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Research Article

Capillary Gas Chromatography and Thermionic N–P-Specific Detection of 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) or 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in Stability and Pharmacokinetic Studies

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An expedient, rapid, and sensitive capillary gas chromatographic method for the analysis of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in plasma is described. Separation of the underivatized nitrosourea compounds was performed on a 0.33-mm-i.d., 25-m fused-silica, SE-30 capillary column, and detection was carried out using a thermionic N–P-specific detector. The compounds were extracted from plasma with benzene with a yield of >87%. The assay was linear in the ranges of 0.001 to 0.5 and 0.5 to 25 μg/ml for CCNU or 0.003 to 0.50 and 0.5 to 25 μg/ml for BCNU, with correlation coefficients from 0.9914 to 0.9999 and coefficients of variation (CV) of <3.3%. Other antineoplastic agents did not interfere in the assay. The method was employed to study the pharmacokinetics of BCNU in rabbits. The plasma concentration–time curves were fit to a two-compartment model with a mean (SE) α, β, and total-body clearance of 2.898 (0.913) hr^{-1}, 0.1228 (0.0179) hr^{-1}, and 7.211 (2.862) liters/hr · kg, respectively. Further, the stability of BCNU and CCNU in solution was examined at different temperatures. Both compounds were stable in benzene or acetone (4 to 37°C) but labile in plasma even if refrigerated. The apparent rate constants for degradation of BCNU and CCNU were 0.09921 and 0.02853 hr^{-1} at 4°C and 5.998 and 2.553 hr^{-1} at 37°C, respectively.

KEY WORDS: capillary gas chromatography; analysis; stability; pharmacokinetics; carmustine; lomustine.

INTRODUCTION

BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] or carmustine (I) and CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] or lomustine (II) are drugs used in the treatment of brain tumors, lymphomas including Hodgkin's disease, myeloma, lung cancer, and melanoma (1). Because of their high lipid solubility and small molecular weight, these drugs can penetrate the blood–brain barrier and exhibit their cytotoxic activities on malignant brain cells; however, their chemical stability is limited.

Recently, more aggressive high-dose (2–5) chemotherapy including intracarotid administration of I and II has been employed (6–8). This modality, however, is of high risk, which makes it important to monitor these agents in cancer patients. Several techniques have been utilized for the analysis of I and II in plasma and other biological samples. These comprise spectrophotometry (9,10), gas chromatography–mass spectrometry (11,12), pulse polarography (13), chemical ionization–mass spectrometry (14,15), thin-layer chromatography of radiolabeled entities (16), and high-performance liquid chromatography with ultraviolet (17,18) or electrochemical (18) detection. The use of capillary gas chromatography with thermionic nitrogen–phosphorus-specific detection has not been previously described.

In this report, a simple, accurate, and rapid gas chromatographic assay is presented. The use of thermionic nitrogen–phosphorus-specific detection makes this assay highly sensitive, with a minimum analyzable limit matched only by mass spectrometry. The described assay was utilized to study the stability of both drugs and the pharmacokinetics of BCNU using the rabbit as an *in vivo* model.

MATERIALS AND METHODS

Materials. Analytical samples of BCNU and CCNU were kindly provided by Laboratoire Roger Bellon (Neuilly-Paris, France). Methotrexate (American Cyanamid Co., Pearl River, N.Y.), vincristine-sulfate (Eli Lilly & Co., Indianapolis, Ind.), etoposide (Mead Johnson, Evansville, Ind.), doxorubicin-HCl (Farmitalia Carlo Erba, Milan, Italy), *cis*-platinum (Sandoz Ltd., Basel, Switzerland), bleomycin-sulfate (Bristol Laboratories, Syracuse, N.Y.), cytarabine (Upjohn Co., Kalamazoo, Mich.), prednisone (Philips Roxane Laboratories, Columbus, Ohio), and acetaminophen

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(McNeil Laboratories, Fort Washington, Pa) were either reagent or pharmaceutical grade. Benzene and acetone (Fisher Scientific Co., Fair Lawn, N.Y.) were high-performance liquid chromatographic (HPLC) grade. Acetone was doubly distilled before use.

Animals. Three mature New Zealand albino rabbits weighing 5.1, 5.0, and 4.9 kg were used in this study. Water was offered *ad libitum* for the duration of the experiment. Intravenous infusion and blood collection were performed via intravenous catheters inserted in the marginal ear vein of both ears (19). The dose, 6 mg/kg of I, was infused at a constant rate over 45 min using a variable-speed syringe-type infusion pump (Sage Instruments, Cambridge, Mass.). The blood (1–1.1 ml) was sampled frequently over 8 hr, and the plasma was immediately harvested after centrifugation at 1746*g* and was stored frozen at −20°C until analysis.

