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Assay for the quinocarmycin analog DX-52-1 in human plasma using high-performance liquid chromatography with automated column switching and low wavelength ultraviolet detection

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Abstract

The hydrocyanated derivative of the antitumor antibiotic quinocarmycin, DX-52-1 (I), exhibits impressive activity against human melanoma xenograft models in vivo. Phase I clinical trials to evaluate this compound as an antineoplastic agent have been initiated by the US National Cancer Institute. We have developed an HPLC assay for the determination of I in human plasma involving automated column switching and UV detection at 210 nm. The preparation of samples for chromatographic analysis entails the preliminary removal of plasma proteins by precipitation with acetonitrile, acidifying the clear supernatant to pH 4.5, then extracting twice with *tert*.-butyl methyl ether to recover the drug. A heart-cutting procedure employing two HPLC columns with contrasting retention characteristics under isocratic reversed-phase conditions was used to achieve the selectivity required for low wavelength UV detection of the analyte. The sample extract was initially loaded onto a column packed with a cyanopropyl stationary phase. During the predetermined time interval that I eluted from this column, a fully automated six-position switching valve was used to direct the effluent onto an octadecylsilane analytical column. The assay has been thoroughly validated with regard to linearity, inter- and intra-day accuracy and precision, recovery, selectivity and specificity. Using a sample volume of 1.0 ml, the lowest concentration of I quantified with acceptable day-to-day reproducibility was found to be 2.56 ng/ml (R.S.D. 18.9%, $n=21$, 4 months). This proved to be sufficiently sensitive for pharmacokinetic drug level monitoring in cancer patients treated with a 6-h continuous intravenous infusion of I, even at the starting dose of 3 mg/m^2. The successful performance and reliability of the assay has been demonstrated through extensive application to the routine analysis of plasma specimens acquired during a phase I clinical trial of the drug.

Keywords: Quinocarmycin; DX-52-1

1. Introduction

DX-52-1 (I; Fig. 1) is an investigational anticancer agent which exhibits impressive activity against human melanoma xenograft models [1]. It was prepared by hydrocyanation of quinocarmycin (Fig. 1), an antitumor antibiotic isolated from the culture broths of *Streptomyces melanovinaceus* [2], in an effort to identify derivatives possessing greater chemical stability in aqueous solution with retained antineoplastic activity [3,4]. Bearing two basic and one acidic functional group, the compound is water

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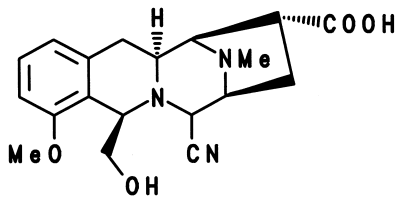
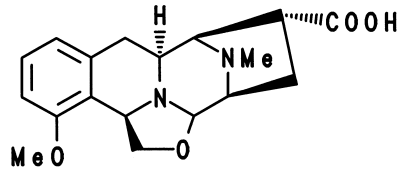


DX-52-1



QUINOCARMYCIN

Fig. 1. Chemical structures of DX-52-1 (I) and quinocarmycin.

soluble and presumably ionized in aqueous media at essentially all pH values. Analytical methods based upon reversed-phase HPLC with UV detection for the determination of I in mouse plasma with lower limits of quantitation in the 0.1–0.5 μg/ml range have been reported [5,6]. Whereas these assays served to demonstrate that I was eliminated very rapidly by mice, plasma levels of the unchanged drug could only be monitored for about 30 min following bolus intravenous injection of 12–75 mg/m^2 doses. In comparison, the drug will be administered as a 6-h continuous infusion with a starting dose of 3 mg/m^2 during the initial phase I clinical trials of I. Consequently, a marked improvement in assay sensitivity will most likely be necessary to detect even peak plasma levels of the drug achieved in patients at the end of the infusion.

The work described in this report was undertaken to develop an alternative analytical method for I with the sensitivity and selectivity required to characterize its pharmacokinetic behavior in cancer patients. As anticipated, this proved to be a particularly challenging problem. The polar nature of the drug presented difficulties in isolating it from numerous endogenous compounds present in plasma. It also lacked structural features amenable to the more sensitive and selective means of direct detection during HPLC, such as fluorescence and electrochemical oxidation. Furthermore, in common with many polar plasma constituents, a relatively weakly absorbing unconjugated phenyl ring represented the principal chromophore of the drug. In addition to these considerations, technical procedures and instrumentation were confined to those that would allow the analytical method be readily implemented at other institutions participating in the clinical evaluation of this chemotherapeutic agent.

2. Experimental

2.1. Reagents and chemicals

Analytical reference samples of I (NSC 607097; 4-cyano-6-hydroxymethyl-3,12-imino-7-methoxy-13-methyl-1,2,3,4,5,11,11a,12-octahydroazepino[1,2-b]isoquinoline-1-carboxylic acid) and quinocarmycin monocitrate (NSC 601422) were generously provided by the Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis, and Centers, National Cancer Institute (Bethesda, MD, USA). Distilled water was deionized and stripped of dissolved organics by passage through a Picosystem Ultrapure Water System consisting of mixed-bed resins and activated carbon (Hydro Service and Supply, Weymouth, MA, USA). Outdated frozen human plasma was obtained from the Blood Transfusion Service, Massachusetts General Hospital (Boston, MA, USA). Additional solvents, reagents, and chemicals were obtained from commercial sources in grades appropriate for direct use.

2.2. Analytical solutions

Stock solutions of I (0.10 mg/ml) were prepared by accurately weighing the compound on a Cahn C-34 analytical microbalance (ATI Orion, Beverly, MA, USA). The sample was dissolved in acetone within a silanized class A borosilicate glass volumetric flask (Kontes, Vineland, NJ, USA). These solutions were stored in a refrigerator (5°C) and used for approximately 1 month. Standard solutions were made daily by serially diluting the stock solution of I

with human plasma to provide concentrations of about 250, 175, 100, 50, 25, 10, 5 and 2.5 ng/ml.

