Specific high-performance liquid chromatographic assay with ultraviolet detection for the determination of 1-(2-chloroethyl)-3-sarcosinamide-1-nitrosourea in plasma

J.G. Supko*, L.R. Phillips, L. Malspeis

Laboratory of Pharmaceutical Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Frederick, MD 21701-8527, USA

Abstract

A facile, sensitive and highly specific HPLC method for assaying 1-(2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) in plasma has been developed. The drug was efficiently isolated from plasma by extraction with *tert.*-butyl methyl ether. A structurally related compound with similar physicochemical properties served as the internal standard (I.S.). Following evaporation of the organic solvent, the extract was reconstituted with 0.05 M ammonium acetate buffer, pH 5.0, and loaded onto a 4 μm Nova-Pak C_{18} column (15 cm×3.9 mm), which was preceded by a 7 μm Brownlee RP-18 precolumn (1.5 cm×3.2 mm). Chromatography was performed at ambient temperature using a mobile phase of methanol–0.1 M ammonium formate buffer, pH 3.7 (25:75, v/v). UV absorbance of the effluent was monitored at 240 nm. A flow-rate of 1.0 ml/min was used for analyzing mouse and dog plasma extracts. Under these conditions, the drug eluted at 4.0 min and was followed by the I.S. at 6.1 min. An automatic switching valve was employed to allow the precolumn to be flushed 1.5 min into the run, without interrupting the flow of the mobile phase to the analytical column, thereby preventing the apparent build-up of extractable, strongly retained, UV-absorbing components present in mouse and dog plasma. Operating in this manner, more than 100 samples could be analyzed during a day using a refrigerated autosampler for overnight injection. The method was readily adapted to the determination of SarCNU in human plasma by simply decreasing the eluent flow-rate to 0.6 ml/min, whereby SarCNU and the I.S. eluted at approximately 5.8 and 9.1 min, respectively. Furthermore, the switching valve was not necessary for the analysis of human plasma samples. With a 50-μl sample volume, the lowest concentration of SarCNU included in the plasma standard curves, 0.10 μg/ml, was quantified with a 7.8% R.S.D. ($n=27$) over a 2 month period. Plasma standards, with concentrations of 0.26 to 5.1 μg/ml, exhibited R.S.D. values ranging from 1.3 to 4.7%. Thermospray-ionization MS detection was used to definitively establish the specificity of the method. The sensitivity of the assay was shown by application to be more than adequate for characterizing the plasma pharmacokinetics of SarCNU in mice.

Keywords: 1-(2-Chloroethyl)-3-sarcosinamide-1-nitrososurea

---

*Corresponding author. Present address: Massachusetts General Hospital, Cox Building, Room 640, 100 Blossom Street, Boston, MA 02114, USA.

1. Introduction

Although N-nitroso compounds are known to be potent carcinogens [1], a number of 1-(2-chloro-ethyl)-1-nitrosourea (CENU) derivatives exhibit broad spectrum activity against experimental tumors and human neoplasms [2,3]. The cytotoxic CENUs readily decompose under physiological conditions to afford a variety of reactive species that alkylate and carbamoylate essential cellular macromolecules [4]. Whereas their antitumor activity [2,4] and dose-limiting hematological toxicity [4] appear to be exclusively attributable to the alkylating inter-mediate, carbamoylation may be responsible for the pulmonary toxicity observed during prolonged therapy with some of these drugs [5]. Unlike the CENUs presently in clinical use that enter cells by passive diffusion [2], the uptake of 1-(2-chloro-ethyl)-3-sarcosinamide-1-nitrosourea (SarCNU, Fig. 1) by glioma cells is augmented by catecholamine transport mechanisms [6] and it lacks carbamoylating activity [7]. In addition, SarCNU is more efficacious against cultured human glioma cells and substantially less toxic to normal bone marrow tissue *in vitro* than 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) [8,9], the comparative standard for new CENU anticancer agents [10].

SarCNU was recently selected for development as a clinical candidate by the National Cancer Institute on the premise that its unique physicochemical and pharmacological properties may prove therapeutical-ly advantageous in the treatment of malignant glioma, against which currently available drugs demonstrate only limited effectiveness [3]. In con-junction with the preclinical evaluation of SarCNU, we embarked upon the development of an assay for this compound in plasma that would be suitable for use during pharmacokinetic studies in small animals. As described herein, the procedure involves isocratic reversed-phase HPLC with UV detection following isolation of the drug by extraction with an immisc-ible organic solvent. The assay has been shown to be specific for SarCNU, has been thoroughly validated, and has been proven to be highly reliable, through extensive application during preclinical phar-macokinetic studies.

2. Experimental

2.1. Reagents and chemicals

Analytical reference samples of SarCNU (NSC 364432) and 1-(2-chloroethyl)-3-prolinamide-1-ni-trosourea (ProCNU; NSC 673531) were provided by the Pharmaceutical Resources Branch, Developmen-tal Therapeutics Program, Division of Cancer Treat-ment, National Cancer Institute (Bethesda, MD, USA). 'Baker Analyzed' HPLC-grade *tert.*-butyl methyl ether (TBME; J.T. Baker, Phillipsburg, NJ, USA) was further purified by fractional distillation prior to use. Distilled water was deionized and stripped of dissolved organics by passage through mixed-bed resins and activated carbon (Hydro Water Systems, Rockville, MD, USA). Additional solvents, reagents and chemicals were obtained from commer-cial sources in grades appropriate for direct use. Mouse plasma was purchased from Harlan Biop-roducts for Science (Indianapolis, IN, USA) and human plasma was procured from the American Red Cross (Baltimore, MD, USA).

