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A column-switching high-performance liquid chromatographic assay for a novel cytotoxic thioxanthone derivative (WIN 33377) in mouse plasma with toxicokinetic results from a mouse LD_{10} study

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Abstract

WIN 33377 (I) is a member of a novel class of cytotoxic antitumor agents, 4-aminomethyl thioxanthone derivatives. A simple, rapid and reproducible method has been developed for the assay of I in mouse plasma using a high-performance liquid chromatographic method utilizing a column-switching technique. The method involves direct injection of buffered plasma to the extraction column for sample clean-up followed by switching onto an analytical column for analysis with UV detection at 256 nm. The method has demonstrated accuracy and precision over the range 10–2500 ng/ml using a 100-μl plasma sample with a minimum quantifiable level at 10 ng/ml. Stability of mouse plasma samples was demonstrated after storage for 4 weeks at −15 to −20°C, as was the ability of samples to be accurately quantified after a maximum of three freeze–thaw cycles. Recovery was greater than 87% for the compound and the internal standard. The assay was accurate and reproducible with measured values lying within the limits of defined acceptance criteria. The utility of the method was demonstrated by analyzing plasma samples obtained from mice dosed with I as part of a pre-clinical safety study intended to assist in the design of a pharmacokinetically guided dose escalation strategy.

1. Introduction

WIN 33377, N-[[1-[[2-(diethylamino)ethyl]amino]-9-oxo-9H-thioxanthen-4-yl]methyl]methanesulfonamide (I) (Fig. 1), is a novel cytotoxic antineoplastic agent which has shown a broad spectrum of anti-tumor activity and is being developed for the treatment of solid tumors. In order to support clinical development of this molecule through a pharmacokinetically guided dose escalation (PGDE) strategy in phase-I trial design [1–4], an accurate and sensitive method for the quantitation of I in mouse plasma was required. The method used WIN 33422, a structurally related analogue of I, as the internal standard (I.S.) (Fig. 1) and was based

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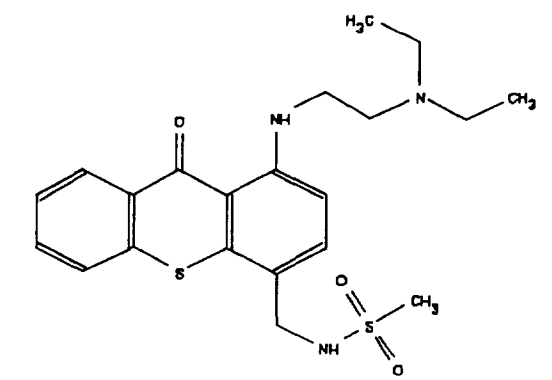
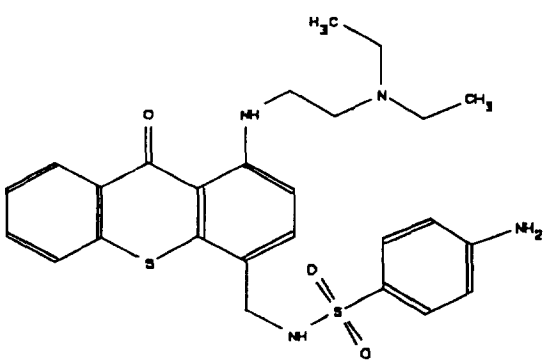


(I)
WIN 033377



(IS)
WIN 033422

Fig. 1. Chemical structures of I and I.S. I = N[[1-[[(2-diethylamino)ethyl]amino]-9-oxo-9H-thioxanthen-4-yl]methyl]methanesulfonamide; molecular mass 433.6. I.S. = N[[1-[[(2-diethylamino)ethyl]amino]-9-oxo-9H-thioxanthen-4-yl]methyl]methanesulfonilamide; molecular mass 510.7.

on HPLC separation with sample clean-up achieved through column switching. The method was successfully used to analyze samples obtained from a pre-clinical mouse study designed to give target area under the plasma concentration time (AUC) data that will later be applied in clinical development.

2. Experimental

2.1. Reagents and chemicals

I and I.S. were obtained from Sterling Winthrop Pharmaceutical Research Division, Rensselaer, NY, USA. Water, acetonitrile, methanol and ammonium acetate were all of HPLC grade and sodium bisulfite of reagent grade and were used without further purification. Control mouse plasma (potassium oxalate, sodium fluoride anticoagulant) was obtained from Rockland, Gilbertsville, PA, USA, and stored frozen at −15 to −20°C prior to use.

