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Note

High-performance liquid chromatographic determination of terephthalamidine in plasma

WILLIAM R. WAUD* and DONALD L. HILL

Southern Research Institute, P.O. Box 55305, Birmingham, AL 35255-5305 (U.S.A.)

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Terephthalamidine (N,N″-bis[*p*-(N′-methylamidino)phenyl]terephthalamidine, tetrahydrochloride; NSC 57155), the structure of which is shown in Fig. 1, is representative of a unique class of antitumor compounds that have impressive activity against a broad spectrum of murine leukemias and lymphomas [1]. Terephthalamidine was highly active against murine leukemias with acquired resistance to actinomycin D, mitomycin C, methylglyoxal-bis(guanylhydrazone) and vincristine [1]. Furthermore, it had good activity against the mouse M5076 ovarian tumor [2] and, in man, a metastatic mediastinal germ cell tumor and Hodgkin's lymphoma [3]. Phase I evaluation, however, revealed acute and chronic effects, including nausea, vomiting, anorexia, ophthalmoplegia and central nervous system toxicity [3]. As a result of the toxicity, clinical trials were terminated.

The toxicity of terephthalamidine and other phthalanilides could be prevented by complexing these agents with various anionic compounds, including di- or polysulfonic and diphosphoric acid derivatives and pamoic acid [4–6]. In mice, however, these compounds also blocked the antileukemic activity of terephthalamidine. A positive observation was that complexing with suramin, a polysulfonate antitrypanosomal agent, reduced toxicity, without preventing the antileukemic activity. Because of the evidence of clinical activity and because of the possibility of controlling the toxicity, the Developmental Therapeutics Pro-

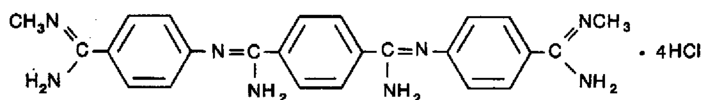

Fig. 1. Structure of terephthalamidine.

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gram of the Division of Cancer Treatment, NCI, has renewed interest in terephthalamidine.

High-performance liquid chromatographic (HPLC) procedures have been reported for the antibacterial agent pentamidine [7, 8], which shares some structural characteristics with terephthalamidine. An HPLC method for the quantitation of terephthalamidine in bulk preparations of drug has been reported [9]. The method, however, is not applicable to the determination of drug in biological samples. Kreis et al. [10] have reported the disposition of ^{14}C-label in two adult patients who received intravenous injections of [^{14}C]terephthalamidine (1 mg/kg) over a period of 15 min. The half-life for the elimination of radioactivity from blood was approximately 45 min.

This paper describes an HPLC method for the quantitation of terephthalamidine in plasma and its application to the measurement of drug levels after intraperitoneal and intravenous dosing in mice.

EXPERIMENTAL

Chemicals

Organic solvents, HPLC grade, were purchased from American Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Purified water was obtained from a Milli-Q reagent water system equipped with ion-exchange, organic and carbon filters (Millipore, Bedford, MA, U.S.A.). The ion-pairing agent, 1-heptanesulfonic acid, sodium salt, and triethylamine (TEA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Terephthalamidine was supplied as a tetrahydrochloride salt by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment (NCI, Bethesda, MD, U.S.A.). With absorbance detection at 254 nm, the purity of our sample of terephthalamidine was determined to be greater than 99% by the HPLC method described below.

Standard curve

A terephthalamidine stock solution (1 mg/ml) was prepared weekly by dissolving the drug in an appropriate amount of water. The stock solution was stored at $-20°C$ and maintained at $4°C$ when in use. Dilutions of the stock solution were used to establish a standard curve. A standard for injection on the HPLC system was prepared by diluting 0.15 ml of a standard solution with an equal volume of acetonitrile and adding the preparation (0.25 ml) to an equal volume of 40 m*M* sodium heptanesulfonate, pH 3, containing 20 m*M* TEA. Standard solutions of drug prepared for injection were found to be stable during overnight, automated HPLC runs.

Equipment

A Waters chromatograph equipped with a Model 6000A pump, a Model 441 absorbance detector, a Model 730 data module and a WISP Model 710A autosampler was used in the analyses (Chromatography Division, Milford, MA, U.S.A.). The column used was a 300 mm × 3.9 mm I.D. Waters μBondapak C_{18} analytical column (10 μm particle size) fitted with a 25 mm × 2.0 mm I.D. SGP ODS guard

column (Phase Separations, Norwalk, CT, U.S.A.). The column was used at ambient temperature.