2.3. Sample preparation

Plasma samples were prepared for analysis in disposable 10-ml borosilicate glass round-bottomed culture tubes and conical-bottomed centrifuge tubes with PTFE-lined phenolic screw caps (Fisher Scientific, Pittsburgh, PA, USA). The tubes were washed by overnight soaking in a 5% (v/v) solution of Contrad 70 cleaning agent (Fisher Scientific) in deionized water, then thoroughly rinsed with distilled water, and oven dried. All glassware was deactivated by treatment with a freshly prepared solution of 1% (v/v) Surfasil siliconizing fluid (Pierce Chemical, Rockford, IL, USA) in HPLC grade hexane and oven drying at 120°C.

Frozen plasma was thawed, vortex mixed, and centrifuged for 10 min at 11 750 *g* to effect the separation of particulates and lipids. Plasma (1000 μl) was pipeted into a 10-ml centrifuge tube and thoroughly mixed with acetonitrile (1000 μl) on a vortex stirrer for 15 s. The mixture was centrifuged for 10 min at 3500 *g*. The clear supernatant was transferred into another 10-ml centrifuge tube, mixed with 100 μl of 1.0 *M* formic acid, then extracted with hexane (5 ml) using a model 6010 reciprocating shaker (Eberbach, Ann Arbor, MI, USA) set at high speed for 10 min. After centrifugation (1300 *g*, 5 min), the upper organic phase was completely removed using a Pasteur pipet and discarded. The sample was then extracted twice with 5 ml of methyl *tert*.-butyl ether with centrifugation between successive extractions as described in the above. The methyl *tert*.-butyl ether extracts were combined in a culture tube and the solvent removed, using either an N-EVAP model 112 nitrogen evaporator (Organomation Associates, Berlin, MA, USA) with a bath temperature of 40–45°C, or a Heto CS3 vacuum concentration system (Appropriate Technical Resources, Laurel, MD, USA). The residue was reconstituted in 250 μl of 0.05 *M* potassium phosphate buffer, pH 2.1 with vortexing for 10 s and centrifuged (11 750 *g*, 10 min) in a polypropylene tube. The supernatant was transferred into a borosilicate glass insert which was sealed in an autosampler vial (12×32 mm). A 200-μl aliquot of the sample was injected into the chromatograph.

2.4. Chromatographic conditions

The chromatographic system consisted of two 1050 Series isocratic pumps and a 1050 Series autosampler fitted with a 500-μl sample loop and a 100 vial external tray (Hewlett-Packard, Wilmington, DE, USA). A model 7000 six-port switching valve coupled to a model 5701 two-position pneumatic actuator was operated automatically with a type 7163 dual three-way solenoid valve (Rheodyne, Cotati, CA, USA) controlled through the time-programmable +24 V DC relay contact on each HPLC pump. The system was configured for heart-cutting as follows: port 1 of the switching valve (SV) was connected to a waste container; flow from pump A was directed to the autosampler, then to SV port 2; a Zorbax (MAC-MOD Analytical, Chadds Ford, PA, USA) SB-CN guard cartridge (12.5×4.6 mm I.D., 5 μm) and SB-CN Rapid Resolution column (75×4.6 mm I.D., 3.5 μm) were connected in series between SV ports 3 (inlet) and 6 (outlet); pump B was connected to SV port 4; SV port 5 was connected to a Zorbax SB-C_{18} guard cartridge coupled to a stainless steel column (15 cm×3.9 mm I.D.) packed with 4 μm Nova-Pak C18 (Waters, Milford, MA, USA). Both precolumns were preceded by 0.5 μm in-line filters (Upchurch Scientific, Oak Harbor, WA, USA). The precolumns were routinely replaced after running approximately 36 plasma extracts.

The mobile phase used for the clean-up separation (pump A) was methanol–0.05 *M* ammonium phosphate buffer, pH 3.0 (25:75, v/v). The analytical mobile phase (pump B) was methanol–0.05 *M* ammonium phosphate buffer, pH 3.0 (35:65, v/v) containing 1.5 m*M* octyl sodium sulfate. These solutions were degassed in an ultrasonic bath for 15 min before use. Separations were performed at ambient temperature with both pumps operated at a flow-rate of 1.0 ml/min. The switching valve was initially positioned with ports 2–3, 4–5 and 1–6 connected to allow effluent from the clean-up column to flow to waste when injecting the sample. The valve was switched at 3.50 min postinjection to divert flow from the clean-up column to the analytical column for a period of 1.00 min, at which

time the valve was switched back to the initial position. UV absorbance of the effluent from the analytical column was monitored at 210 nm (6.5 nm bandwidth), with a 1-s response time, using a Hewlett-Packard model 79853C variable wavelength detector fitted with a 14-μl flow cell (8 mm path-length). The system was controlled through HP CHEMSTATION for LC software, rev. A.04.02, operating under Microsoft Windows 95 on a Vectra XM 5/90 Series 3 computer (Hewlett-Packard). The chromatograms were integrated to provide peak areas using the data analysis functions of the software.

2.5. Quantitation

Standard curves were constructed by plotting the chromatographic peak area of I as a function of its concentration in plasma. Linear least squares regression was performed using a weighting factor of $1/y_{obs}$, without inclusion of the origin, to determine the slope, y-intercept, and correlation coefficient of the best-fit line. Analyte concentrations in pharmacokinetic plasma specimens were calculated using results of the regression analysis. Pharmacokinetic samples were initially assayed in duplicate, with additional analyses performed if the replicate determinations deviated from their average by more than 10%. Specimens with concentrations exceeding the upper limit of the standard curve were reassayed upon appropriate dilution with drug-free plasma.

2.6. Accuracy and precision

Accuracy and precision of the assay were evaluated by analyzing the backcalculated sample concentrations and regression parameters from standard curves of I in human plasma. The R.S.D. of the mean predicted concentration for the independently assayed standards provided the measure of precision. The lower limit of quantitation was defined as the minimum concentration amenable to analysis with an inter-day R.S.D. not exceeding 20% [7]. The accuracy of the assay was assessed by expressing the mean predicted concentration of I as a percentage of the known concentration in the standard solutions.