2.2. Analytical solutions

Stock solutions (1.0 mg/ml) of SarCNU and ProCNU, the internal standard (I.S.), were prepared by dissolving the compounds, accurately weighed on a Cahn C-31 microbalance (Cahn Instruments, Cer-ritos, CA, USA), in dimethyl sulfoxide (DMSO) within class A borosilicate glass volumetric flasks (Kontes, Vineland, NJ, USA). Fresh stock solutions were prepared weekly and stored at 5°C. The solu-tions were protected from light, allowed to thaw at ambient temperature and then thoroughly mixed before each use. Standard solutions were made daily by serially diluting the SarCNU stock solution with chilled mouse plasma to provide concentrations of 5.0, 4.0, 3.0, 2.0, 1.0, 0.75, 0.5, 0.25 and 0.1 μg/ml.

$$
\begin{array}{c}
O \\
\parallel \\
N \quad O \quad CH_3 \quad O \\
| \quad \parallel \quad | \quad \parallel \\
Cl\text{-}CH_2CH_2\text{-}N\text{-}C\text{-}N\text{-}CH_2\text{-}C\text{-}NH_2
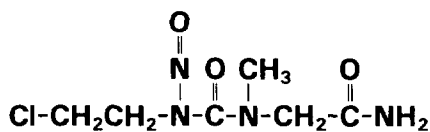\end{array}
$$

Fig. 1. Chemical structure of SarCNU.

2.3. Sample preparation

Samples were prepared for analysis in disposable 10-ml borosilicate glass culture tubes (Sun Brokers, Wilmington, NC, USA) and centrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA) with Teflon-lined phenolic screw caps. The tubes were washed with Alconox detergent (Alconox, New York, NY, USA), thoroughly rinsed with distilled water, and oven dried. The sample extraction solution used in the assay was made daily, by diluting the ProCNU stock solution (20 μl, 1.0 mg/ml) with distilled TBME in a volumetric flask (500 ml), to a concentration of 40 ng/ml. The TBME–I.S. solution was dispensed directly from the volumetric flask using a class A borosilicate glass volumetric pipette.

A 50-μl aliquot of plasma and 3 ml of TBME–I.S. solution were pipetted into a 10-ml centrifuge tube and vigorously mixed on a vortex action stirrer for 1.0 min. After centrifugation (2500 g, 3 min), the upper organic phase was removed and concentrated to dryness in a round-bottomed culture tube under a stream of nitrogen gas using a Meyer N-EVAP (Organomation Assoc., Berlin, MA, USA) at a bath temperature of 45–50°C. The residue was reconstituted in 250 μl of 0.05 M ammonium acetate buffer, pH 5.0 by vortexing for 10 s. The solution was transferred into a borosilicate glass insert and sealed within an amber crimp-top autosampler vial (12 × 32 mm) containing 1 ml of ice-cold water. A 200-μl aliquot of the solution 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, Palo Alto, CA, USA). Temperature control of the sample vials was accomplished by circulating a 5°C mixture of distilled water–ethylene glycol (5:1, v/v) through the external tray at 5 l/min using a Model 9101 isotemp digital circulator (Fisher Scientific). 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 d.c. relay contact on each HPLC pump. The system was configured for precolumn flushing as follows: flow from pump A was directed to the autosampler then to port 1 of the switching valve; a 1.5 cm × 3.2 mm Brownlee RP-18 (7 μm spherical silica, 300 Å pore size) precolumn (Rainin Instrument, Woburn, MA, USA) was placed between ports 2 and 5; port 6 was directed to the inlet of a 15 cm × 3.9 mm stainless-steel column packed with 4 μm Nova-Pak C_{18} (Millipore, Milford, MA, USA), preceded by a 0.5 μm in-line filter (Rainin); pump B was connected to port 3; and port 4 was connected to waste. The analytical mobile phase (pump A) was methanol–0.10 M ammonium formate buffer, pH 3.7 (25:75, v/v) and the solution used for precolumn flushing (pump B) was acetonitrile–0.10 M ammonium formate buffer, pH 3.7 (25:75, v/v). These solutions were degassed in an ultrasonic bath for 15 min before use. In addition, the analytical mobile phase was continuously sparged with a gentle stream of helium during chromatography. Separations were performed at ambient temperature and both pumps were operated at a flow-rate of 1.0 ml/min. The switching valve was initially positioned with ports 1–2, 3–4 and 5–6 connected, to situate the precolumn between the autosampler and the analytical column, when injecting the sample. The valve was switched at 1.5 min post-injection, which brought the precolumn in line with pump B, directing the precolumn's effluent to waste and maintaining flow from pump A to the analytical column. The switching valve was reset to its original position at 8 min post-injection to allow the precolumn time (3 min) to equilibrate with the analytical mobile phase before the subsequent injection. UV absorbance of the effluent from the analytical column was monitored at 240 nm (6.5 nm bandwidth) using a Hewlett-Packard model 79853C variable-wavelength detector fitted with a 14 μl flow cell (8 mm path-length). The 1 V output of the detector was provided as the signal to an HP 3396 Series II integrator, configured to report peak height using a 0.2 min peak width at a threshold setting of 1 with baseline construction through each detected valley point.

To assess specificity of the method, the effluent from a similarly configured HPLC system, but without the switching valve, was introduced directly

into a Hewlett-Packard 5989A mass spectrometer equipped with a thermospray LC–MS interface. Chromatographic conditions were the same as described above, except that the mobile phase flow-rate was reduced to 0.7 ml/min to accommodate the interface. The thermospray ion source was operated in the filament-assisted mode with positive ion detection. Operating temperatures for the interface were as follows: ion source, 230°C; probe stem, 122°C; and probe tip, 228–232°C. Nominal resolution mass spectra (140–500 u) were acquired at the rate of 2.44 s/scan with the electron multiplier set at 2400 V. Selected-ion monitoring (SIM) was performed by measuring the ions at m/z 223 and 240 sequentially using a mass width of 0.07 u and a dwell time of 310 ms with the electron multiplier at 2400 V.