2.2. Assay procedure

Plasma samples (100 μl) were spiked with internal standard (20 μl of internal standard at a concentration of 5 ng ml^{-1}) and 200 μl of 0.01 *M* $NaHSO_3$. The sample was vortex-mixed for 5–10 s on a VWR Model G560 vortexer to insure adequate mixing, and centrifuged at 4000 *g* for 5 min to remove particulate matter. Samples were then subjected to automated HPLC analysis.

2.3. Instrumentation

Samples were loaded onto the sample clean-up column (column 1, 4 mm × 3 cm with Alltech C3 Pellicular packing 37–50 μm) using a Varian Model 9090 autoinjector. Mobile phases were delivered with a Varian Model 5560 pump (loading and clean-up system) and a Waters 510 pump (analytical system). The column-switching apparatus used consisted of a Rheodyne valve Model 7000P with pneumatic actuator (Rheodyne Model 7163 solenoid valve). The analytical separation system consisted of a guard column (Alltech Universal Guard Column, 3 cm × 2 mm I.D., with Pellicular C_{18} packing) placed before the analytical column (column 2, Beckman Ultrasphere ODS 5 μm, C_{18}, 25 cm × 4.6 mm I.D.). Detection was achieved with an Applied BioSystems 783A Variable Wavelength UV Absorbance Detector with Model 283 flow cell set at 256 nm. The detector output signal was

captured on the Fisons Multichrom data capture system. A block diagram of the column-switching configuration is given in Fig. 2.

2.4. Chromatographic conditions

Analysis incorporated the use of two mobile phases. Mobile phase 1 consisted of acetonitrile–water (7:3, v/v) delivered at a flow-rate of 1 ml min^{-1} through column 1. Acetonitrile–0.05 *M* ammonium acetate adjusted to pH 4.5 with glacial acetic acid (38:62, v/v) was mobile phase 2 and was delivered through column 2 at a flow of 1.0 ml min^{-1}. The two columns were connected in tandem via the switching apparatus. All flows were in a forward flush mode.

Samples (100 μl) injected onto column 1 were "washed" with mobile phase 1 for 1 min in a manner such that the eluate was directed to waste (Fig. 2). Concurrently, mobile phase 2 was directed through the analytical column. At the end of the 1-min wash cycle, mobile phase 2 was re-directed through column 1 onto column 2. The flow was maintained for 1 min, at the end of which time, the switching valve completed its cycle and column 1 was re-equilibrated with mobile phase 1 in preparation for the next sample injection, while mobile phase 2 continued to pass through column 2 to the end of the analytical run.

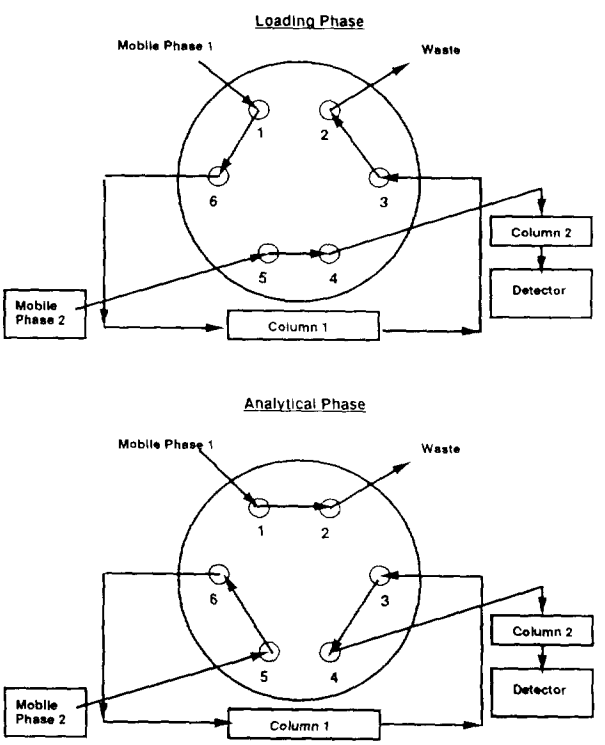


Fig. 2. The Rheodyne Model 7000 ARV pneumatically activated switching valve (rear view).

