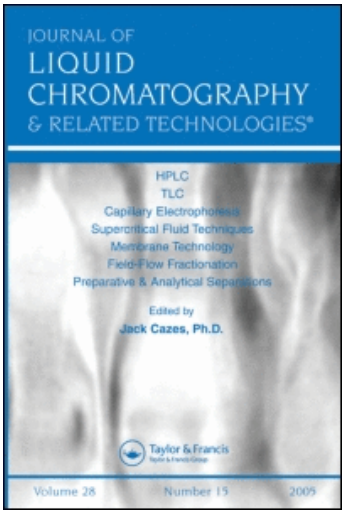

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S. D. Studenberg[a]; D. V. DeAngelis[a]; J. L. Woolley[a]
[a] Division of Pharmacokinetics and Drug Metabolism, Wellcome Research Laboratories, Research Triangle Park, North Carolina

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH FLUORESCENCE DETECTION FOR THE ANALYSIS OF 1954U89, 1,3-DIAMINO-7-(1-ETHYLPROPYL)-8-METHYL-7H-PYRROLO-(3,2-f)-QUINAZOLINE, IN PLASMA

S. D. Studenberg, D. V. DeAngelis, J. L. Woolley

Division of Pharmacokinetics and Drug Metabolism
Wellcome Research Laboratories
3030 Cornwallis Rd.
Research Triangle Park, North Carolina 27709

ABSTRACT

1954U89, 1,3-diamino -7 - (1-ethylpropyl) -8- methyl-7H-pyrrolo(3,2-f)-quinazoline, is a potent, lipid-soluble inhibitor of dihydrofolate reductase that is under preclinical evaluation as an anticancer agent. A rapid and selective high performance liquid chromatographic assay with fluorescence detection was developed for the quantitation of 1954U89 in rat and dog plasma. The compound was removed from plasma by solid phase extraction, and the extracts were chromatographed on a Hypersil C_1 column (4.6 mm x 15 cm) under isocratic conditions. The HPLC mobile phase consisted of methanol and 0.02 M ammonium acetate buffer (pH = 4.5) delivered in a ratio of 70:30 and at a flow rate of 1.0 mL/min. The compound was quantitated by fluorescence detection with excitation and emission wavelengths set at 335 and 460 nm, respectively. The quantitation range of the assay was 0.01 to 2.0 μg/mL. The intra- and interassay precision of the method were approximately

4 and 6%, respectively, in rats, and approximately 7 and 4%, respectively, in dogs. The accuracy (% bias) ranged from -10 to +2% across the concentration range in both species. This assay has been used to support nonclinical pharmacokinetic and bioavailability studies of 1954U89 in rats and dogs.

INTRODUCTION

The importance of folate antagonists in cancer chemotherapy has been recognized since the introduction of methotrexate (MTX) in the 1950s, and the role of nonclassical, especially lipid-soluble, antifolates in overcoming the limitations presented by MTX has been the subject of extensive research during the past four decades.[1-3] Lipid-soluble inhibitors of dihydrofolate reductase (DHFR) continue to show potential in cancer chemotherapy, and compounds that may show clinical utility based on their favorable disposition profile or their ability to overcome some forms of resistance have been identified.[4,5]

The diaminoquinazoline, 1954U89, 1,3-diamino-7-(1-ethylpropyl)-8-methyl-7H-pyrrolo-(3,2-f)-quinazoline (Figure 1), is a potent DHFR inhibitor that arose from an extensive research program that also produced pyrimethamine, metoprine, and piritrexim.[6] It is a small (MW 283), lipophilic (log P 2.7) compound with a K_i against human DHFR of 1.4 pM. The free base has limited aqueous solubility (<0.1 mg/mL), but the mesylate salt is soluble up to approximately 2.0 mg/mL in aqueous solutions and buffers. The compound achieves relatively high brain to plasma and lung to plasma concentration ratios in mice, rats, and monkeys, and shows activity against several tumor cell lines in culture.[6]

In conjunction with preclinical development of the compound, an analytical method with sufficient sensitivity, specificity, accuracy, and precision to support pharmacokinetic, disposition, and toxicology studies was required. Previous assay methods in our laboratory for compounds of this type included quantitative thin layer chromatography (TLC),[7] high performance liquid chromatography (HPLC),[8] and competitive protein binding.[9]

For the quantitation of 1954U89 in rat and dog plasma, an HPLC method with fluorescence detection was developed, validated, and applied successfully to rat and dog pharmacokinetic studies. The method and the results of the validation are reported here.