Instrumentation. A Vista 6000 gas chromatograph (Varian Co., Palo Alto, Calif.) equipped with a split/splitless capillary injector, a thermionic nitrogen–phosphorus-specific detector, and a Vista 402 data module was employed. The chromatography was performed on a 25-m, 0.33-mm-i.d., fused-silica SE-30 capillary column (Varian Co., Palo Alto, Calif.).

Chromatographic Conditions. The temperatures of the injection port and the detector were set at 200 and 300°C, respectively. To ensure full separation of the compounds, the temperature of the oven was time-programmed. Initially, a temperature of 35°C was held for 5.5 min, then raised at a rate of 5°C/min to 45°C. This temperature was held for 0.1 min, then raised at a rate of 0.2°C/min to 50°C, which was maintained for the remainder of the run. The carrier gas was nitrogen (flow rate, 2.57 ml/min), and the detector gases were hydrogen and air at flow rates of 4 and 175 ml/min, respectively.

Extraction of Plasma Samples. The extraction was performed by adding 6 ml of benzene to the plasma sample (0.5 to 1 ml) and vortex-mixing for 1 min. After the tube was centrifuged for 5 min at 1746*g*, the benzene layer was transferred to a clean tube and brought to dryness under a gentle stream of nitrogen gas. The residue was reconstituted with 35 μl of doubly distilled acetone by sonication for 3 min, and 1 to 2 μl of the solution was injected.

Calibratión Curves. Stock solutions of appropriate concentrations of I and II in doubly distilled acetone were prepared to construct calibration curves for both I and II at low and high ranges. For I, the calibration curves were established by supplementing 1-ml portions of blank human plasma with appropriate amounts of I to yield concentrations in the ranges of 0.003 to 0.5 μg/ml (low) and 0.5 to 25 μg/ml (high). To each tube either 0.03 μg (low range) or 8 μg (high) of II (internal standard) was added and the sample was brought to a final volume of 1.13 or 1.285 ml with water for the low and high ranges, respectively. For II, the internal standard used was I and the calibration curves were constructed in a similar fashion. The amount of I added as an internal standard was 0.070 μg for low range (0.001 to 0.5 μg/ml) and 5 μg for the high range (0.5 to 25 μg/ml), and the final volumes were 1.17 and 1.175 ml, respectively. After vortex-mixing for 30 sec, the samples were extracted and analyzed as described above.

Analysis of Rabbit Plasma Samples. The plasma samples (0.5 ml) collected at different time intervals from the three rabbits used for the pharmacokinetics study were anlayzed as described above after the addition of the appropriate amount of II as internal standard.

Stability Study. The stability of I and II in plasma at 4 and 37°C was studied over periods of 6 and 1.5 hr, respectively. Initially, sampling was carried out at both temperatures for 6 hr. However, because of rapid degradation observed at 37°C, sampling at this temperature was terminated after 1.5 hr. In these experiments, samples supplemented with 0.25 μg/ml of I or II were subjected to the described extraction procedure and the residue was reconstituted with 50 μl of 5 μg/ml solution of the respective internal standard (II or I) in doubly distilled acetone.

Also, the stability of I and II was examined in the organic solvents employed at the various stages of the analysis (i.e., benzene and doubly distilled actone) at 4°C (refrigerator) and 22°C (room temperature). Additionally, because of absolute stability observed at these temperatures, it was of interest to study the degradation of either one of these drugs (i.e., II) in benzene at higher temperature (viz. 45°C). In these experiments, 2 μl of a 25-μg/ml solution of I or II in benzene or acetone was withdrawn at appropriate intervals and injected into the column under the conditions described above. The concentrations were calculated using calibration curves.

Pharmacokinetic Calculations. The postinfusion plasma concentration vs time data obtained for I were fitted by nonlinear regression analysis to Eq. (1) (20). This equation describes the concentration (C) as a function of time (t) after cessation of infusion for drugs which exhibit the characteristics of the two-compartment model,

$$C = R\,e^{-\alpha \cdot t} + S\,e^{-\beta \cdot t} \qquad (1)$$

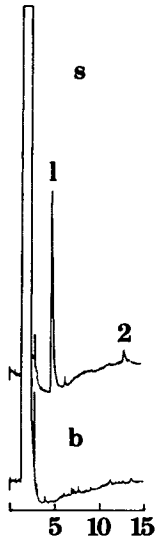

Fig. 1. Representative capillary gas chromatograms of an intact blank human plasma sample (b) and blank human plasma sample spiked with 0.07 μg/ml of I and 0.001 μg/ml of II (s), the sensitivity limit of the assay for II. 1, BCNU; 2, CCNU.