2.5. Preparation of standards

Calibration standards were prepared by addition of I stock solutions to control mouse plasma to give final concentrations of 10, 25, 50, 100, 250, 500, 1000, 2000, and 2500 ng ml^{-1}. In no case did spiking volumes exceed 25 μl ml^{-1} plasma. The calibration curve was run singly, with triplicate samples being run at the upper and lower concentrations. Two plasma blanks without internal standard were included in each calibration run. The calibration line was determined by a weighted ($1/Y^2$) linear regression analysis.

2.6. Assay validation

Validation samples were prepared under single blind conditions by addition of I to control plasma to give final concentrations of 10, 25, 250, 1000, and 2500 ng ml^{-1}. The low and the high concentrations correspond to the minimum quantifiable level (MQL) and the upper limit of the assay, respectively. The MQL is the lowest validation concentration with precision and accuracy within the 15% acceptance limits. Aliquots (100 μl) were distributed to glass autosampler vials for immediate assay for intra-day analysis and the remainder (for inter-day and satellite studies) was stored frozen at −15°C to −20°C.

Intra-day variability samples consisted of 6 levels (0, 10, 25, 250, 1000, and 2500 ng ml^{-1}) of I with 6 replicates at each level. The levels chosen reflect the entire range of the calibration

curve and the samples were analyzed immediately following preparation. A total of 80 samples were processed for this study, composed as follows: 36 validation samples (6 replicates at 6 concentrations), 13 calibration curve standards and 31 plasma blanks (added to mimic a typical analytical run of 62 samples in total). Between-day validation was performed by assaying one sample at each concentration level along with a freshly prepared calibration curve for five separate days.

2.7. Satellite studies

The stability of I in mouse plasma was assessed at 25 and 1000 ng ml^{-1}. Plasma samples were examined after 2 and 4 weeks of storage at −15 to −20°C. Additional plasma pools at 25 and 1000 ng ml^{-1} I were prepared and triplicate aliquots were assayed fresh and after 1, 2, and 3 freeze–thaw cycles. Samples were thawed at room temperature and analyzed against calibration standards on the same day.

The extraction efficiency (recovery) of the method was determined using quintuplicate plasma samples at low, medium and high (25, 250, and 1000 ng ml^{-1}) I concentration levels. These samples (processed) were analyzed in the usual manner. Similar quintuplicate sets of samples prepared in mobile phase 2 (direct) were injected directly onto column 2, bypassing column 1. For each concentration level, the ratios of the mean peak height (processed/direct) × 100 equals the percent extracted.

2.8. Statistical analysis

The acceptance criteria for the intra- and inter-day validation exercises were based on both the mean statistics determined for each group and the deviation of the individual concentrations from their expected values: validations were accepted if the coefficient of variation and accuracy were within ± 15% and 75% of all samples were within 15% of their expected values, with no more than 2 samples for any one group exceeding ± 15% of the expected.

The observed concentration can be either an individual determination or a mean of replicate determinations. The same acceptance criteria were applied to the stability and freeze–thaw experiments, together with an evaluation against run 1 for systemic deviations.

2.9. Mouse toxicokinetic study

The study was part of a development strategy designed to characterize the pharmacokinetics of intravenously administered I in the mouse. Typically, these studies are done at doses equivalent to the LD_{10} and fractions of the LD_{10}. The data generated in this study will be used to guide Phase I human clinical trials. All research involving animals described here was performed in accord with the Sterling Winthrop Pharmaceuticals Research Division (SWPRD) Policy on Animal Use and all national and federal legislation. All SWPRD animal facilities and programs are accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

I was administered intravenously (bolus) to male mice in a lactate buffer vehicle at 0 (vehicle only), 10.7, 53.5 and 107 mg kg^{-1} (3 mice per sampling time for the 10.7 and 53.5 mg kg^{-1} doses and 6 mice per sampling time at the 107 mg kg^{-1} dose). These doses represent 1/10, 1/2 and the LD_{10} values in male mice, respectively. Blood samples (potassium oxalate anticoagulant) were obtained at 0.083, 0.167, 0.5, 0.75, 1, 2, and 5 h post-dose and plasma was stored frozen at −20°C prior to assay. The samples were analyzed according to the described validated method.