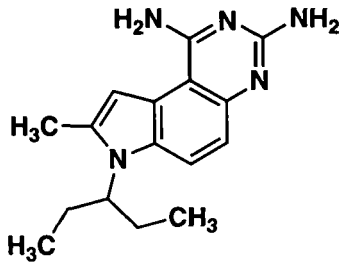

Figure 1. Chemical structure of 1954U89.

MATERIALS AND METHODS

Chemicals, Solvents, and Solutions

The mesylate salt of 1954U89 was obtained from Compound Registration, Burroughs Wellcome Co., Research Triangle Park, NC. Methanol and water were HPLC grade (Omnisolv, EM Science, Cherry Hill, NJ). Ammonium acetate and hydrochloric acid (37%) were A.R. grade (Mallinckrodt, Paris, KY). Rat plasma was obtained from male CD rats (Charles River Laboratories, Raleigh, NC) housed in the Central Animal Facility (Burroughs Wellcome Co.). Dog plasma was obtained from Environmental Diagnostics (Burlington, NC) or the Central Animal Facility. Ammonium acetate buffer (0.02 M, pH = 4.5), prepared by dissolving ammonium acetate (1.54 g) in distilled water (1000 mL) and adjusting the pH with HCl, was used to buffer plasma and to reconstitute extracts after evaporation. Ammonium acetate in methanol (0.02 M), prepared by dissolving ammonium acetate (1.54 g) in methanol (1000 mL), was required to elute the compound from the extraction columns. The HPLC mobile phase consisted of methanol and ammonium acetate buffer in a ratio of 70:30. Stock solutions of 1954U89 in ammonium acetate buffer were used to prepare spiked plasma calibration standards; separate stock solutions were used to prepare the spiked plasma controls. Eight concentrations of calibration standards (0.01 to 2.0 μg/mL) and three spiked plasma controls (2.0, 0.2, 0.02 μg/mL) were prepared, divided into 1-mL portions, and stored at 80°C until assayed.

Experiment

A Perkin Elmer AD-2 analytical balance was used to weigh the compound for the preparation of stock solutions. Micropipettors with glass tips (Scientific

Manufacturing Industries, Emeryville, CA) were used to prepare standard solutions and plasma controls. 1954U89 was extracted from plasma with 100-mg C_2 Bond Elut solid-phase extraction cartridges (Varian Associates, Sunnyvale, CA) and a multiple-cartridge vacuum apparatus (Vac Elut, Varian). Plasma extracts were evaporated in an N-Evap analytical evaporator (Organomation Associates, South Berlin, MA). The HPLC system consisted of a Waters 600 multisolvent delivery system and a Waters 712 WISP auto injector (Waters Associates, Milford, MA).

Samples were injected onto a Hypersil C_1 analytical column (4.6 mm x 15 cm, Phenomenex, Torrance, CA) with a 2-μm precolumn filter (Upcurch Scientific, Oak Harbor, WA) and a C_1 guard cartridge (Keystone Scientific, Bellefonte, PA). Sample fluorescence was quantitated with a Shimadzu RF-530 fluorescence detector (Shimadzu Scientific Instruments, Inc., Columbia, MD). The excitation (or absorption) spectrum of 1954U89 was determined initially, then the excitation wavelength was set at its maximum and the compound was scanned to determine the emission spectrum. The excitation and emission maxima were determined to be 335 and 460 nm, respectively. The mobile phase was delivered at a flow rate of 1.0 mL/min.

Chromatographic data acquisition and peak area analyses were accomplished with VG Multichrom software (Fisons Instruments Inc., Beverly, MA) and a VMS operating system (U.S. 5-20) on a VAX 6320 (Digital Equipment Corp., Maynard, MA).

Sample Preparation and Assay

Portions (0.1 mL) of plasma samples, calibration standards, or plasma control samples were combined with ammonium acetate buffer (0.5 mL) in 12 x 75 mm glass test tubes and vortexed. For each sample, standard, and control sample, an individual solid-phase extraction cartridge was preconditioned with methanol (1.0 mL) followed by ammonium acetate buffer (1.0 mL).

