methyltetrahydrofolate were obtained from Sigma (St. Louis, MO, U.S.A.). Etoposide was obtained from Bristol-Myers (Syracuse, NY, U.S.A.). Methotrexate and leucovorin were supplied by Lederle Labs. (Pearl River, NY,

U.S.A.). Ribavirin was purchased from Virate (Costa Mesa, CA, U.S.A.). Acyclovir and zidovudine were obtained from Burroughs Welcome (Research Triangle Park, NC, U.S.A.) and 3,4,5-trimethoxyaniline was purchased from Aldrich.

Chromatographic equipment and conditions

The liquid chromatograph consisted of a Waters (Milford, MA, U.S.A.) Model 590 pump and a Kratos (Ramsey, NJ, U.S.A.) Spectraflow 773 variable-wavelength detector. Automated sample injection was performed by a Perkin Elmer (Norwalk, CT, U.S.A.) ISS-100 fitted with 200 μl sample loop. The analytical column was a Dupont Zorbax TMS, 5 μm particle size, 250 mm $\times$ 4.6 mm I.D. from MAC-MOD Analytical (Chadds Ford, PA, U.S.A.). Guard columns were Dupont Zorbax TMS, 7 μm particle size, 30 mm $\times$ 4.6 I.D. from Phenomenex (Rancho Palos Verdes, CA, U.S.A.). Column temperature was maintained at 45°C with a Rainin (Woburn, MA, U.S.A.) Model 1061 column oven.

The mobile phase was an acetonitrile–buffer mixture (23:77, v/v) continuously degassed with helium. Mobile phase was prepared fresh daily and filtered through Ultipor N66 0.2-μm Nylon 66 filters purchased from Anspec (Ann Arbor, MI, U.S.A.). Buffer was 0.05 *M* ammonium dihydrogenphosphate containing 0.8% (v/v) triethylamine and 0.2% (v/v) orthophosphoric acid at pH 4.5. Mobile phase flow-rate was 1.2 ml/min with typical back-pressures of 7.6 ± 0.7 MPa. Ultraviolet detection was at 241 nm.

Preparation of stock solutions and standards

A 1.0 mg/ml stock solution of trimetrexate was prepared by dissolving 33.6 mg of the isethionate salt in 25 ml of N,N-dimethylacetamide. A 200 μg/ml working solution of trimetrexate was prepared in N,N-dimethylacetamide by serial dilution. A 0.5-ml aliquot of this standard was added to 10 ml of control human plasma to prepare the 9.524 μg/ml calibration standard. The remaining calibration standards, ranging from 2.4 ng/ml to 9.524 μg/ml, were prepared by serial dilution with control plasma.

A 0.8 mg/ml stock solution of I was prepared by dissolving 24.8 mg of the formate salt in 25 ml N,N-dimethylacetamide. A working solution of 3.2 μg/ml was prepared by serial dilution with N,N-dimethylacetamide. Stock solutions of trimetrexate and I were prepared fresh on each validation day. These standards were stable for at least two weeks if frozen when not in use.

Trimetrexate standards of 1.25, 25.0 and 250 μg/ml were prepared in N,N-dimethylacetamide by serial dilution of the 1.0 mg/ml stock standard. A 2-ml volume of each of these standards was added to 98 ml of human plasma to yield bulk spiked control plasmas with final concentrations of 25 ng/ml, 500 ng/ml and 5.0 μg/ml, respectively. These plasma controls were divided into 1.5-ml

aliquots and stored frozen. The spiked control samples were used to assess reproducibility and quality control for determination of unknowns.