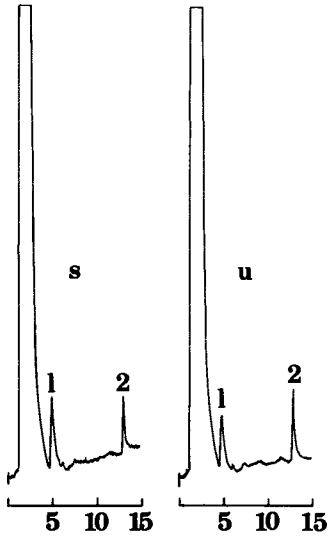

Fig. 2. Chromatograms of blank rabbit plasma sample spiked with 0.075 μg/0.5 ml of I and 0.015 μg/0.5 ml of II (s) and rabbit plasma sample collected 210 min after the termination of 45-min intravenous infusion of 6 mg/kg of I and spiked with 0.015 μg/0.5 ml of II (u). 1, BCNU; 2, CCNU.

where α and β are the apparent first-order rate constants for the distribution and postdistribution phases in the two-compartment open model, respectively. R and S are related to the preexponential coefficients A and B for distribution and postdistribution phases in the two-compartment open model according to the following equations:

$$A = R \cdot \alpha \cdot T \quad (2)$$

and

$$B = S \cdot \beta \cdot T \quad (3)$$

where T is the infusion time and is equal to 0.45 hr in this study. The values of R, α, S, and β generated from the fit were substituted in Eqs. (2) and (3) and estimates for A and B were computed. With the aid of a computer program, the values of A, α, B, and β were used in standard equations (20) and the apparent volume of the central compartment (V_c), apparent beta-phase distribution volume (V-beta), rate constant for transfer of drug from the central compartment to the peripheral compartment (k_{12}), rate constant for transfer of drug from the peripheral compartment to the central compartment (k_{21}), rate constant for elimination of drug from the central compartment (K_{10}), and total-body clearance (TBC) of I were computed.

RESULTS AND DISCUSSION

Most of the gas chromatographic methods reported for the analysis of nitrosourea compounds (11,12) involve a derivatization followed by a conventional gas chromatography. As demonstrated in this report, this step can be eliminated by the use of a fused-silica, SE-30 capillary column. As demonstrated in Figs. 1, 2, and 4, the peaks generated for the underivatized I and II were sharp, as is typical of capillary gas chromatography. The retention times for I and II were 4.7 and 12.8 min, respectively.

Table I. Extraction Efficiency (%)

Drug	Concentration (μg/ml) 0.01	0.5	10
BCNU	96.9	86.5	88.5
CCNU	91.7	92.2	94.8

The one-step extraction of the sample with benzene yielded clean chromatograms (Figs. 1, 2, and 4) and reduced the analysis time. The extraction efficiency was examined by supplementing 1-ml portions of plasma with 0.01, 0.5, and 10 μg/ml of I or II and performing the analysis after the addition of the respective internal standard. The peak height drug/internal standard ratio obtained at each concentration was divided by the peak height ratio obtained from injecting acetone solutions containing the same amounts of drug and internal standard. The extraction efficiency at different concentrations is presented in Table I. The values obtained are higher than or similar to those previously reported (11–18) for I or II.

The analytical recovery and intrarun precision of the assay were investigated at 0.01, 0.25, and 10 μg/ml of I or II, which represent low, medium, and high concentrations. To 1-ml portions of blank plasma appropriate amounts of I or II were added to yield the above concentrations, and the analysis was performed as described after the addition of the respective internal standard. The percentage analytical recovery at each concentration was calculated as 100 × amount found/amount added and is presented along with the coefficient of variations (CV) for I or II in Table II. As demonstrated in this table, the values of the analytical recovery ranged between 99 and 107%, which signifies a good assay accuracy.