The buffered samples were loaded onto the cartridges while the packing was wet, and washed sequentially with water (1.0 mL) and methanol (1.0 mL). 1954U89 was eluted from the cartridges with ammonium acetate in methanol (1.0 mL). The eluates were evaporated under nitrogen at 50°C, and the residues were reconstituted with ammonium acetate buffer (0.2 mL) and vortexed. Reconstituted extracts were loaded into autosampler vials, and the autosampler was programmed to inject 0.1 mL at 7-min intervals.

Calculations

A least-squares linear regression model (weighted $1/c^2$) was selected and fitted to the peak area and concentration data obtained from the calibration standards. The concentrations of 1954U89 in plasma control samples were calculated from the equation of the regression line.

Assay Validation

The calibration model was selected after the analysis of calibration standards at eight concentrations (0.01 - 2.0 μg/mL). Standards were assayed in triplicate during six assay runs (rat plasma) and in duplicate during twelve assay runs (dog plasma), and a least-squares linear regression model with four weighting schemes (unweighted, $1/c$, $1/c^2$, and log-log transformed) was fitted to the concentration-peak area data. The residuals (the difference between the observed peak area and the peak area predicted by the regression equation) at each concentration were calculated and plotted as a function of concentration. The residuals plots were inspected for heteroscedasticity and for random distribution of the residuals around a residual value of zero.[10] The weighting scheme that resulted in homogeneous and normally-distributed variance of the residuals was chosen as the calibration model.[11,12] Assignment of the upper and lower limits of quantitation of the assay, defined as those concentrations at either end of the calibration curve that maintained the variance characteristics of the rest of the calibration curve, were made from the plot of the residuals.

The extraction efficiency of the assay was assessed by the comparison of extracted samples to unextracted calibration standards. Spiked rat plasma controls (0.02, 0.2 and 2.0 μg/mL) were extracted and assayed. Measured concentrations were determined with a calibration curve derived from the direct injection of 1954U89 stock standards and compared to their theoretical (nominal) concentrations to estimate the recovery from plasma.

Plasma from rats (n = 6) and dogs (n = 6) without added 1954U89 was extracted and assayed as described to ascertain that the method was specific for 1954U89. Chromatograms from these experiments were examined to determine if endogenous substances would interfere significantly with the integration of the compound peak. In addition, extracts of *in vitro* incubations of 1954U89 with hamster liver homogenates, which contained five (as yet unidentified) metabolites of 1954U89, were injected onto the HPLC system to check the retention times of the putative metabolites relative to the unchanged compound.

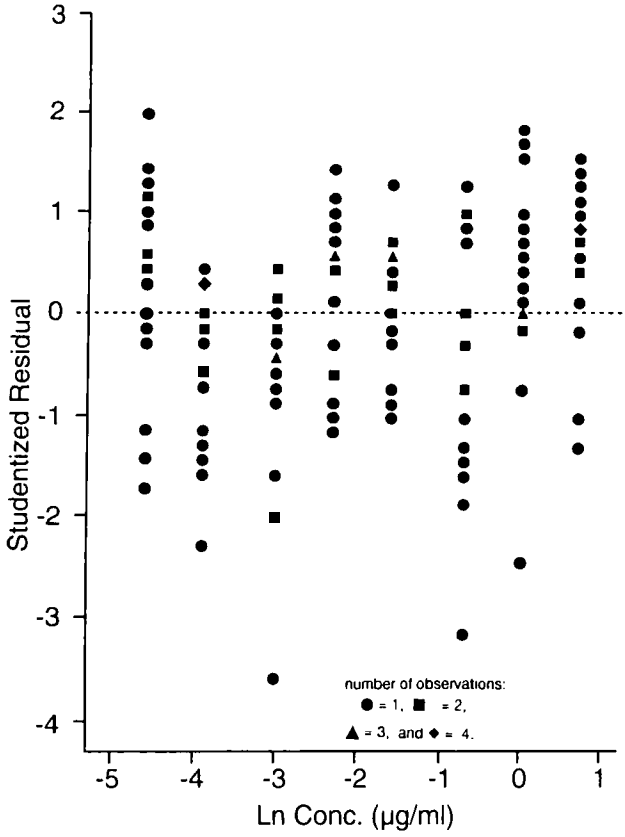

Figure 2. Plot of the Studentized residuals for the weighted ($1/c^2$) least-squares linear regression on the calibration standards in rat plasma (n = 3 x 6 at each concentration).