3. Results

3.1. Assay validation

The assay was successfully validated within the acceptable limits for mouse plasma. Complete chromatographic resolution was achieved for I with approximate retention times of 5.7 and 7.5 min for I and I.S., respectively. At these times, no endogenous components eluted from control

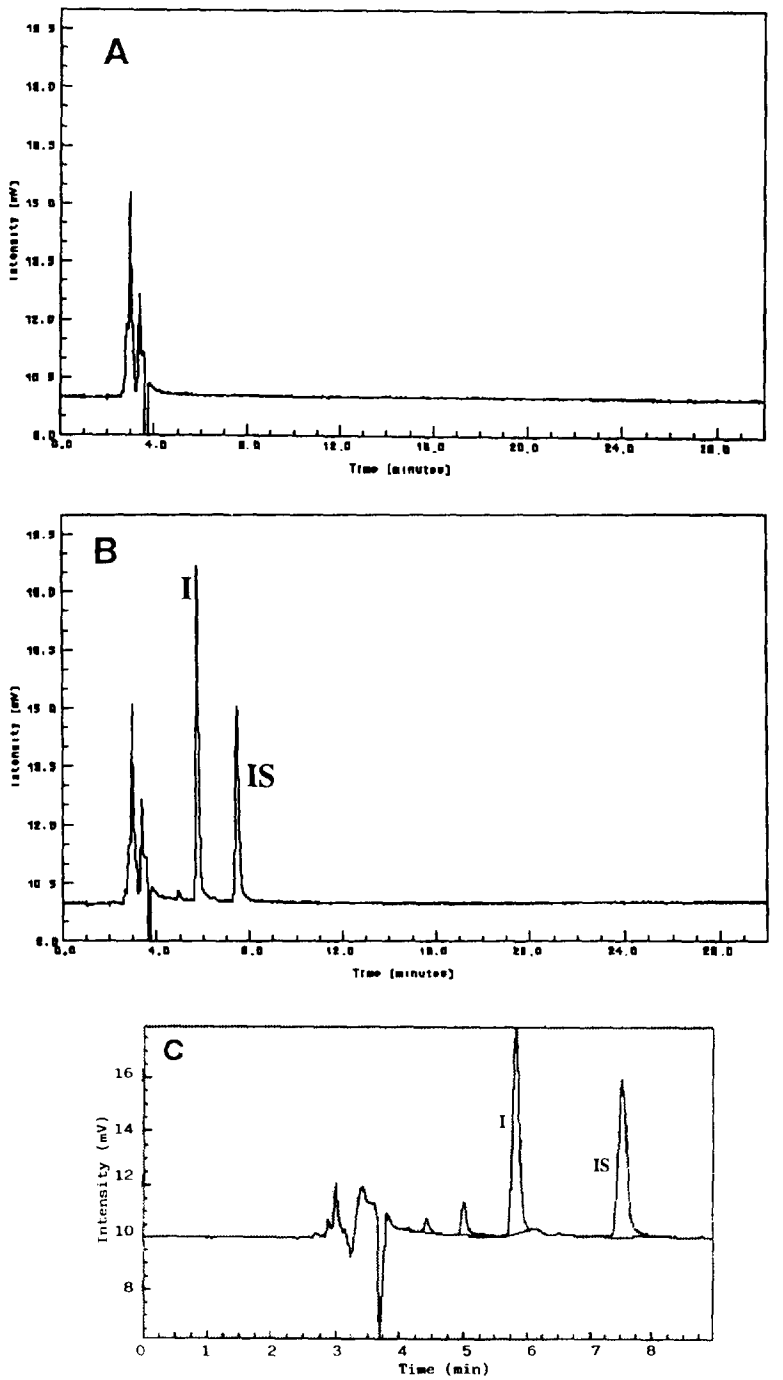


Fig. 3. Representative computer-generated chromatograms. (A) Control mouse plasma. (B) Control mouse plasma "spiked" with 1000 ng ml^{-1} I and 500 ng ml^{-1} internal standard. (C) Mouse plasma from the toxicokinetic study with a concentration of 550 ng/ml of the analyte.

mouse plasma which would interfere with the analysis of either I or the internal standard (Fig. 3).

The coefficients of variation (C.V.) of the intra-day validation samples (10, 25, 250, 1000, and 2500 ng ml^{-1}) ranged between 3.2 and 8.3%. Accuracy ranged from −5.3 to 4.9% of nominal values. The C.V. of the inter-day validation samples (at the same concentrations as the intra-day) ranged from 4.6 to 8.8%. Accuracy ranged from −7.7 to 1.1%.