2.7. Absolute recovery

Reference solutions of I having concentrations comparable to plasma standards following preparation for HPLC analysis, assuming quantitative recovery, were made by serially diluting the stock solution directly with 0.05 *M* potassium phosphate buffer, pH 2.1. During the course of a single day, three aliquots from each of four different plasma standards (2.5–250 ng/ml) were prepared for analysis and sequentially chromatographed with a corresponding reference solution. Absolute recovery was calculated by comparing the chromatographic peak area of the drug observed in each plasma standard to its paired reference solution.

2.8. Stability studies

The degradation of I was evaluated at ambient temperature in 0.05 *M* potassium phosphate buffer, pH 1.55, 2.14 and 2.75. An Orion model 920A pH meter equipped with a Ross semimicro combination pH electrode (Orion Research, Boston, MA, USA) was used for pH measurements. Kinetic runs were initiated by adding a stock solution of the drug (20 μl) to 5.98 ml of buffer, with mixing, to provide a concentration of 427 ng/ml. The solution was pipeted into 12 borosilicate glass autosampler vials for injection directly onto the Nova-Pak C_{18} analytical column (i.e., no column switching) approximately once an hour for 12 h. Mobile phase B (see Section 2.4) was used to separate I (t_R 9.1 min) from quinocarmycin (t_R 11.2 min), its principal degradation production in aqueous solution.

The stability of I in human plasma was determined at temperatures of −80, −30 and 37°C. Stability studies in nonfrozen plasma were initiated by pipeting an appropriate volume of a stock solution of I (0.1 mg/ml) into a 50-ml polypropylene tube containing 25 ml of plasma, equilibrated to 37.0°C in a shallow form shaking water bath (Precision Scientific, Chicago, IL, USA), to achieve concentrations of 50, 100 and 250 ng/ml. The tubes were capped, mixed by vortexing and replaced in the constant temperature bath. Subsequently, at eight time intervals ranging from 5 min to 6 h, two 1 ml samples were removed from each reaction mixture, flash

frozen (dry ice–isopropanol bath) within a glass centrifuge tube, and stored at −70°C until assayed during the following 2 days. To assess drug stability in frozen plasma, solutions of I were similarly prepared using ice-cold plasma. Aliquots of each solution (1000 μl) were pipeted into chilled 10-ml glass centrifuge tubes and flash frozen. The tubes were divided between dedicated laboratory sample storage freezers (Revco Scientific, Asheville, NC, USA) maintained at constant temperatures of −30° and −80°C. Both freezers were equipped with a digital temperature display and the reading was recorded on a daily basis. Two aliquots of each frozen solution were removed from the freezers, rapidly thawed, and immediately prepared for analysis every 4–5 days for 4 weeks. Apparent first-order rate constants for the loss of I (k_{obs}) were determined by nonlinear regression analysis of the chromatographic peak area–time profiles using the WINNONLIN software package (Scientific Consulting, Apex, NC, USA).

2.9. Dosing and sample collection

Plasma specimens were acquired from a cancer patient who received 6 mg/m^2 of I as a 6-h continuous intravenous infusion during a phase I clinical trial conducted by the Harvard/Boston Phase I Oncology Group. The study was sponsored by the National Cancer Institute and approved by the MGH Institutional Review Board for Human Studies. The patient was a 62-year-old female with metastatic cholangiocarcinoma that had failed to respond to all standard therapeutic interventions. All of the eligibility criteria for entry into the trial were satisfied and the patient signed an institutional consent form. Drug was supplied by the Cancer Therapy Evaluation Program, National Cancer Institute in a 5-mg vial as a lyophilized powder for reconstitution with 5 ml of Sterile Water for Injection, USP. This solution was further diluted with 0.9% Sodium Chloride Injection, USP to deliver the desired dose in a volume of 480 ml. Blood specimens (5 ml) were collected in Vacutainer Brand plasma tubes with freeze dried-sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA) from a peripheral veinous catheter placed in the arm opposing that used for dosing. Samples were acquired shortly before treatment, 3 and 6 h after starting the infusion, and at 5, 10, 20, 30, 60, 120 and 180 min postinfusion. The sample tubes were immediately placed on ice and centrifuged (800 *g*, 10 min, 10°C) within 1 h of collection. The plasma was separated from blood cells and maintained at −70°C until assayed.

3. Results and discussion

3.1. Detection

Although I lacks structural features that would facilitate direct detection during HPLC with a high degree of sensitivity and selectivity, it has several functional groups that could be exploited for an analytical derivatization with a fluorogenic reagent. These include the carboxylic acid moiety at C-1, the α-amino nitrile function at C-4, and the 1° hydroxyl group at C-14, among which reactions directed at the carboxyl group were considered to be the most promising. Numerous analytical reagents have been developed to generate fluorescent amide and ester derivatives of carboxylic acids under a broad range of conditions [8]. In addition, a method based upon derivatization of the carboxyl group would provide the capability to quantitate quinocarmycin, a known degradation product in aqueous solution [3] and possible metabolite of the drug.

A series of preliminary experiments revealed that derivatization of the C-1 carboxylic acid function of I with 4-bromomethyl-6,7-dimethoxycoumarin proceeded more favorably than several other commercially available fluorogenic reagents that were available. Conditions were established to derivatize I with an efficiency of about 70% following isolation of the compound from plasma by liquid–liquid extraction. However, interference from derivatized endogenous compounds became significant when the concentration of I in plasma was less than 1 μg/ml (data not shown). Efforts to resolve the drug derivative from interferences both prior to and during HPLC were unsuccessful. Precolumn clean-up techniques that were evaluated in this capacity included solid-phase extraction (SPE) in the normal and reversed-phase modes, thin-layer chromatography, and liquid–

liquid extraction (LLE). Furthermore, in each case, the overall procedure would be technically intensive and very time consuming.