Intra- and interday precision and accuracy of the assay also were determined. Rat plasma was spiked with 1954U89 at three concentrations (0.02, 0.2, and 2.0 µg/mL). Each sample was divided into 1.0-mL portions and stored at -80°C. Eighteen samples from each group were analyzed over a six-week period. Analysis of variance was used to partition the total observed variance of the assay into its two components, intra-assay variability (random error) and interassay variability, or error associated with differences in day to day conditions.[13] Accuracy (% bias) was calculated as the percentage difference between the mean measured concentrations for each group of control samples and their theoretical (nominal) values.

Table 1

Extraction Efficiencies of 1954U89 from Rat Plasma

Normal Concentration (μg/mL)	Assayed Concentration[a] (μg/mL)	Recovery (%)
0.02	0.020 ± 0.0011[b]	100 ± 6
0.20	0.204 ± 0.0119	102 ± 6
2.00	2.027 ± 0.0804	101 ± 4

[a]n = 9 at each concentrtion
[b]Mean ± S.D.

Several studies were conducted to observe the effect of various storage conditions and experimental treatments on the stability of 1954U89. The stability of an analytical standard solution of 1954U89 stored at approximately 25°C in the dark was evaluated by assaying portions (50 μL, 0.5 μg, n = 4) at two, four, and six weeks after preparation. The concentration of the standard solution was determined with a standard curve derived from a freshly prepared standard solution. The stability of 1954U89 in plasma stored at -80°C was determined with rat plasma spiked at three concentrations (0.02, 0.2, and 2.0 μg/mL). Three replicates from each concentration were assayed on the day of preparation, frozen, and then re-assayed at one, three, and six weeks after preparation with freshly prepared calibration standards. The stability of the compound in plasma that underwent repeated freeze-thaw cycles also was studied. Dog plasma was spiked with 1954U89 (2.0 μg/mL), and duplicate portions were assayed immediately. The remainder was stored at -80°C, allowed to thaw, and then reassayed; this freeze-thaw-assay cycle was repeated three times. The stability of 1954U89 during the period between extraction and injection of the samples also was investigated. Fresh calibration standards and a spiked dog plasma sample (5.0 μg/mL) were prepared and extracted as described above. The extract was divided into six autosampler vials, and injected at 4-h intervals over a 21-h period, which was the maximum expected assay run time.