Assay procedure

Spiked control samples were thawed at 37°C for 10 min and mixed for 30 s prior to analysis. Aliquots (1 ml) of calibration standards and spiked control samples were placed in 100 mm×13 mm disposable glass culture tubes. Internal standard, 160 ng, was added to each tube, and samples were thoroughly mixed using a vortex mixer. The C_2 cartridges were set up in a vacuum manifold and preconditioned with two 1-ml methanol rinses, followed by two 1-ml water rinses. A small amount of solvent was left on top of each cartridge to keep it wet between each rinse. Sample was applied to and aspirated through the cartridge at a flow-rate of approximately 0.5 ml/min. Tubes were rinsed with 1 ml of water and this rinse was aspirated through the cartridge. The cartridge was washed with another 1 ml of water and dried for 1 min using a vacuum of approximately 68 kPa. Dry cartridges were rinsed with 0.25 ml of acetonitrile and dried for 20 s by applying vacuum. Trimetrexate was eluted with 0.5 ml methanol–water (95:5, v/v) and the eluate was collected in 2-ml polypropylene centrifuge tubes. Unless stated otherwise, a vacuum of 10–17 kPa was used throughout the procedure.

The methanol eluate was evaporated to dryness in a water bath at 37°C under a gentle stream of nitrogen. Plasma residues were stored in the dark at room temperature (20–23°C) until all samples were extracted.

Samples were reconstituted in 0.4 ml of mobile phase, except for the 9.524 and 4.762 μg/ml calibration standards and the 5.0 μg/ml spiked control sample which were reconstituted in 2, 1 and 1 ml, respectively. This avoided sample carry-over by the injector. Reconstituted samples were mixed on a vortex mixer for 30 s and centrifuged for 15 min at 1800 *g*. A 0.2-ml aliquot of the supernatant was carefully transferred to polypropylene injector vials and 0.1 ml was injected.

Calculations

Twelve concentrations ranging from 2.4 ng/ml to 9.524 μg/ml trimetrexate were used to determine the calibration curve. Linear regression analysis (weighting factor $1/\text{concentration}^2$) of calibration curve data was performed by regressing peak-height ratios of trimetrexate to I on the concentration of trimetrexate in plasma. The $1/\text{concentration}^2$ weighting factor was chosen to achieve homogeneity of variance.

Assay validation

Trimetrexate and I recovery from human plasma was determined by comparing peak heights of seven samples obtained from extracted plasma to unextracted controls prepared in mobile phase at three different concentrations.

Specificity of the assay was assessed by analysis of drug-free human plasma and by injection of potential metabolites and therapeutic agents that might be encountered in a clinical setting. Compounds interfered with the assay if they eluted at the same retention time as either trimetrexate or I and were not removed by the extraction procedure.

The reproducibility of the HPLC system was determined from nine consecutive, replicate injections of standards containing 25 ng/ml and 5.0 μg/ml of both trimetrexate and I. System reproducibility was expressed as the percentage coefficient of variation of the mean peak-height ratio.

Evaluation of the calibration curve was carried out by analyzing the calibration standards in triplicate on three separate days.

Assay accuracy and precision were assessed by assaying spiked control samples in triplicate at 25 ng/ml, 500 ng/ml and 5.0 μg/ml on three different days. Assay was expressed as the percentage difference between the mean assayed concentrations ($n=9$) and nominal values. Assay precision was expressed as the percentage coefficient of variation of the nine replicates.

Method application

Suitability of the method to study pharmacokinetics of trimetrexate was assessed by analyzing plasma samples from a patient with metastatic cancer who received a 140 mg/m^2 intravenous dose of trimetrexate by 30 min continuous infusion. Heparinized blood samples were obtained prior to and serially up to 142 h post-dose. Plasma was harvested by centrifugation and stored frozen. Plasma concentration–time data were analyzed by non-compartmental methods with the aid of the LAGRAN computer program [13].

RESULTS AND DISCUSSION

Retention times of trimetrexate and I were 9.4 ± 0.2 and 12.0 ± 0.2 min, respectively. Capacity factors (k') for trimetrexate and I were 2.7 and 3.8, respectively. Relative retention of trimetrexate to I was expressed by the separation factor (α) which was 1.4. The reproducibility of the HPLC system was 0.8 and 0.2% at 25 ng/ml and 5.0 μg/ml, respectively. Mean recovery of trimetrexate was 93.4, 89.2 and 100.7% at 23.8 ng/ml, 238 ng/ml and 2.381 μg/ml, respectively. Mean recovery of I was 99.0, 96.8 and 99.5% at 19.2 ng/ml, 192 ng/ml and 1.917 μg/ml, respectively.