2.5. Quantitation

Standard curves were constructed by plotting the SarCNU to I.S. chromatographic peak height ratio against the concentration of SarCNU. 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 analyses. 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 back-calculated sample concentrations and regression parameters from standard curves of SarCNU in mouse plasma that were prepared and assayed on separate days. 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 a R.S.D. not exceeding 10%. The accuracy of the assay was assessed by expressing the mean predicted concentration of SarCNU as a percentage of the known concentration in the standard solutions.

2.7. Absolute recovery

Reference solutions of SarCNU and the I.S. at concentrations comparable to plasma standards following preparation for HPLC analysis, assuming quantitative recovery of the two compounds, were made by diluting their stock solutions directly with 0.05 M ammonium acetate buffer, pH 5.0. During the course of a single day, five aliquots from each of six different plasma standards (0.1–5.1 μg/ml) were prepared for analysis and sequentially chromatographed with a corresponding reference solution. Absolute recovery was calculated by comparing the chromatographic peak height of the drug or I.S. observed in each plasma standard to its paired reference solution.

2.8. Stability studies

The degradation of SarCNU was evaluated at ambient temperature (22±1°C) in the following buffer solutions: 0.05 M potassium phosphate (pH 2.23, 3.06, 5.61, 6.59 and 7.57), 0.05 M ammonium formate (pH 4.13); and 0.05 M ammonium acetate (pH 4.96). Ionic strength of the solutions was not adjusted. Kinetic runs were initiated by adding a solution of SarCNU in acetonitrile (~1.25 mg/ml) to the buffer, with mixing, to achieve an initial concentration of 2.0 μg/ml. Aliquots (30 μl) of the solution, contained within an amber borosilicate glass autosampler vial, were loaded directly into the liquid chromatograph approximately once an hour for 22 h.

At initial concentrations of 5, 10 and 25 μg/ml, the stability of SarCNU in fresh mouse plasma was determined at temperatures of 5, 15, 25 and 37°C. The plasma was obtained from male CD2F₁ mice (National Cancer Institute, Frederick, MD, USA), using heparin sodium as the anticoagulant, and kept ice-cold until used. To 1500 μl of temperature-equilibrated plasma, 5 μl of SarCNU stock solution (1.5, 3.0 or 7.5 mg/ml in DMSO) was added. The

sample was mixed by vortexing and replaced in the constant temperature bath. Subsequently, at 12 time-intervals ranging from 5 min to 54 h, 50 μl of the reaction mixture were removed, quenched by flash-freezing in a 10-ml centrifuge tube, and immediately prepared for HPLC analysis. In another experiment, SarCNU was added to ice-cold mouse plasma to afford a concentration of 10 μg/ml. Aliquots of the solution (50 μl) were pipetted into chilled 10-ml glass centrifuge tubes and flash-frozen in a dry ice-isopropanol bath. The tubes were maintained at approximately -70°C in a low-temperature freezer (Revco Scientific, Asheville, NC, USA). Aliquots from three separate solutions were removed for analysis on days 1, 2, 3, 4 and 8.

Apparent first-order rate constants for the loss of SarCNU (k_{obs}) were determined by linear regression analysis of a semi-log plot of its chromatographic peak height (for buffer solutions) or the drug-to-I.S. peak height ratio (for plasma samples) as a function of time. Graphical estimates of the rate constants in the theoretical equation that best described the functional relationship between k_{obs} and pH were improved by unweighted non-linear least squares regression using the PCNonlin software package (Statistical Consultants, Lexington, KY, USA). Hydronium ion activities (a_{H^+}) were calculated directly from the measured pH. The corresponding hydroxyl ion activities, $a_{OH^-} = K_w/a_{H^+}$, were calculated using a value of $8.0211 \cdot 10^{-5}$ for the ion product of water (K_w) at 22°C without correction for ionic strength [11].

2.9. Dosing and sample collection

Male CD2F$_1$ mice were treated by intraperitoneal injection with SarCNU (25.6 mg/kg) dissolved in 1.0 μl of DMSO/g body weight. At thirteen time intervals ranging from 3 min to 2 h after dosing, groups of five animals were anesthetized with methoxyfluorane and bled by retro-orbital puncture using heparinized capillary tubes into Eppendorf heparin-coated microcentrifuge tubes (Curtin Matheson Scientific, Jessup, MD, USA). The plasma afforded by immediate centrifugation (12 000 g, 2 min) of the whole blood from individual mice was flash-frozen, stored at -70°C, and processed for analysis within 48 h. Time points were determined as

the difference between the blood collection interval mid-point and the starting time of dose administration. The average sample time and geometric mean plasma concentration for the individual mice in each group were calculated. The model-independent equation which most adequately described the plasma concentration-time profile [12],

$$C = C_i(e^{-S_z t} - e^{-S_1 t}) \tag{1}$$

was ascertained by weighted (y_{obs}^{-2}) non-linear regression analysis (PCNonlin). Final values of the iterated parameters (C_i, S_1, S_z) were used to calculate all pharmacokinetic terms.

3. Results

3.1. Analysis of SarCNU in plasma

A liquid chromatogram of drug-free mouse plasma determined by UV detection at 240 nm is shown in Fig. 2A. Comparison with chromatograms of plasma to which SarCNU was added at concentrations of 0.10 and 5.1 μg/ml, respectively (Fig. 2B–C), clearly demonstrates the absence of endogenous components eluting in the presence of the drug (t_R 4.0 min) or I.S. (t_R 6.1 min). An automatic switching valve was employed to prevent contamination of the analytical column with strongly retained UV-absorbing substances, apparently of endogenous origin,
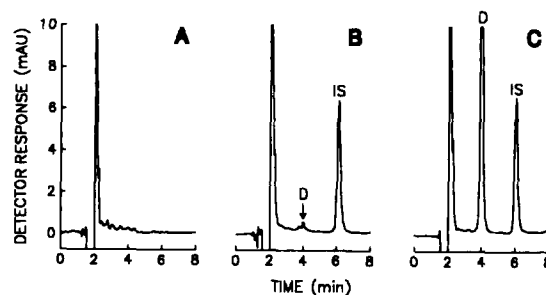

Fig. 2. Representative liquid chromatograms of drug-free mouse plasma assayed without the internal standard (A) and standard solutions of SarCNU in mouse plasma at concentrations of 0.10 μg/ml (B) and 5.10 μg/ml (C). Samples were prepared for analysis and separated as described under experimental Sections 2.3 and 2.4. Peak identification: D, SarCNU; I.S., internal standard (ProCNU).

present in the plasma extracts [13]. The time required for the drug and I.S. to completely elute from the precolumn onto the analytical column was 1.5 min. The switching valve was then activated to allow the precolumn to be flushed without interrupting flow of the mobile phase to the analytical column. The normal path of the mobile phase was restored following elution of the I.S. from the analytical column. Since equilibrium of the chromatographic system was re-established within 3 min, the total run time for a single sample was 11 min, thereby allowing more than 100 samples to be analyzed during a single day using an autosampler for overnight injection.