Analysis of plasma samples

An equal volume of acetonitrile was added to 0.17 ml of plasma. After incubation at 4 °C for 30 min, the preparation was centrifuged at 12 800 *g* for 15 min and the supernatant was removed. Supernatant (0.25 ml) was added to a 1-ml micro insert (Phase Separations) containing 0.25 ml of an aqueous solution (pH 3) that was 40 m*M* in sodium heptanesulfonate and 20 m*M* in TEA, and the contents were mixed. Sample or standard (0.3 ml) was injected into the HPLC system by means of the autosampler equipped with a 2-ml sample loop.

The solvent for HPLC was prepared by mixing 290 ml of acetonitrile and 710 ml of an aqueous solution that was 25 m*M* in sodium heptanesulfonate and 14 m*M* in TEA, with the pH of the aqueous solution adjusted to 3.0 with phosphoric acid. The flow-rate was 1 ml/min, and absorbance detection was at 280 nm.

Quantitation of terephthalamidine in plasma was accomplished by comparing sample peak areas with those obtained by analyzing known amounts of drug in water. Values were corrected for efficiency of recovery of terephthalamidine from plasma samples.

Mouse studies

Male CD2F1 mice weighing 20–23 g received an intraperitoneal dose of a freshly prepared aqueous solution of terephthalamidine (15 mg/kg). At intervals of 0.125, 0.25, 0.5, 1, 2, 6 and 24 h, blood from four mice was collected in Microtainer plasma separator tubes (Becton Dickinson, Rutherford, NJ, U.S.A.). The tubes were centrifuged at 12 800 *g* for 90 s. The plasma was removed and stored at −20 °C until analyzed.

Male CD2F1 mice weighing 21–24 g received an intravenous dose of a freshly prepared aqueous solution of terephthalamidine (2.4 mg/kg). Blood was collected from four mice at intervals of 0.083, 0.25, 0.5, 1, 2 and 3 h. Plasma samples were obtained and stored as described above.

Male CD2F1 mice weighing 21–24 g were fasted overnight and then hydrated, each with 0.5 ml water. After 90 min, the mice received an oral dose of a freshly prepared aqueous solution of terephthalamidine (60 mg/kg). Blood was collected at intervals of 0.083, 0.25, 0.50, 1 and 2 h. Plasma samples were obtained and stored as described above.

Pharmacokinetic analysis

Pharmacokinetic half-lives were estimated with modified forms of the NONLIN [11] and CSTRIP [12] programs. The data were fitted to one-, two- and three-compartment open models. A model was accepted as the best fit if an additional term, or compartment, failed to reduce significantly ($p < 0.05$) the weighted sums of squared errors, as estimated by F-test with appropriate degrees of freedom. Statistical weights were determined from the measured concentrations and were the same for each model.

RESULTS AND DISCUSSION

Development and characterization of the method

The reversed-phase HPLC procedure developed was adapted from those reported for pentamidine [7, 8]. As found for the chromatography of pentamidine, an ion-pairing agent was necessary to elute the terephthalamidine with reasonable peak shape, and TEA was necessary to reduce tailing of the peak. The pH of the eluting solvent (3.0) ensured that the drug was completely protonated. Acetonitrile was selected as the organic component, since it produced a better peak shape than methanol.

Occasionally, during HPLC analysis of plasma samples containing drug, the drug peak was either broad or eluted as a doublet. This problem was eliminated by reducing the concentration of acetonitrile in the sample in comparison to the eluting solvent system (i.e., from 29 to 25%). This change sharpened the drug peak while permitting an injection volume of 0.3 ml.

Standard curves were linear over the range 0.75–30 μg/ml. Least-squares analysis, however, consistently yielded a negative value for the y-intercept (Fig. 2). For concentrations below 0.75 μg/ml, values were determined visually from a smooth curve constructed with the experimentally derived data points. A standard curve was determined for each set of samples. The limit of detection was approximately 5 ng of drug or 67 ng/ml of standard solution.

Analysis of plasma samples containing no drug often revealed a small peak with the same retention time as the drug (Fig. 3). Alteration of chromatographic conditions did not permit separation of the contaminating component(s) from the drug. Detection by absorbance at other wavelengths, by fluorescence or by electrochemical means proved unsuccessful in analyzing the drug in the presence of the contaminating component(s). From the results of two recovery studies of terephthalamidine from plasma, the contaminating peak was found to be equivalent to 0.08 and −0.04 μg/ml. Because of the presence of the contaminant(s), the lower limit of quantitation of drug was approximately 0.3 μg/ml of plasma.