Subsequently, the feasibility of determining I by HPLC with low wavelength UV detection was evaluated. The most intense absorption band in the UV spectrum of I in phosphate buffered aqueous solution exhibits a maximum at 197 nm (not shown). This is somewhat below the practical wavelength limit for UV detection during reversed-phase HPLC, which tends to be near 210 nm with the use of transparent mobile phase components, such as phosphate buffer, acetonitrile and methanol. To ascertain whether the elution of I could be monitored at 210 nm with the desired sensitivity, signal-to-noise ratios were determined for the chromatographic peak resulting from decreasing amounts of I loaded onto a Nova-Pak C_{18} column and separated using a mobile phase composed of methanol–50 m*M* ammonium phosphate buffer, pH 3.1 (32:68, v/v) at a flow-rate of 1.0 ml/min. The signal-to-noise ratio afforded by 1.0 ng of the drug was 7.9 (not shown), thereby suggesting that it should indeed be possible to detect I at a concentrations approaching 1 ng/ml in plasma, assuming at least 50% recovery of the drug from a sample volume of 1 ml. It was recognized that the ability to actually achieve this level of sensitivity would require extensive sample clean-up and establishing chromatographic conditions to completely resolve the drug from all potentially interfering endogenous compounds.

3.2. Sample preparation

To accommodate the relatively large sample volume mandated by the sensitivity requirement of the assay, SPE and LLE were evaluated for the initial isolation of drug from plasma specimens and to provide a concentrated sample solution for chromatographic analysis. In a series of preliminary experiments, the recovery of I from plasma or aqueous buffer solutions by SPE, using a variety of bonded-phase cartridges (C_8, C_{18}, CN) from commercial sources, was found to be inefficient and highly variable. Attempts to isolate the drug by ion-pair extraction from alkaline solutions (pH 9–11) into immiscible organic solvents (*tert*.-butyl methyl ether, dichloromethane, ethyl acetate) using a variety of quaternary ammonium salts (tetramethyl, tetrapropyl, tetrabutyl, tetradecyltrimethyl, dodecyltrimethyl) at concentrations ranging from 1 to 100 m*M* also yielded unsatisfactory recoveries (<17%). Similarly, I was inefficiently extracted (<10%) from acidic aqueous solution (pH 2–3) in the presence of anionic ion-pairing agents (1-decanesulfonic acid sodium salt, octyl sodium sulfate, heptafluorobutyric acid) at concentrations of 1–10 m*M*.

In consideration of these results, the influence of pH on the amount of drug recovered from 50 m*M* aqueous buffer solutions by a single extraction with 5 ml of *tert*.-butyl methyl ether was determined. As shown in Fig. 2A, I was recovered with poor efficiency from neutral solutions and those more acidic than pH 2.5. In the region from pH 3 to 5, the extraction efficiency was approximately constant at 50–55%, with the notable exception of formate buffer, for which the recovery increased to a maximum near 65%. Although a highly reproducible effect, the nature of the interaction responsible for the enhanced drug recovery in the presence of formate buffer is not obvious. The bell-shaped profile for the extraction efficiency of I from aqueous solution as a function of pH is consistent with the probable ionization state of the molecule [9]. Dissociation of the carboxylic acid moiety affording an anionic species, which is not readily extractable, would predominate in neutral and alkaline solutions. The observed increase in partition coefficient that occurs as the pH is decreased below 7 very likely results from protonation of one of the two weakly basic nitrogen atoms, thereby making the molecule an effectively neutral zwitterion. The molecule would be expected to assume a net positive charge when the pH is further decreased below the pK_a of the carboxylic acid function and it again becomes less readily extractable into an organic solvent.

During adaptation of the LLE procedure to the isolation of I from plasma, it was observed that removing plasma proteins by precipitation prior to extraction with *tert*.-butyl methyl ether realized a significant improvement in drug recovery. However, the pH of the protein-free supernatant could not be adjusted with acceptable reproducibility when perchloric acid was used for protein precipitation. As an alternative, precipitating proteins with acetonitrile and adjusting the supernatant pH with dilute formic

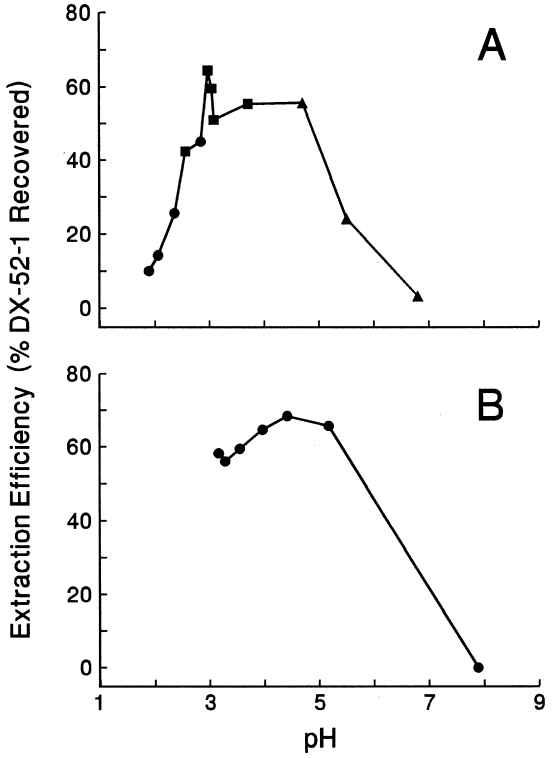


Fig. 2. Influence of pH on the relative amount of I extracted from (A) 50 m*M* aqueous buffer solutions and (B) deproteinized plasma into *tert*.-butyl methyl ether. Potassium phosphate (●), ammonium formate (■) and ammonium acetate (▲) buffers containing 10 μg/ml of I were directly extracted with 5 ml *tert*.-butyl methyl ether for 10 min. Solutions of I in human plasma (10 μg/ml, 1.0 ml) were first treated with acetonitrile (1.0 ml) and centrifuged. The protein-free supernatant was acidified with aqueous formic acid solution (0.1 ml, 0.1–10 *M*) to provide the indicated pH values, washed with hexane (5 ml), then extracted with *tert*.-butyl methyl ether (5 ml). In both cases, the solvent was evaporated and the extract reconstituted in buffer (1.0 ml) for chromatographic analysis. Recovery was based upon the combined peak areas of I and quinocarmycin relative to a 10 μg/ml reference solution of I. Data points represent the average of at least two determinations.

acid prior to extraction afforded greater and more consistent drug recoveries. Optimum extraction efficiencies resulted when the apparent pH of the supernatant was adjusted to approximately 4.5 using dilute formic acid (Fig. 2B). Pre-extraction of the acidified protein free supernatant with hexane also proved advantageous in several respects, such as decreasing the time required for solvent evaporation, affording clear solutions upon reconstitution of the extract, and removing very strongly retained endogenous compounds. Extracting plasma specimens with *tert*.-butyl methyl ether twice in succession significantly improved the reproducibility of drug recovery as well as the lower limit of quantitation.