The analysis of plasma specimens acquired from ten human donors by this procedure showed several prominent chromatographic peaks eluting prior to the I.S., which were absent in chromatograms of mouse or dog plasma. However, as illustrated in Fig. 3, both SarCNU and the I.S. were adequately resolved from these endogenous components. The separation was improved markedly by decreasing the flow-rate of the mobile phase to 0.6 ml/min, whereupon SarCNU and the I.S. eluted at approximately 5.8 and 9.1 min, respectively. Furthermore, unlike mouse and dog plasma, it was found that utilization of the switching valve was unnecessary for assaying human plasma samples.

3.2. Standard curves and assay validation

The SarCNU to I.S. chromatographic peak height ratio was directly proportional to the added concentration of drug in mouse plasma over a 50-fold range from 0.10 to 5.1 μg/ml. Residual analysis revealed that the reciprocal of the observed peak height ratio was the optimal weighting factor for fitting the standard curves by linear regression. Values (mean $\pm$ S.D.) of the regression parameters for 27 standard curves of SarCNU in mouse plasma, assayed over an 8 week period, were: slope, 0.6196 ± 0.0573 ml/μg; y-intercept, -0.00404 ± 0.014; correlation coefficient, 0.9997 ± 0.0003. The y-intercept did not deviate significantly from the origin and the R.S.D. of the averaged slopes was 9.3%, indicative of the consistency of the assay over an extended period of time. The plasma standard with the lowest drug concentration included in the calibration curves, 0.10 μg/ml, was quantified with an R.S.D. of 7.8%. The R.S.D. for replicate determination of standard solutions with added drug concentrations of 0.26–5.1 μg/ml was 1.3–4.7%. As summarized in Table 1, the mean predicted concentration of SarCNU in the plasma standards was independent of concentration and ranged from 96.4 to 103.0% of the known drug concentration with an aggregate mean value of $100.1 \pm 1.7\%$ (standard error, $n = 9$). Accordingly, the analytical method is considered accurate and reproducible for quantifying SarCNU in 50 μl of plasma at concentrations of 0.10 to 5.1 μg/ml.

3.3. Extraction efficiency

Fig. 3. Chromatograms of drug-free human plasma prepared for analysis without the internal standard (A) and human plasma containing 1.0 μg/ml SarCNU (B). The separation was performed as described in Section 2.4, at a flow-rate of 0.6 ml/min, without the switching valve. Peak assignments are as indicated in Fig. 2.

SarCNU was efficiently isolated from mouse plasma by liquid–liquid extraction with TBME throughout the range of concentrations included in the standard curve (Table 1). Values of the absolute recovery (mean $\pm$ S.D., $n = 5$) determined at six individual concentrations ranged from $89.1 \pm 4.8\%$ to $96.6 \pm 2.5\%$ with an aggregate mean value of $93.6 \pm 4.0\%$ ($n = 29$). Absolute recovery of the I.S.,
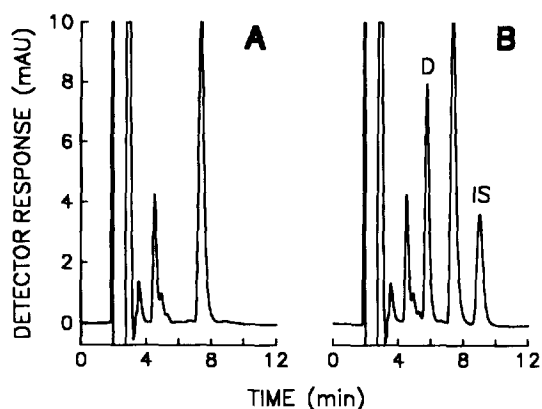
Table 1
Accuracy, precision and absolute recovery of SarCNU from mouse plasma

Amount added (μg/ml)	Accuracy [a] (%)	Precision [a] (%)	Absolute recovery [b] (%)
0.10	103.0	7.8	96.6±4.9
0.25	96.4	3.2	89.1±4.8
0.51	100.0	4.7	94.8±2.6
1.02	100.8	2.8	93.3±3.5
3.06	99.6	1.8	95.4±2.4
5.10	100.2	1.8	92.1±1.8

[a] Accuracy and precision of the assay were assessed from 27 standard curves of SarCNU in plasma analyzed over an eight-week period, as described in Section 2.6.
[b] Tabulated values are the mean ± S.D. of five replicate determinations performed on a single day; exception, n = 4 for the 1.02 μg/ml concentration.

88.2±3.0 (mean ± S.D., n = 30), was not significantly different from that of the drug.