RESULTS AND DISCUSSION

The approach taken in the development of this method reflected both the

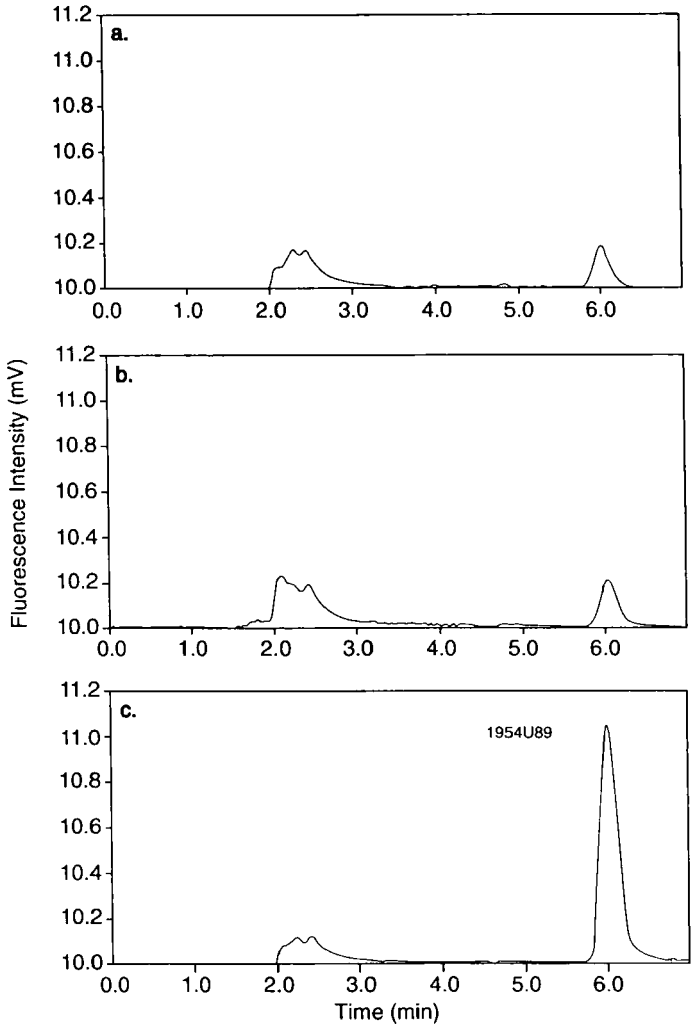

Figure 3. Concentration-peak area profiles of extracted (a) rat plasma, (b) dog plasma and (c) 0.01 μg/mL (LLOQ) calibration standard prepared in rat plasma. The retention time of 1954U89 was 6.0 min.

anticipated requirements of the assay as well as the early stage in the development of 1954U89 for which the assay was being used. Previous

Table 2

Accuracy and Precision for the Analysis of 1954U89 in Rat Plasma

Nominal Concentration (μg/mL)	Assayed Concentration[a] (μg/mL)	Bias (%)	Intraday CV (%)	Intraday CV (%)
0.02	0.018 ± 0.0010[b]	-10.0	3.5	5.3
0.20	0.202 ± 0.0118	+ 1.0	3.5	5.4
2.00	2.024 ± 0.803	+ 1.2	2.9	3.1

[a]n = 9 at each concentration
[b]Mean ± S.D.

Table 3

Stability of 1954U89 in Standard Solution

Time (Week)	Concentration (μg/mL)	Ratio
2	0.52 ± 0.002	1.04
4	0.48 ± 0.005	0.96
6	0.49 ± 0.002	0.98

[a]Mean ± S.D.

experience with compounds having similar physico-chemical properties (lipid solubility, strong chromophore/fluorophore), biochemical properties (potent DHFR inhibition), and pharmacokinetic properties (high volume of distribution and extensive metabolism), suggested that assay limits of detection below 0.1 μg/mL would be required; previous toxicity studies with similar compounds suggested that concentrations greater than 2 μg/mL would be near a toxic range of 1954U89. Consequently, the projected analytical range of 0.01 to 2 μg/mL was set for the method. Previous experience in assaying this class of compounds included quantitative TLC,[7] HPLC,[8] and competitive protein binding assays.[9] Although each of these approaches had potential, HPLC was chosen for its versatility and applicability. Preliminary development work on the method incorporated an internal standard, but the accuracy and precision of

the assay was not improved significantly when internal standarization was used. The excellent accuracy and precision of the data obtained without an internal standard, coupled with the potential problems of internal standard stability, specificity, and precision,[14] made internal standardization unnecessary.

Data from calibration curves were compiled from six assay runs (rats) or twelve assay runs (dogs) and examined to determine the most appropriate model selection and weighting scheme. A plot of the Studentized residuals from the least-squares linear regressions of the rat calibration curve data (weighted $1/c^2$) is shown in Figure 2. A similar plot was obtained for the residuals analysis of the dog calibration curve data. The plot demonstrates that the residuals were distributed normally around a residual value of zero and that the variance was homogeneous over the concentration range. This showed that $1/c^2$ weighted least-squares linear regression was an appropriate model to use for the estimation of 1954U89 concentrations in plasma.[10] Unweighted and 1/c weighted least-squares linear regression were unable to correct for heteroscedastic variance; log-log transformed weighting yielded results similar to the $1/c^2$ weighted regression. The upper and lower limits of quantitation were assigned as 2.0 and 0.01 μg/mL, respectively, based on this analysis of the residuals.[12]

The recovery of 1954U89 from rat plasma is shown in Table 1. The extraction efficiency of the assay ranged from 100 to 102%. Similar results were observed in the recovery of the compound from dog plasma (data not shown). Analysis of variance indicated that the recovery of 1954U89 did not differ significantly at different concentrations.