The recovery of terephthalamidine from mouse plasma was 90.3 ± 0.4% (mean ± S.D., $n=2$) for concentrations of drug from 0.3 to 30 μg/ml. The drug was stable in mouse plasma at 37°C for up to 6 h. For initial terephthalamidine concentrations of 1.3 and 10.9 μg/ml of plasma, drug levels varied from 91 to 105% over a period of 5 min to 6 h. After 24 h at 37°C, approximately 87% of the drug remained.

Application of the method to experimental samples

Experiments utilizing oral, intraperitoneal and intravenous dosing of mice were performed to evaluate the HPLC method and to determine various pharmacokinetic parameters for terephthalamidine.

For mice receiving an oral dose of 60 mg/kg, a maximum plasma drug concentration of 0.5 μg/ml was obtained at 15 min after dosing. As a result of the low concentrations, no pharmacokinetic analysis was performed on the data.

For mice receiving an intraperitoneal dose of 15 mg/kg, the plasma concentration of drug was 30.8 ± 1.7 μg/ml (mean ± S.D., $n=4$) at 7.5 min after dosing

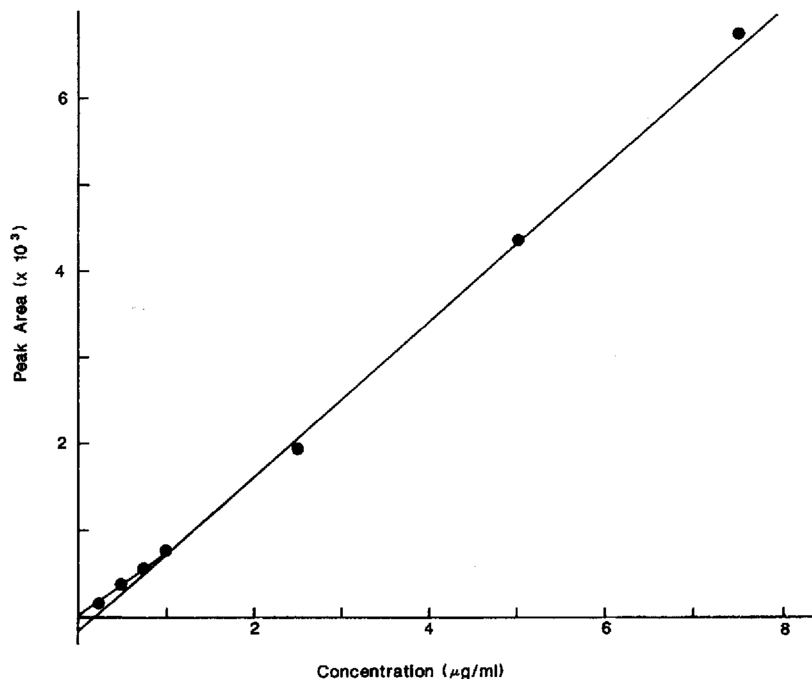

Fig. 2. Standard curve for intravenous dosing with terephthalamidine. Least-squares analysis of the peak areas for concentrations of standard from 0.75 to 10 μg/ml ($n=6$) yielded a straight line, $y=mx+b$ ($m=894$, $b=-208$), with a correlation coefficient of 0.9946.

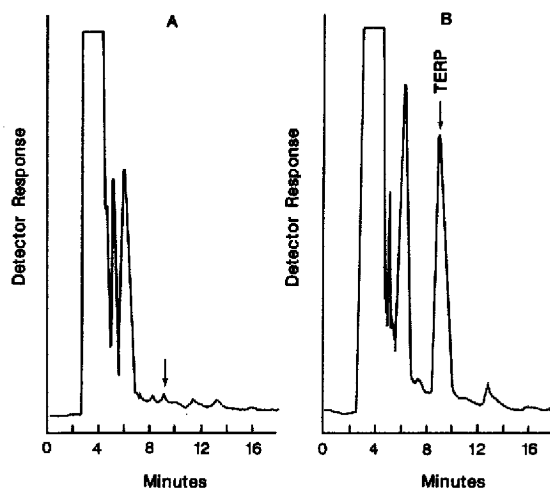

Fig. 3. Representative chromatographic traces. (A) Plasma from a control mouse; (B) plasma obtained from a mouse 15 min after intravenous dosing with drug (2.4 mg/kg). The retention time of terephthalamidine (TERP) was 8.8 min.