3.4. Stability of SarCNU in buffers and plasma

The degradation of SarCNU in buffer solutions exhibited pseudo first-order kinetics as indicated by linear semi-log plots of the chromatographic peak height of SarCNU as a function of time (not shown). In the pH range of 2.23 to 7.57, the k_{obs} values exhibited a simple U-shaped functional dependence upon hydrogen ion activity (Fig. 4). Accordingly, the equation [14],

$$k_{obs} = k_1 a_{H+} + k_2 + k_3 a_{OH-} \qquad (2)$$

was fit to the experimental data by non-linear regression, affording values of 4.85 $M^{-1}h^{-1}$ and 1.73 × 10^4 $M^{-1}h^{-1}$ for the apparent second-order rate constants for hydronium (k_1) and hydroxyl (k_3) ion catalyzed degradation, respectively. The first-order rate constant for the uncatalyzed reaction (k_2) was 1.92 × 10^{-2} h^{-1}. The minimum in the pH-rate profile calculated from these values of the rate constants occurred at pH 5.22. Thus, at the pH of maximum stability, the predicted time for 5% degradation of the drug was 2.7 h at ambient temperature. During a period of 23.3 h, the concentration of SarCNU decreased by 4.37% and the I.S. decayed by 5.03%, when solutions in ammonium acetate buffer (0.05 M, pH 5.0) were maintained at 6–10°C within the sample compartment of the autoinjector.

Apparent first-order kinetics were also observed for the loss of SarCNU in fresh mouse plasma. At a given temperature, k_{obs} values, determined in solutions with initial drug concentrations of 5 to 25 μg/ml were highly consistent (Table 2). The $t_{1/2}$ (harmonic mean ± S.D.) of SarCNU ranged from
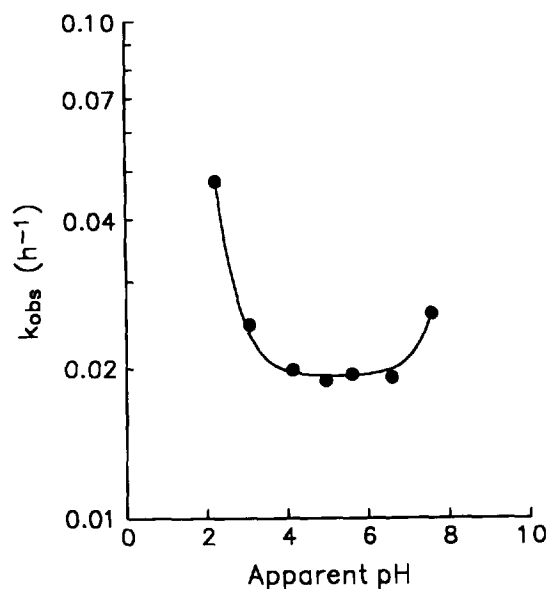

Fig. 4. pH–rate profile for the degradation of SarCNU in aqueous solution containing 0.05 M buffer at ambient temperature (22±1°C). The solid curve was generated from the non-linear regression fit of Eq. 2 to the experimental values (points).

Table 2

Apparent first-order rate constants for SarCNU degradation in fresh mouse plasma

Temperature (°C)	C(0) [a] (μg/ml)	$10^2 \ k_{obs}$ (h^{-1})	
		Individual	Mean
5.0	5.0	0.37	0.35
(0.1)[b]	10.0	0.34	(0.02)[b]
	25.0	0.33	
15.0	5.0	1.32	1.27
(0.1)	10.0	1.21	(0.04)
	25.0	1.27	
26.6	5.0	5.47	5.44
(0.4)	10.0	5.37	(0.05)
	25.0	5.49	
37.3	5.0	26.35	25.80
(0.4)	10.0	26.75	(1.31)
	25.0	24.31	

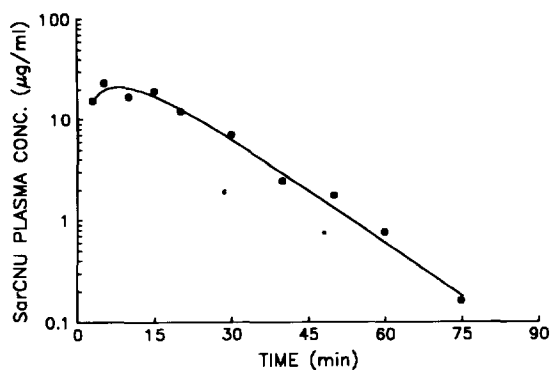[a]Initial SarCNU concentration.
[b]Numbers in parentheses, S.D.

Fig. 5. Plasma concentration–time profile of SarCNU in mice after intraperitoneal administration of a 25.6 mg/kg dose. The plot shows the experimental geometric mean plasma concentrations of five mice per time point (●) and the best-fit line generated by non-linear regression analysis.

200 ± 12 h at 5.0°C to 2.69 ± 0.14 h at 37.3°C. When solutions of SarCNU in fresh mouse plasma were stored at -69 ± 1°C, $4.2 \pm 1.8\%$ (S.D., $n = 3$) of the drug degraded after 3.2 days, and the amount remaining after 7.2 days was only $87.7 \pm 2.9\%$ of the initial concentration (9.9 μg/ml).

3.5. Plasma pharmacokinetics of SarCNU in mice

Although soluble in aqueous vehicles, SarCNU was not sufficiently stable to allow a single dosing solution to be used for an entire day (see Section 3.4). Since solutions in DMSO kept at ambient temperature for 8 h showed no evidence of degradation, the drug was formulated in this vehicle to deliver the intended dose in a volume (1.0 μl/g body weight) that could be given safely to mice [15,16]. A single dose of 25.6 mg/kg SarCNU administered by intraperitoneal injection appeared to be well tolerated. The animals exhibited no outward symptoms of toxicity during a 24 h observation period. Liquid chromatograms determined with UV detection at 240 nm of plasma samples from treated mice showed no peaks consistent with drug metabolites (not shown). The assay was sufficiently sensitive to quantitate SarCNU in plasma specimens obtained at 3 min (15.3 μg/ml) and permit drug levels to be monitored for 75 min after dosing. The time course

of observed SarCNU geometric mean plasma concentrations and the best-fit curve determined by non-linear regression analysis are presented in Fig. 5. The plasma profile was characterized by an initial region where the drug concentration increased in an apparent first-order manner ($t_{1/2} = 3.8$ min) to a 21.2 μg/ml peak at 8.0 min. Plasma levels subsequently declined mono-exponentially with an 8.7 min half-life. The mean residence time of the drug in the body was 18.0 min.