3.3. Liquid chromatography

Compound I eluted with a retention time of 7.6 min from a Nova-Pak C_{18} column using a mobile phase composed of methanol–50 m*M* ammonium phosphate buffer, pH 3.1 (32:68, v/v) at a flow-rate of 1.0 ml/min, monitored by UV detection at 210 nm (Fig. 3A). Separating an extract of drug-free human

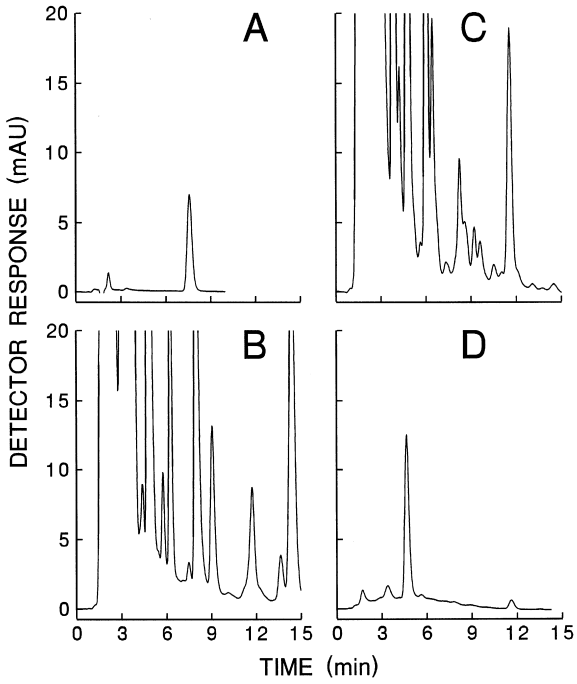


Fig. 3. Liquid chromatograms demonstrating applicability of the heart-cutting technique to improve the selectivity for monitoring I in plasma extracts. (A) Reference solution of 0.5 μg/ml I in 50 m*M* ammonium phosphate buffer (pH 3.1). Extracts of drug-free human plasma separated directly on (B) a Nova-Pak C_{18} column or (C) a Zorbax SB-cyano column. (D) Fraction of effluent collected 3.0 to 4.0 min after injecting a blank plasma extract onto the cyano column reanalyzed on the Nova-Pak C_{18} column. Plasma was extracted as described in Section 2.3. Chromatographic conditions: sample injection volume, 200 μl; mobile phase, methanol–50 m*M* ammonium phosphate buffer, pH 3.1, (C_{18} column, 32:68, v/v; cyano column, 25:75, v/v); flow-rate, 1.0 ml/min; detection, UV at 210 nm.

plasma under these conditions showed a number of endogenous peaks in the region of the chromatogram where I eluted (Fig. 3B). The samples were then analyzed using a Zorbax Rapid Resolution Stable Bond cyano column with the same mobile phase. With a retention time of 3.5 min (not shown), I eluted among an abundance of endogenous peaks (Fig. 3C). To ascertain whether the retention characteristics of the extractable plasma components differed significantly on the cyano and C_{18} columns, another extract of blank human plasma was initially loaded on the cyano column. The fraction eluting from the cyano column between 3.0 and 4.0 min during the run was collected and manually loaded onto the Nova-Pak C_{18} column. The resulting chromatogram showed no endogenous peaks in the region from 7.0 to 8.5 min where I eluted (Fig. 3D).

These observations suggested that a heart-cutting technique, which can be readily automated using a pneumatically operated switching valve, could be used to facilitate the selective quantitation of I in plasma [10,11]. The configuration of the HPLC system, consisting of an autosampler, two isocratic pumps, cyano cleanup column, C_{18} analytical column, six-position automated switching valve, and a variable wavelength UV detector, is shown in Fig. 4. Initially, methanol–50 m*M* ammonium phosphate buffer, pH 3.1 (25:75, v/v) at flow-rate of 1.0 ml/min was used as the mobile phase for both the clean-up and analytical separations. Samples were loaded onto the Zorbax cyano column with the effluent flowing to waste. The switching value was actuated at 4.0 min postinjection, thereby directing the effluent from the cyano column to the analytical column for a period of 1.0 min, after which the valve was switched back to the initial position. Chromatograms of plasma extracts showed several prominent endogenous peaks eluting between 7.5 and 10.0 min, with some comparatively small peaks eluting in the presence of I, which exhibited a retention time of 11.4 min (not shown).

Establishing chromatographic conditions to completely resolve the drug from these relatively minor endogenous peaks was desired, as their presence would affect the lower limit of detection. Since the drug was positively charged in the mobile phase (apparent pH 3.67), the addition of anionic ion-pairing agents to the analytical mobile phase was