(Fig. 4). No drug was detected at 6 or 24 h. The data for elimination of drug from plasma best fitted a two-compartment open model ($p=0.007$). The half-lives of the α- and β-phases were 14 and 205 min, respectively.

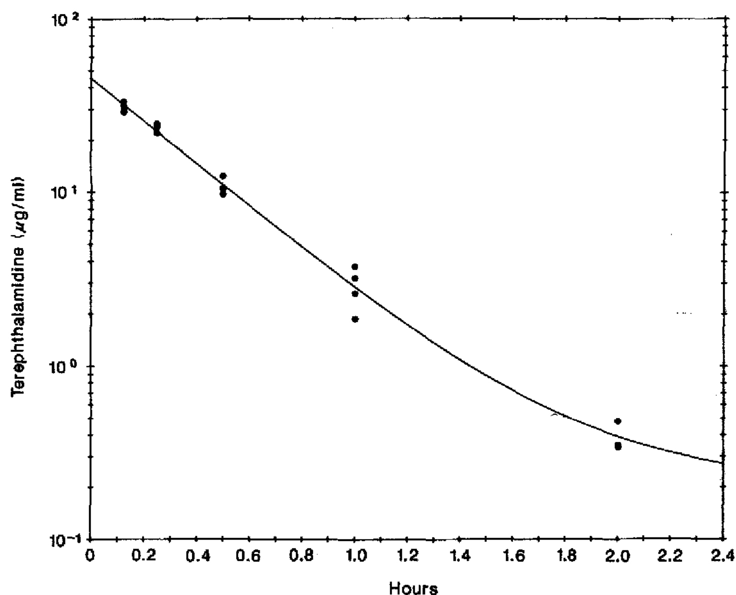

Fig. 4. Elimination of terephthalamidine from plasma of mice receiving an intraperitoneal dose of 15 mg/kg. Each point represents one mouse.

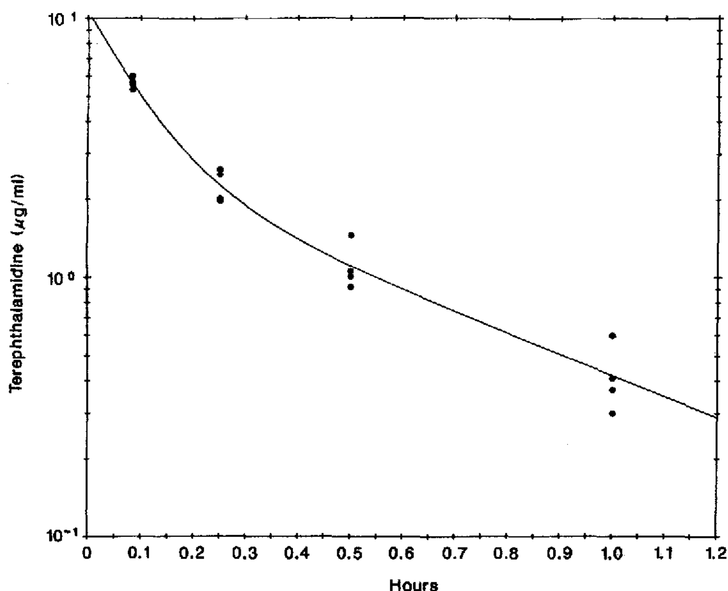

Fig. 5. Elimination of terephthalamidine from plasma of mice receiving an intravenous dose of 2.4 mg/kg. Each point represents one mouse.

In a preliminary experiment, an intravenous dose of 5 mg/kg was toxic to the mice. For mice receiving an intravenous dose of 2.4 mg/kg, the plasma concentration of drug was 5.6 ± 0.3 μg/ml (mean ± S.D., $n=4$) at 5 min after dosing (Fig. 5). No drug was detected at 2 or 3 h. As found for intraperitoneal dosing,

the data for the elimination of drug from the plasma best fitted a two-compartment open model ($p=0.006$). The half-lives of the α- and β-phases were 4 and 22 min, respectively.

In summary, the present assay method, which utilizes a simple extraction procedure, appears to be applicable for the determination of terephthalamidine in plasma.

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