3.6. Specificity of the analytical method

Thermospray-ionization mass spectrometry (TSP-MS) was employed to confirm the identity of the peak that possessed the same k' as authentic SarCNU in chromatograms of plasma acquired from mice following drug administration. The partial positive ion mass spectrum (150–300 u) acquired during chromatographic analysis of a 1-μg reference sample of SarCNU is shown in Fig. 6B. The salient features of the spectrum include abundant ion peaks at m/z 158, 194, 223 and 240. There were no significant ions in the region from m/z 300–500. Identification of the ions at m/z 223 and 240 as the respective $[M + H]^+$ and $[M + NH_4]^+$ adducts was supported by the presence of peaks at m/z 225 and 242, in abundance ratios characteristic of a single chlorine atom. The peak at m/z 194 is consistent with the species resulting from denitrosation of the
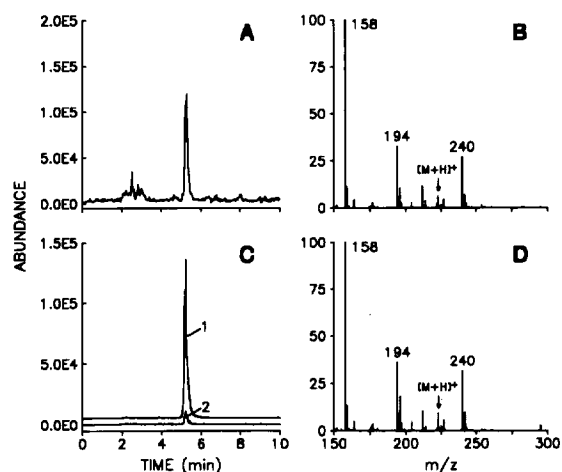
Fig. 6. (A) Total ion liquid chromatogram of a 1-μg sample of SarCNU determined by filament-assisted TSP-MS detection with continuous scanning over the mass range 140–500 u in the positive ion mode. (B) Averaged TSP mass spectrum acquired across the chromatographic peak eluting at 5.3 min in panel A. (C) Liquid chromatograms of plasma specimens collected from mice 15 min (1) and 60 min (2) after 26 mg/kg of SarCNU were given by intraperitoneal injection. TSP–MS detection was performed in the SIM mode by monitoring the positive ions at m/z 223 and 240 sequentially. (D) Averaged TSP mass spectrum measured with positive ion mass scanning detection (140–500 u) of the peak at 5.3 min in the chromatogram of plasma collected from a mouse 15 min after dosing with SarCNU. Chromatography was performed at 25°C on a 4 μm Nova-Pak C_{18} column (15 cm × 3.9 mm) eluted with methanol–0.10 M ammonium formate buffer, pH 3.7 (25:75, v/v) at a flow-rate of 0.7 ml/min, without utilization of a switching valve.

parent molecule. Additional fragmentation of this product ion via dehydrohalogenation would afford the base peak observed at m/z 158. Examination of plasma samples acquired from mice 15 and 60 min after dosing with SarCNU, using SIM at m/z 223 and 240 to enhance sensitivity as well as selectivity, clearly showed chromatographic bands eluting with the same t_R as authentic SarCNU (Fig. 6C). The mass spectra acquired on the up-slope, apex and down-slope of the peak, in the specimen collected 15 min after dosing, were comparable to authentic SarCNU (Fig. 6D). Furthermore, sample concentrations estimated from the TSP-MS response, 20.7 μg/ml at 15 min and 1.6 μg/ml at 60 min, were in excellent agreement with the values determined by UV detection.

4. Discussion

Difficulties encountered in establishing suitable methodology for the specific quantitation of CENU antineoplastic agents in biological fluids have significantly encumbered the acquisition of reliable pharmacokinetic data for these drugs [4,17,18]. The CENUs are inherently unstable in aqueous solution at physiological pH and interactions with protein may accelerate their decomposition in plasma [1,2,4,19]. Thus, the potential for degradative loss of drug during sample collection, storage and preparation for analysis are paramount considerations in assessing the accuracy and precision of results derived from any procedure for assaying these compounds [18,19]. Moreover, the sensitivity required to adequately define plasma concentration-time profiles of unchanged drug is dictated by the rapid systemic clearance characteristic of the CENUs coupled with the relatively low peak concentrations afforded by therapeutic doses [4]. Although a number of chromatographic methods have been reported that are sufficiently sensitive for characterizing CENU plasma pharmacokinetics [4,17–31], issues pertaining to sample stabilization have been almost entirely ignored [18]. Furthermore, as described later, assay specificity has been compromised to facilitate analysis by GC, whereas LC-based procedures have been utilized without substantive physicochemical evidence to verify that the parent drug is the actual species being measured.

Although direct GC analysis of CENU compounds is precluded by their characteristic thermal lability [4], several reactions that afford derivatives possessing physicochemical properties compatible with GC have been identified. The procedure based upon conversion to an O-methylcarbamate [20] is not applicable to SarCNU because, unlike a 1,3-dialkyl-1-nitrosourea, its decomposition under basic conditions will not afford the requisite isocyanate due to the presence of a methyl group at N-3 [7]. Alternatively, substitution of the nitroso group with a trifluoroacetyl moiety may be accomplished by the action of trifluoroacetic anhydride [17]. We found that SarCNU reacted similarly and that the resulting trifluoroacetyl derivative was amenable to GC separation. Whereas mass scanning detection (EI) revealed the presence of multiple chromatographic

peaks, presumably arising from incomplete reaction or degradation of the compound during the derivatization procedure [17], chromatograms acquired with SIM at m/z 202 showed a single peak for the derivative. Nevertheless, this reaction yields the same derivative for the parent drug as its potential denitrosated metabolite, thereby necessitating a preliminary separation step to enhance specificity [17].