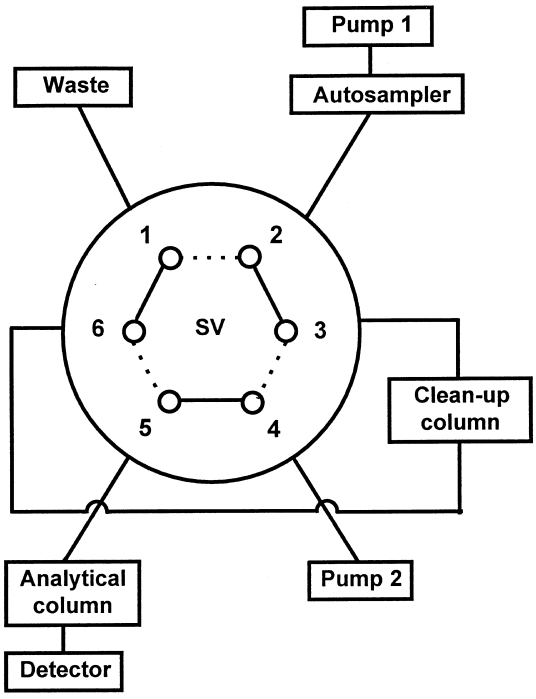


Fig. 4. Diagram depicting the chromatographic system and connections to the six-port switching valve (SV).

explored in an attempt to selectively enhance the retention of I. A mobile phase prepared with 1-hexane sulfonic acid sodium salt at a concentration of 15 m*M* not only resulted in a dramatic increase in the retention time of I, but very effectively resolved the drug peak from endogenous compounds, which were evidently unaffected by the presence of the ion-paring agent. Although symmetrical, the chromatographic peak for I was somewhat broader than desired. A considerably sharper peak was afforded by changing the ion-pairing agent to 1.5 m*M* octyl sodium sulfate and increasing the amount of organic modifier in the mobile phase. With an eluent composed of methanol–50 m*M* ammonium phosphate buffer, pH 3.0 (35:65, v/v), I eluted at 12.8 min and was well resolved from all peaks of endogenous origin, as demonstrated by the chromatograms of drug-free human plasma and standard solutions prepared by adding I to plasma at concentrations of 2.56, 25.6 and 256.0 ng/ml shown in Fig. 5.

The performance of the chromatographic system was verified routinely by injecting a reference solu-

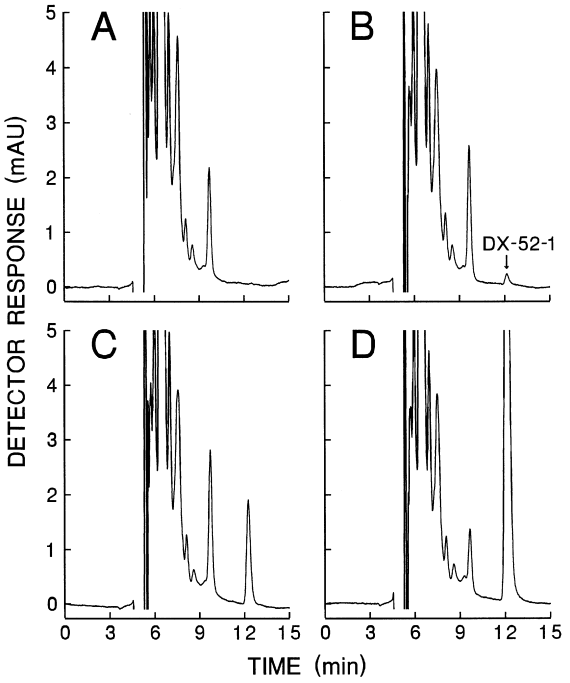


Fig. 5. Typical chromatograms of drug-free human plasma (A) and standard solutions of I in plasma at concentrations of 2.56 ng/ml (B), 25.6 ng/ml (C) and 256.0 ng/ml (D). Samples were prepared for analysis and separated as described in Sections 2.3 and 2.4.

tion of I in 50 m*M* potassium phosphate buffer, pH 2.1 (0.5 μg/ml, 200 μl) onto the clean-up column connected directly to the detector or coupled to the analytical column by the automated switching procedure. Retention times of I (mean±S.D.) determined during a period of 5 months were 3.89±0.12 min (n=19) on the clean-up system alone and 12.78±0.68 min (n=41) using the heart-cutting procedure with a 3.5 to 4.5 min window. Corresponding mean values of the peak width at half-height were 0.222±0.033 min and 0.345±0.100 min for the clean-up column alone and coupled to the analytical column, respectively.

3.4. Assay selectivity and specificity

A partially degraded solution of I containing a detectable amount of quinocarmycin was chromatographed independently on the cleanup and analytical columns. Quinocarmycin eluted from the cyano column prior to I, with a retention time of 3.2 min, but was more strongly retained on the analytical column, eluting at 11.2 min (Fig. 6). Thus, with the initial switching time set at 3.5 min, the peak for quinocarmycin would be almost completely eluted from the clean-up column before directing flow from it to the analytical column.

Chromatograms of human plasma from more than eight different anonymous donors showed no peaks which interfered with the detection of I (e.g. Fig. 5A). Furthermore, plasma specimens obtained from more than 20 cancer patients shortly before the administration of I showed no potentially interfering chromatographic peaks (e.g. Fig. 7A). These samples were generally acquired after the patients received pretreatment medications, including dexamethasone phosphate, granisetron, thiethylperazine, ondansetron, and perphenazine. On the basis of these observations, the analytical method is considered to be highly specific for the determination of I.

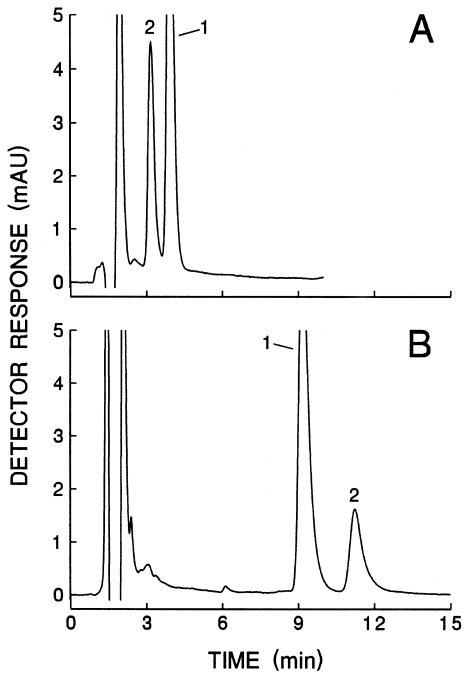


Fig. 6. Liquid chromatograms of a partially degraded solution of I in 50 m*M* potassium phosphate buffer (pH 8.1), separated directly on either the Zorbax SB cyano clean-up column (A) or the Nova-Pak C_{18} analytical column (B). Chromatographic conditions as described in Section 2.4. Peak assignments: 1=DX-52-1 (I); 2=quinocarmycin.