The assay was adequately selective for trimetrexate. Chromatograms of human plasma taken from metastatic cancer patients prior to receiving trimetrexate were free of significant peaks at the retention times of trimetrexate and I. Chromatograms of drug-free human plasma, a 25 ng/ml spiked control and a 25 ng/ml calibration standard are shown in Fig. 2. Of the therapeutic agents and potential metabolites tested only melphalan and chlorambucil interfere with the assay.

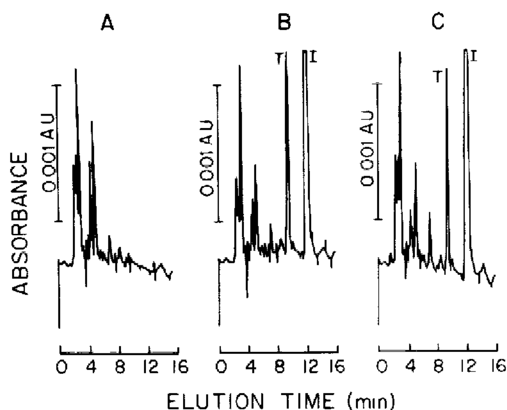


Fig. 2. Chromatograms of (A) a control human plasma, (B) a 25 ng/ml spiked control and (C) a 25 ng/ml calibration standard. Trimetrexate and internal standard are labeled as T and I, respectively.

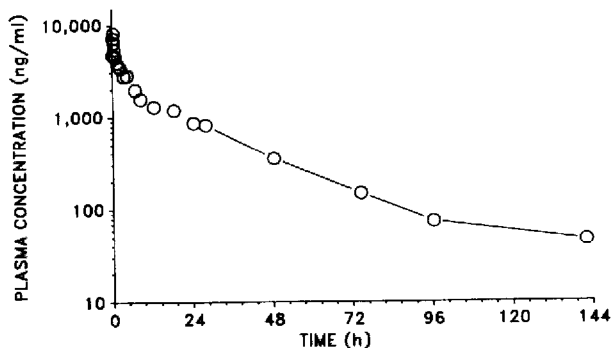


Fig. 3. Trimetrexate plasma concentration–time curve obtained following administration of a 140 mg/m^2 intravenous dose by continuous infusion to a metastatic cancer patient.

Peak-heights ratios of calibration standards were proportional to the concentration of trimetrexate in plasma over the range tested. The calibration curve was linear and well described by a least-squares regression line with a mean ($n=3$) coefficient of determination of 0.998. The limit of quantitation was 2.4 ng/ml based on a signal-to-noise ratio of 2.5:1. Accuracy was 5.2, 4.0 and 4.1% and precision was 3.3, 2.0 and 2.5% for spiked control samples at 25 ng/ml, 500 ng/ml and 5.0 μg/ml, respectively.

The trimetrexate plasma concentration–time curve obtained after a continuous intravenous infusion of a 140 mg/m^2 dose in a patient with metastatic cancer is depicted in Fig. 3. A peak concentration of 8.196 μg/ml was reached at the end of the infusion. The plasma concentration–time curve subsequently declined in a triphasic manner to a plasma concentration of 46 ng/ml at 142 h post-dose. The sensitivity of the method would have permitted characteriza-

tion of the plasma concentration–time curve for several more days after the 142-h sample. The terminal elimination phase half-life value was 27.4 h. Steady state volume of distribution and total plasma clearance values were 38.5 l/m^2 and 22.5 $ml/min/m^2$, respectively.

CONCLUSIONS

This HPLC assay for trimetrexate in human plasma is selective, precise, accurate and linear over the trimetrexate concentration range studied. The method is suitable to study the clinical pharmacokinetics of trimetrexate.

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