An assay for SarCNU based upon HPLC was subsequently pursued with the objective of developing a method with absolute specificity for the parent drug and greater technical convenience than the GC procedures. Isocratic reversed-phase HPLC with UV detection has been employed for the quantitative determination of BCNU and several other CENUs in plasma [18,19,22–31]. The principal chromophore in these compounds is typically the N-nitrosourea group, which exhibits an absorption band in the region of 230–240 nm with a molar absorptivity of approximately 6000 $M^{-1}cm^{-1}$ in aqueous solution [1]. Restricted to detection at a low intensity, short wavelength band, most of the reported methods use a sample volume of approximately 1 ml to achieve the sensitivity necessary to measure CENU plasma concentrations following the administration of therapeutic doses [18,22,23,25,26,29–31]. Although suitable for clinical drug level monitoring, such a large sample volume does not accommodate the analysis of plasma from an individual mouse or serial specimens from a rat or dog. With regard to the present method, the sample volume was restricted to 50 μl, since it was being developed specifically for an investigation of the pharmacokinetic behavior of SarCNU in small animals.

Extraction with an immiscible organic solvent has been commonly used to isolate CENUs from plasma prior to chromatographic analysis [17,19,22,24,26, 28,31]. Similarly, we found that SarCNU could be efficiently extracted from plasma by methylene chloride, ethyl acetate or TBME, although TBME afforded markedly cleaner liquid chromatograms of plasma extracts. On evaporation of the solvent, the drug was quantitatively recovered by reconstituting the extract in the aqueous component of the mobile phase. Volumes of this solution as large as 250 μl could be loaded onto the analytical column, without adversely affecting the separation. A variety of bonded stationary phases were evaluated with eluents composed of methanol or acetonitrile and ammonium formate, ammonium acetate or potassium phosphate buffers ranging from pH 3.0 to 7.2. The best separation of SarCNU and the I.S. from each other and extractable endogenous components present in mouse, dog and human plasma was achieved on a Nova-Pak C_{18} column (4 μm, 15 cm × 3.9 mm) using a mobile phase composed of methanol–0.1 M ammonium formate buffer, pH 3.7 (25:75, v/v). UV absorption of the column effluent was monitored at 240 nm, the wavelength of maximum absorbance of SarCNU in the mobile phase.

During the course of these studies, it became apparent that the drug and I.S. were unstable in the presence of the mobile phase buffer at ambient temperature. Thus, the influence of pH on SarCNU stability in aqueous solution was evaluated to identify conditions that would enable the use of an automatic injector for unattended sample analysis. As observed with other CENUs [19], SarCNU was most stable over a relatively broad pH range that extended from approximately 4 to 6. Beyond this pH-independent region, it was subject to specific acid and base catalyzed decomposition in solutions of lower and higher pH, respectively. Whereas the time for 5% degradation of SarCNU at pH 4.93 (0.05 M ammonium acetate buffer) and 25.1°C was only 2.27±0.03 h (mean ± S.D., n = 3; data not shown), maintaining solutions of the drug and I.S. in this same buffer, but at 6–10°C, diminished their rates of decomposition sufficiently to allow samples to be injected for at least 18 h after reconstituting.

Information on the stability of SarCNU in plasma was required to establish validated protocols for sample acquisition, storage and processing during pharmacokinetic studies. As observed with other CENUs [4], SarCNU degraded more rapidly in fresh plasma ($t_{1/2}$ = 2.69±0.14 h) than in pH 7.4 buffer at 37°C ($t_{1/2}$ = 5.5 h) [7]. Nevertheless, SarCNU was stable enough to allow plasma to be separated from whole blood without preliminary temperature reduction [18,22,24–26] or pH adjustment [27,32,33] for enhanced stability, which could alter the drug plasma concentration. Specifically, a negligible amount (*i.e.*, ≤2.1%) of the drug will be lost to degradation if the time from the beginning of sample collection to freezing the separated plasma does not exceed 5 min.

This can be readily accomplished by sequentially collecting blood directly into a heparinized micro-centrifuge tube, immediately spinning the sample in a standard microcentrifuge (~ 10 000 g) for 2 min, and flash freezing the plasma upon separation from the blood cells. In our experience, plasma processed from blood in this manner shows no evidence of erythrocyte disruption. We found that plasma specimens could be kept for only three days at −70°C before the extent of degradation became significant. Furthermore, frozen plasma specimens were individually thawed for extraction within 5 min by placing the sample tube in a constant temperature bath at approximately 40°C, thereby preventing the occurrence of significant degradation of the drug, as previously commented upon [18]. The extraction procedure, which is performed in only 1 min, was also devised to minimize the possibility of degradation. In contrast to the instability of the drug in plasma, organic extracts of plasma specimens can be stored at −20°C for at least several weeks before, or after, removal of the solvent.

ProCNU, an N-3 disubstituted structural congener of SarCNU, was selected as the I.S. for the assay based upon its chromatographic behavior and similar physicochemical properties as the drug. The half-lives of the two compounds in aqueous solution under physiological conditions are not significantly different [7]. Structurally related compounds have been used infrequently as internal standards in HPLC methods for the analysis of CENUs. A chemically stable I.S. enhances the precision of quantitation by facilitating compensation for variability introduced during the preparation of samples for analysis and their injection into the chromatograph. In addition to this function, an I.S. with stability characteristics similar to the analyte makes it possible to assess whether or not conditions of stability are maintained from the point at which it is added to the sample. This is particularly important during unattended overnight analysis of potentially unstable samples. Evidence of degradation during workup and subsequent chromatographic analysis of a series of samples can be readily discerned by inconsistency or a temporal decrease in the magnitude of chromatographic peak for the I.S.