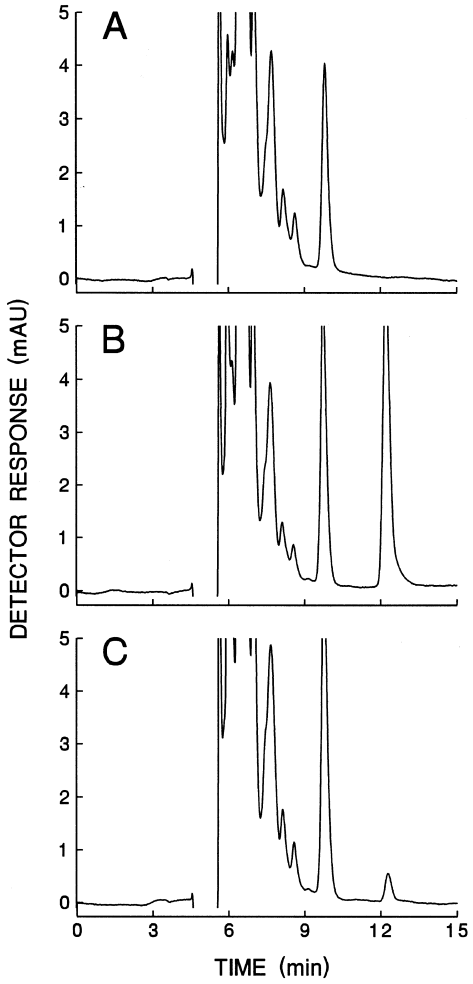


Fig. 7. Representative liquid chromatograms of plasma samples acquired from a cancer patient (A) prior to treatment with 6.0 mg/m^2 of I given as a 6-h continuous intravenous infusion, (B) 10 min postinfusion, 107.1 ng/ml, and (C) 3 h after dosing, 9.8 ng/ml.

3.5. Assay validation

The chromatographic peak area for I increased proportionately to the added concentration of drug in human plasma ranging from 2.56 to 256.0 ng/ml (Fig. 5). Linear regression performed with weighting according to the reciprocal of the observed peak area yielded the best fit of the standard curves. A total of 21 standard curves of I in human plasma were prepared and assayed during a 4-month period by several different technicians. Mean values (±S.D.) of the regression parameters for these standard curves were as follows: slope, 1.150±0.149; *y*-intercept, −0.661±0.874; correlation coefficient, 0.999± 0.002. Within-day accuracy and precision for determinations of the low, high and two intermediate drug concentrations comprising the standard curve are presented in Table 1. The drug was quantitated with a mean accuracy of 100.1±1.8% (S.D.) and the lowest concentration included in the standard curve, 2.56 ng/ml, was determined with a 4.8% R.S.D. during a single day. Backcalculated drug concentrations from 21 standard curves assayed over 4 months were used to assess between-day accuracy and precision of the analytical method (Table 1). Inter-day accuracy of the assay ranged from 99.5 to 103.4% and did not appear to be dependent upon the concentration of I. In contrast, the precision showed a trend toward improvement with increasing drug concentration, with R.S.D. values ranging from a minimum of 2.7% for the 256.0 ng/ml plasma standard to 13.7% at 10.2 ng/ml. The 2.56 ng/ml plasma standard, which had a signal-to-noise ratio of approximately 10 (Fig. 5B), was quantitated with an inter-day precision of 18.9%. In accordance with recommended criteria [7], this concentration was therefore established as the lower limit of quantitation of the assay. As indicated in Table 2, the absolute recovery of I from human plasma was very consistent and reproducible at concentrations ranging from 10.2 to 256.0 ng/ml, with an average value of 77.8±2.5% (S.D.), and slightly greater at 2.56 ng/ml (85.6±6.2%). These results serve to demonstrate that the assay is accurate, reproducible and robust.

3.6. Stability studies

The stability of I in aqueous solution was examined to ascertain whether an autosampler could be used for overnight analysis of reconstituted plasma extracts. Preliminary studies revealed that a peak corresponding to quinocarmycin became detectable within 3 h of the addition of I to buffer solutions ranging from pH 3.1 to 9.1. In each case, the reaction appeared to achieve an equilibrium when monitored for a sufficiently long time. Although these pilot studies where not conducted in a manner which would provide quantitative kinetic data, it did appear

Table 1
Accuracy and precision for the determination of I in human plasma

DX-52-1 plasma concentration (ng/ml)	Within-day[a]		Between-day[b]	
	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
2.56	102.3	4.8	103.4	18.9
10.2	98.0	1.2	99.5	13.7
51.2	N.D.[c]	N.D.	101.8	6.3
102.4	99.7	2.5	100.4	5.0
256.0	100.2	0.5	99.8	2.7

[a] Number of replicate determinations=3.
[b] Determined from the backcalculated concentrations for 21 standard curves of I in human plasma prepared and analyzed during a 4-month period.
[c] N.D., not determined.

that the initial rate of quinocarmycin formation was greatest near pH 7 and considerably slower in weakly acidic and alkaline solutions. The formation of quinocarmycin was not evident 3 h after adding I to pH 2.75 phosphate or pH 1.5 perchlorate. Accordingly, more detailed kinetic studies were performed within this pH range. Also, in contrast to alkaline solutions, moderately acidic solutions can be loaded directly onto silica-based columns without damaging the stationary phase. The degradation of I in phosphate buffers ranging from pH 1.55 to 2.75 at ambient temperature, monitored at hourly intervals for 12 h, exhibited pseudo first-order kinetics as indicated by linear semi-log plots of the chromatographic peak area as a function of time (not shown). Rate constants for the loss of I determined by nonlinear regression analysis of the peak area time courses were 0.032 h^{-1} at pH 2.75 (10 m*M* potassium phosphate buffer), 0.0038 h^{-1} at pH 2.14 (50 m*M* potassium phosphate buffer), and 0.0023 h^{-1} at pH 1.55 (100 m*M* phosphoric acid). Calculated values of the time for 5% degradation of the drug are 1.6 h at pH 2.75, 13.5 h at pH 2.14 and 22.4 h at pH 1.55. Selecting 50 m*M* potassium phosphate buffer, pH 2.14 as the vehicle for the final sample solution facilitated chromatographic analysis of plasma extracts for 12 h after reconstitution when maintained at room temperature.