The specificity of the assay was assessed with plasma samples from untreated animals. Figure 3 contains concentration-peak area profiles from untreated rat and dog plasma, and the concentration-peak area profile of the lowest calibration standard (0.01 μg/mL). Although a small endogenous peak present in blank rat and dog plasma had a retention time similar to 1954U89, the contribution of this peak to the area of the lowest calibration standard was less than 10% and therefore did not present a significant interference to the quantitation of 1954U89. This endogenous peak was not present in human plasma samples.

Five putative metabolites of 1954U89 obtained from *in vitro* incubations with hamster liver homogenates were examined to determine their retention times relative to unchanged 1954U89. All metabolites eluted before, and were

Table 4

Stability of 1954U89 in Rat Plasma Stored at -80°C

Time (Week)	Concentration[a] (μg/mL)	Ratio
0.02μg/mL		
0	0.019 ± 0.0058[b]	0.94
1	0.023 ± 0	1.15
3	0.022 ± .0029	1.10
6	0.021 ± 0.0012	1.05
0.20 μg/mL		
0	0.206 ± 0.0096	1.03
1	0.213 ± 0.0040	1.06
3	0.208 ± 0.0070	1.04
6	0.19 ± 0.0058	0.96
2.00 μg/mL		
0	2.016 ± 0.075	1.01
1	2.038 ± 0.084	1.02
3	1.984 ± 0.031	0.99
6	2.094 ± 0.046	1.05

[a]n = 3 at each concentration
[b]Mean ± S.D.

resolved completely from, the parent compound. After intravenous administration of 1954U89 to rats and dogs, several (three to five) metabolites were present in plasma. These metabolites eluted before the parent compound and did not interfere with quantitation of 1954U89.

The intra- and interday precision and accuracy data for the assay of 1954U89 in rat plasma are shown in Table 2. The intraday CV was less than 4% over the examined concentration range in rats, and less than 7% in dogs. The interday precision ranged from 3 to 5% in rats, and from 1 to 6% in dogs. The percent bias ranged from -10.0 to +1.2 in rats, and -5.5 to +1.0 in dogs.

The concentration of 1954U89 in a standard solution stored at approximately 25°C remained stable during a six-week period. Table 3 shows

Table 5

Stability of 1954U89 After Freeze-Thaw Cycles

Cycle	Asseyed Concentration[a] (μg/mL)	Ratio
Initial	1.95 ± 0.06[b]	0.98
1	2.05 ± 0.16	1.02
2	1.92 ± 0.13	0.96
3	2.02 ± 0.04	1.01

[a]n = 4; nominal concentration = 2.00 μg/mL
[b]Mean ± S.D.

Table 6

Stability of 1954U89 in Processed Samples

Time[a] (h)	Assayed Concentration[b] (μg/mL)	Ratio
1	4.88	0.98
5	4.80	0.96
9	4.83	0.97
13	4.88	0.98
17	4.89	0.98
21	4.80	0.96

[a]Time elapsed between sample preparation and sample analysis
[b]Nominal concentration = 5.00 μg/mL

the average measured concentrations of the compound and the ratios of the assayed spiked concentrations to the nominal concentration on the days the assay was run. The mean concentrations of 1954U89 and the assayed concentration/nominal concentration ratios obtained from the assay of spiked plasma stored frozen (-80°C) during a six-week period are presented in Table 4. No trend in concentration during the time period was apparent. Likewise, no change was evident in the measured plasma concentrations of 1954U89 after

three freeze-thaw cycles (Table 5). The results of the experiment to determine the stability of the compound in processed samples (Table 6) indicated that 1954U89 remained stable at room temperature for at least 21 h after extraction from plasma.

In summary, a rapid, precise, and specific HPLC method for measuring 1954U89 concentrations in rat and dog plasma was developed and validated. The compound was removed from plasma by solid-phase extraction, chromatographed by isocratic reversed-phase HPLC, and quantitated by fluorescence. Least-squares linear regression with $1/c^2$ weighting was used as the calibration model. This assay is useful for the measurement of 1954U89 concentrations in plasma from nonclinical studies, and preliminary work suggests that it could also provide the basis for a bioanalytical method should the compound proceed to clinical trials.

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