As the plasma concentrations of I or II in patients treated with these agents in a single or combined chemotherapeutic modality extend over a wide range, calibration curves in low and high ranges had to be constructed and employed. The drug/internal standard peak height ratios were plotted against concentrations and the slope, intercept, and correlation coefficient (r) for each curve was calculated by linear regression analysis. As shown in Table III, the cor-

Table II. Intrarun Precision and Accuracy of the Described Method

Drug	Amount added (μg/ml)	Amount found (μg/ml)	Coefficient of variation (%)	Number of experiments	Analytical recovery (%)
BCNU	0.01	0.0985	2.2	8	98.5
	0.25	0.25	3.1	5	100.4
	10.0	10.97	3.0	4	99.7
CCNU	0.01	0.0938	2.8	7	93.8
	0.25	0.247	3.3	5	98.9
	10.00	10.44	3.1	4	100.4

Table III. Linearity of the Described Assay

Drug	Concentration range (μg/ml)	Mean (SD) of correlation coefficient	Range of correlation coefficient	Number of experiments
BCNU	0.003–0.5	0.9969 (0.0028)	0.9914–0.9998	13
	0.5–25	0.9951 (0.0034)	0.9914–0.9987	7
CCNU	0.001–0.5	0.9972 (0.0022)	0.9944–0.9999	7
	0.5–25	0.9969 (0.0029)	0.9929–0.9997	7

relation coefficient *(r)* for I or II at high and low ranges in no case was <0.9914, indicating a good assay linearity.

The minimum analyzable concentrations for I and II were 0.003 and 0.001 μg/ml, respectively. Figure 1 demonstrates a typical chromatogram of a human plasma sample supplemented with 0.001 μg/ml of II and 0.07 μg/ml of I (internal standard). These analyzable limits are lower than most of those reported (12,13,17,18) previously for I or II using different analytical techniques and are equivalent to that reported by Smith *et al.* (11), who employed mass spectrometry for detection.

The specificity of the assay was investigated by determining the retention times of anticancer drugs commonly used with I or II in combined chemotherapy. As demonstrated in Table IV, none of these drugs was detected under the described chromatographic conditions.

This method was employed to study the pharmacokinetics of I using the rabbit as an *in vivo* model. As mentioned above, a dose of 6 mg/kg of I was infused over 45 min, and blood was sampled from each rabbit at different intervals and analyzed according to the described procedure. A representative chromatogram of a sample collected from rabbit I 3.5 hr after the termination of the infusion is presented in Fig. 2. Semilogarithmic plots of the concentration–time data obtained for all three rabbits are provided in Fig. 3. As shown in this figure, the data fitted the two-compartment model well. Indeed, the sums of the square of the observed minus predicted residuals, as determined by nonlinear regression analysis, were randomly distributed and reasonably small.

The pharmacokinetic parameters for I obtained in each animal are presented in Table V. The mean (SE) apparent

Table IV. Specificity of the Assay

Drug	Retention time (min)
BCNU	4.7
CCNU	12.8
Etoposide	ND
Methotrexate	ND
Cyclophosphamide	ND
Adriamycin	ND
Cis-platinum	ND
Bleomycin	ND
Vincristine	ND
Cytarabine	ND
Prednisone	ND
Acetaminophen	ND

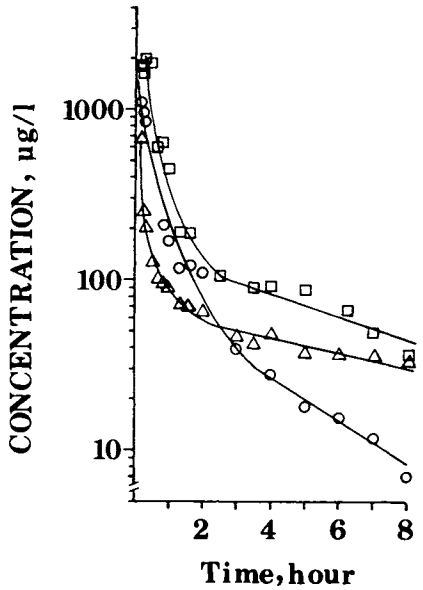

Fig. 3. Semilogarithmic plots of postinfusion BCNU plasma concentrations vs time acquired in the three rabbits used in this study. Animal I (□); animal II (○); animal III (△). See Table III for estimates of pharmacokinetic parameters.

first-order rate constants for the distribution (α) and postdistribution (β) phases were 2.898 (0.913) and 0.1228 (0.0179) hr^{-1}, respectively. The mean beta-phase volume of distribution, volume of distribution of the central compartment, and total-body clearance were 55.89 (15.38) liters/kg, 2.616 (0.505) liters/kg, and 7.211 (2.862) liters/hr · kg, respectively. k_{12} was 0.2588 (0.1860) hr^{-1}, k_{21} was 0.1341 (0.0248) hr^{-1}, and k_{10} was 2.628 (0.7199) hr^{-1}.