Table 2
Absolute recovery of I from human plasma

DX-52-1 plasma concentration (ng/ml)	Absolute recovery[a] (%)
2.56	85.6±6.2
10.2	78.2±3.1
102.4	78.8±2.1
256.0	76.5±1.0

[a] Tabulated values are the mean±S.D. of three replicate determinations.

Information on the temperature dependence of the stability of I in plasma was required to develop sample handling and storage protocols for pharmacokinetic studies. The results of these experiments are summarized in Table 3. Plasma samples containing added concentrations of I ranging from 42.6 to 255.5 ng/ml showed no evidence of degradation when maintained at −30 or −80°C during a period of 29 days. Thus, plasma specimens can be stored at temperatures ≤ −30°C for at least 1 month prior to analysis. Compound I appeared to degrade very slowly in human plasma at 37.2°C during a 6-h observation period. Under these conditions, estimated values (harmonic mean±S.D., $n=3$) of the apparent first-order half-life and time for 5% degradation of the drug were 62.1±11.3 and 4.6±0.8 h, respectively. As such, blood specimens acquired during the course of clinical pharmacokinetic studies can be handled and processed without implementing procedures to further enhance drug stability. Plasma could even be separated from blood at 37°C if protein binding or drug partitioning into blood cells proves to be temperature dependent.

Table 3
Stability of I in human plasma

Temperature (°C)	Time interval	Initial DX-52-1 plasma conc. (ng/ml)	Mean conc. found/added[c] (%)	k_{obs}^{d} (h^{-1})
−79.2±0.8[a]	29 days (7)[b]	42.6	96.7±4.9	
		85.2	104.4±5.0	
		255.5	100.3±3.6	
		Mean	100.5±3.9	
−30.3±0.2	29 days (7)[b]	42.6	99.1±6.9	
		85.2	100.3±4.6	
		255.5	101.6±4.7	
		Mean	100.3±1.3	
37.2±0.1	6 h (8)[b]	52.2		0.0128
		105.7		0.0119
		254.9		0.0088
		Mean		0.0112±0.0021

[a] Mean±S.D. of temperature readings recorded throughout the observation period.
[b] Numbers in parentheses: number of time points.
[c] Mean±S.D. of the concentration determined at each time point for conditions in which no evidence of degradation during the observation period was evident.
[d] Apparent first-order rate constant for the loss of I in plasma.

3.7. Application of the assay to the analysis of patient samples

The sensitivity and specificity of the assay were found to be sufficient for characterizing the plasma pharmacokinetics of I in cancer patients that received doses of 3 mg/m^2 and greater given as a 6 h continuous intravenous infusion. Representative chromatograms of plasma specimens acquired from a patient before, during and after treatment with a 6 mg/m^2 dose of I are shown in Fig. 7. The time course of observed plasma concentrations of I in this patient and the best-fit curve determined by nonlinear regression analysis are presented in Fig. 8. Plasma levels of I increased rapidly during the infusion to a steady state concentration of 117 ng/ml. Upon terminating the infusion, the drug concentration declined monoexponentially with a 51-min half-life and remained above the 2.56 ng/ml lower limit of quantitation of the assay for more than 3 h thereafter. The total plasma clearance and apparent volume of distribution, determined from nonlinear regression analysis of the plasma profile, were 8.5 $l/h/m^2$ and 10.4 l/m^2, respectively.

4. Conclusion

An HPLC assay involving automated column switching and UV detection at 210 nm has been developed for the determination of I in human plasma. The preparation of samples for chromatographic analysis entails the preliminary removal of plasma proteins by precipitation with acetonitrile,

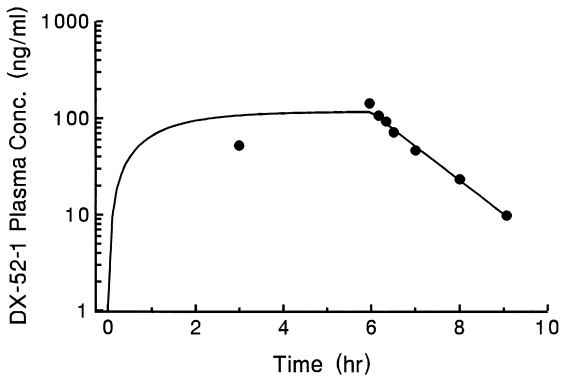


Fig. 8. Plasma concentration–time profile of I in a cancer patient who received a 6.0 mg/m^2 dose of drug I given as a 6-h continuous intravenous infusion.

acidifying the clear supernatant to pH 4.5, then extracting twice with *tert.*-butyl methyl ether to recover the drug. A heart-cutting procedure employing two HPLC columns with contrasting retention characteristics under isocratic reversed-phase conditions was used to achieve the selectivity required for low wavelength UV detection of the analyte. The sample extract was initially loaded onto a column packed with a cyanopropyl stationary phase. During the predetermined time interval that I eluted from this column, a fully automated six-position switching valve was used to direct the effluent onto an octadecylsilane analytical column. The assay has been thoroughly validated and shown to be specific, accurate and reproducible. Using a sample volume of 1.0 ml, the lowest concentration of I quantified with acceptable day-to-day reproducibility was found to be 2.56 ng/ml. This proved to be sufficiently sensitive for pharmacokinetic drug level monitoring in cancer patients treated with a 6 h continuous intravenous infusion of I. The successful performance and reliability of the assay has been demonstrated through extensive application to the routine analysis of plasma specimens acquired during a phase I clinical trial.

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