3.2. Satellite studies

The stability of mouse plasma samples prepared at nominal concentrations of 25 and 1000 ng ml^{-1} and stored at −15 to −20°C was examined after 2 and 4 weeks. For each level, the C.V. ranged from 4.9 to 6.5% and the accuracy ranged from −6.5 to 1.3%.

Up to three freeze–thaw cycles were examined for effect on stability at 25 and 1000 ng ml^{-1} concentrations. For each level, the C.V. ranged from 0.7 to 13.2% and accuracy ranged from −5.2 to 12.2. Stability was not affected under these conditions.

The overall recovery of I from solutions "spiked" at 25, 250, and 1000 ng ml^{-1} was 105%, 91% and 87.1%, respectively. The recovery of the internal standard was 93.3%.

3.3. Mouse toxicokinetic profiles

The developed method was successfully applied to generating mouse toxicokinetic data, with no endogenous interference found in any sample. Following intravenous administration, plasma exposure to I increased with dose. Mean plasma concentrations are graphed in Fig. 4. Mean plasma concentrations were found to decrease rapidly with time at all dose levels. Mean plasma concentrations at 0.083 h post-dose ($C_{0.083}$) increased in a linear and proportional manner and were 324, 1600 and 3264 ng ml^{-1} at the 10.7, 53.5 and 107 mg kg^{-1} doses, respectively. Total exposure as assessed by curves of area under mean plasma concentration versus time also increased linearly, but in a greater than proportional manner with a 10-fold increase in dose producing approximately a 20-fold increase in AUC(0–t); i.e. 116, 1043, and 2572 ng h ml^{-1} at the 10.7, 53.5, and 107 mg kg^{-1} doses, respectively. Corresponding AUC(0–inf) values were 122, 1046, and 2590 ng h ml^{-1}. Plasma half-lives were 0.2, 0.6 and 0.7 h at the low-, mid- and high-dose groups, respectively.

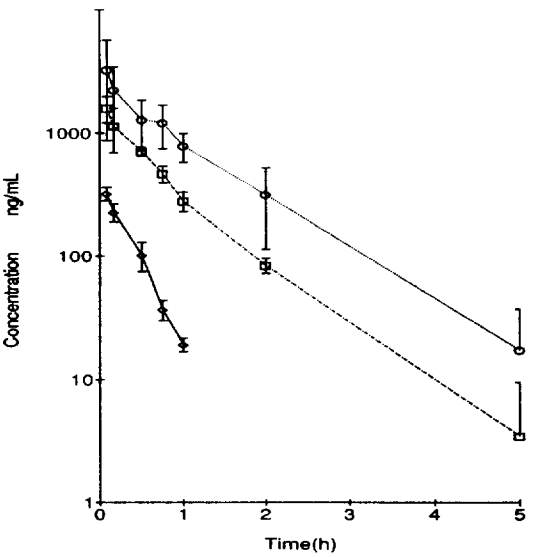


Fig. 4. Mean plasma levels (±S.D.) in mice following intravenous administration of I: (◇) dose = 10.7 mg/kg (n = 3); (□) dose = 53.5 mg/kg (n = 3); (○) dose = 107.0 mg/kg (n = 6).

4. Discussion

A simple, rapid and reproducible method is described for the assay of I in mouse plasma using an HPLC method with UV detection at 256 nm. A structurally related compound, WIN 33422, was used as the internal standard. The method uses 0.1 ml of plasma and sample preparation is minimal resulting in minimum exposure of the analyst to potentially hazardous or infectious material.

The current assay has been validated from 10 ng ml^{-1} to 2500 ng ml^{-1} (4000 ng/ml^{-1} if dilution is employed). At the five validation concentrations, the coefficients of variation were less than or equal to 8.8% and accuracies ranged

from −7.7 to 4.9%. Stability data indicated that I is stable in mouse plasma stored frozen for 4 weeks. Although not examined separately, stability of processed samples at room temperature for up to 12 h can be inferred since analysis of samples for intra-day validations required that amount of time.

Application of this assay has been used to determine the pharmacokinetics of I in mice at doses as low as 1/10 LD_{10} in mice. The resulting pharmacokinetic data determined at the LD_{10} dose, determined the "target" AUC for a pharmacokinetically guided dose escalation study in man [5].

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