As described above, the stability of I and II was studied in plasma and two solvents (i.e., benzene and acetone) at 4°C (refrigeration), 22°C (ambient), and 37°C. These conditions are similar to those used in the assay. There was no change of the concentration of I or II in the organic solvents examined as a function of time at the above temperatures or at 45°C. However, their stability in plasma, particularly for I

Table V. Pharmacokinetic Parameters for BCNU Obtained in Rabbits Treated with 6 mg/kg of this Drug by Constant-Rate Intravenous Infusion

	Animal No.			
	I	II	III	Mean (SE)
α, hr^{-1}	2.082	1.891	4.720	2.898 (0.913)
β, hr^{-1}	0.1139	0.0972	0.1572	0.1228 (0.0179)
V-beta, liters/kg	28.0	58.57	81.09	55.89 (15.38)
V_c, liters/kg	1.606	3.106	3.136	2.616 (0.505)
k_{12}, hr^{-1}	0.0902	0.0554	0.6299	0.2588 (0.1860)
k_{21}, hr^{-1}	0.1194	0.1003	0.1825	0.1341 (0.0248)
k_{10}, hr^{-1}	1.986	1.833	4.065	2.628 (0.7199)
TBC, liters/hr · kg	3.191	5.692	12.75	7.211 (2.862)

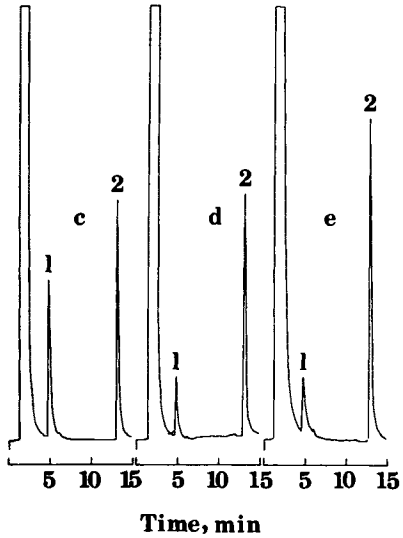

Fig. 4. Typical chromatograms of human plasma samples spiked with 0.25 μg/ml of I and the extract residues reconstituted in 50 μl of 5 ng/μl of II in acetone prior to injection into the capillary column. c, analyzed immediately after spiking; d, analyzed 2 hr after spiking and storing at 4°C; e, analyzed 10 min after spiking and storing at 37°C. 1, BCNU; 2, CCNU.

(Fig. 4), is limited. The percentages remaining intact (undegraded) of 0.25 μg/ml of I or II in human plasma at 4 and 37°C are provided in Fig. 5. As demonstrated in this figure, the degradation of these nitrosourea compounds in plasma followed first-order kinetics. The apparent rate constants for

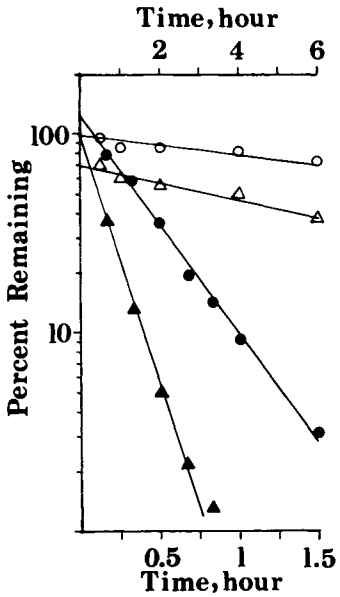

Fig. 5. Stability profiles of BCNU (O) and CCNU (△) in human plasma at 4°C (open symbols and upper time axis) and 37°C (solid symbols). Each point is the mean of four determinations.

Table VI. The Apparent Rate Constants and Activation Energies for Degradation in Plasma

Drug	Degradation rate constant (hr^{-1}) 4°C	37°C	Activation energy (cal · mol^{-1})
BCNU	0.09921	5.998	21,208
CCNU	0.02853	2.553	23,236

degradation of I and II at 4 and 37°C as determined by nonlinear regression analysis of the percentage of concentration intact–time data are presented in Table VI. Using these parameters in the Arrhenius equation, estimates of the activation energies for the degradation of I and II in plasma were computed to be 21,208 and 23,236 cal · mol^{-1} (Table VI), respectively.

In conclusion, the capillary gas chromatographic method described in this report is an improved approach for analysis of BCNU or CCNU in plasma. This assay is highly rapid, accurate, and specific, with minimum analyzable concentrations (viz. 0.001 μg/ml for CCNU or 0.003 μg/ml for BCNU) which are matched only by the more expensive, complicated, and often unavailable gas chromatography–mass spectrometry. The method was employed to study the pharmacokinetics of BCNU in rabbits and the stability of BCNU and CCNU in human plasma and in solution at different temperatures. The data indicate that these compounds are unstable in plasma even if refrigerated (4°C).

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