In summary, a procedure based upon isocratic reversed-phase HPLC has been developed for the determination of SarCNU in plasma. The preparation of samples for analysis simply involves a single extraction with TBME. Following removal of the organic solvent, the reconstituted sample was directly analyzed by liquid chromatography with UV detection at 240 nm. Approximately 1000 biological samples have been run on a single analytical column without significant degradation in its performance. Employing a sample volume of 50 µl to allow replicate determinations of plasma obtained from a single mouse, the lowest concentration of SarCNU quantified with acceptable day-to-day precision was 0.10 µg/ml. This degree of sensitivity permitted the plasma concentration–time profile of intact drug to be definitively characterized in mice following intra-peritoneal administration of a 25.6 mg/kg dose, which is less than one-tenth of the reported value of its median lethal dose [7]. Specificity of the method for the determination of SarCNU was conclusively established through the analysis of plasma specimens, acquired from mice treated with the drug by HPLC with TSP–MS detection. The assay has been thoroughly validated and shown to be accurate and reproducible. The ease of sample preparation and utilization of commonly available chromatographic instrumentation renders the procedure well-suited for monitoring drug levels during preclinical and clinical pharmacokinetic studies.

Acknowledgments

References

[1] W. Lijinsky, Chemistry and Biology of N-Nitroso Compounds, University Press, Cambridge, 1992, pp. 1–18, 60–63.
[2] M. Colvin and B.A. Chabner, in B.A. Chabner and J.M. Collins (Editors), Cancer Chemotherapy: Principles and Practice, Lippincott Company, Philadelphia, PA, 1990, pp. 282–284.
[3] R.W. Weiss and B.F. Issell, Cancer Treat. Rep., 9 (1982) 313–330.

[4] R.J. Weinkam and H.S. Lin, Adv. Pharmacol. Chemother., 19 (1982) 1–33.

[5] W. Stahl and G. Eisenbrand, Free Rad. Res. Commun., 14 (1991) 271–278.

[6] V. Skalski, W. Feindel and L.C. Panasci, Cancer Res., 50 (1990) 3062–3066.

[7] T. Suami, T. Kato, H. Takino and T. Hisamatsu, J. Med. Chem., 25 (1982) 829–832.

[8] L. Panasci, M. Dufour, L. Chevalier, C. Isabel, P. Lazarus, A. McQuillan, E. Arbit, S. Brem and W. Feindel, Cancer Chemother. Pharmacol., 14 (1985) 156–159.

[9] V. Skalski, J. Rivas, L. Panasci, A. McQuillan and W. Feindel, Cancer Chemother. Pharmacol., 22 (1988) 137–140.

[10] A.H. Kaye and J.D. Laidlaw, Curr. Opin. Neurol. Neurosurg., 5 (1992) 526–533.

[11] H.S. Harned and B.B. Owens, in The Physical Chemistry of Electrolytic Solutions, Reinhold, New York, 3rd ed., 1958, p. 645.

[12] R.E. Notari, in Biopharmaceutics and Clinical Pharmacokinetics, Marcel Dekker, New York, 4th ed., 1987, p. 181.

[13] P. Campanis-Falco, R. Herraez-Hernandez and A. Sevillino-Cabeza, J. Chromatogr., 619 (1993) 177–190.

[14] K.A. Connors, G.L. Amidon and V.J. Stella, in Chemical Stability of Pharmaceuticals, John Wiley, New York, 2nd ed., 1986, pp. 43–47, 307–305.

[15] H. Rosenkrantz, Z. Hadidian, H. Seay and M.M. Mason, Cancer Chemother. Rep., 31 (1963) 7–24.

[16] J.E. Willson, D.E. Brown and E.K. Timmens, Toxicol. Appl. Pharmacol., 7 (1965) 104–112.

[17] R.G. Smith, S.C. Blackstock, L.K. Cheung and T.L. Loo, Anal. Chem., 53 (1981) 1205–1208.

[18] B.H. Gordon, R.P. Richard, M.P. Hiley, A.J. Gray, R.M.J. Ings and D.B. Campbell, Xenobiotica, 19 (1989) 329–339.

[19] S. Eksborg and H. Ehrsson, J. Chromatogr., 340 (1985) 31–72.

[20] R.J. Weinkam and T.J.Y. Liu, J. Pharm. Sci., 71 (1982) 153–157.

[21] B. Caddy and O.R. Idowu, Analyst, 107 (1982) 550–555.

[22] R. Lemoine and A. Gouyette, Cancer Treat. Rep., 63 (1979) 1335–1341.

[23] I.S. Krull, J. Strauss, F. Hochberg and N.T. Zervas, J. Anal. Toxicol., 5 (1981) 42–46.

[24] F.Y.F. Lee and P. Workman, Br. J. Cancer, 47 (1983) 659–669.

[25] W.D. Henner, W.P. Peters, J.P. Eder, K. Antman, L. Schnipper and E. Frei III, Cancer Treat. Rep., 70 (1986) 877–880.

[26] J. Polacek, P.O. Gunnarsson and S. Brandin, J. Chromatogr., 425 (1988) 424–428.

[27] J.A. Double, M.C. Bibby, P.M. Loadman and J.C. Bloomer, Eur. J. Cancer Clin. Oncol., 24 (1988) 1355–1360.

[28] A. Meulemans, B. Giroux, P. Hannoun, D. Henzel, J.P. Bizzari and J. Mohler, Chemotherapy, 35 (1989) 313–319.

[29] K. Wong, C.A. Wallen and K.T. Wheeler, Int. J. Radiat. Oncol. Biol. Phys., 17 (1989) 135–143.

[30] J. Polacek, B. Gustafsson, S. Brandin and A. Ottersgard-Brorsson, J. Chromatogr., 526 (1990) 151–157.

[31] I. El Abed, P. Roger, C. Gosse, G. Atassi and A. Gouyette, Cancer Chemother. Pharmacol., 27 (1991) 295–300.

[32] I. Bartosek, R.G. Russo, M.T. Cattaneo, Tumori, 70 (1984) 491–498.

[33] A. Guaitani, M. Corada, C. Lucas, A. Lemoine, S. Garattini and I. Bartosek, Cancer Chemother. Pharmacol., 28 (1991) 293–297.