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Determination of procarbazine in human plasma by liquid chromatography with electrospray ionization mass spectrometry

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Abstract

Procarbazine is a cytotoxic chemotherapeutic agent used in the treatment of lymphomas and brain tumors. Its pharmacokinetic behavior remains poorly understood even though more than 30 years have elapsed since the drug was approved for clinical use. To characterize the pharmacokinetics of procarbazine in brain cancer patients during a phase I trial, a method for determining the drug in human plasma by reversed-phase high-performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry (ESI–MS) was developed and thoroughly validated. Plasma samples were prepared for analysis by precipitating proteins with trichloroacetic acid and washing the protein-free supernatant with methyl *tert*-butyl ether to remove excess acid. The solution was separated on a Luna C-18 analytical column using methanol-25 mM ammonium acetate buffer, pH 5.1 (22:78, v/v) as the mobile phase at 1.0 ml/min. A single-quadrupole mass spectrometer with an electrospray interface was operated in the selected-ion monitoring mode to detect the $[M + H]^+$ ions at *m*/*z* 222.2 for procarbazine and at *m*/*z* 192.1 for the internal standard (3-dimethylamino-2-methylpropiophenone). Procarbazine and the internal standard eluted as sharp, symmetrical peaks with retention times (mean ± S.D.) of 6.3 ± 0.1 and 9.9 ± 0.3 min, respectively. Calibration curves of procarbazine hydrochloride in human plasma at concentrations ranging from 0.5 to 50 ng/ml exhibited excellent linearity. The mean absolute recovery of the drug from plasma was 102.9 ± 1.0%. Using a sample volume of 150 μl, procarbazine was determined at the 0.5 ng/ml (1.9 nM) lower limit of quantitation with a mean accuracy of 105.2% and an interday precision of 3.60% R.S.D. on 11 different days over 5 weeks. During this same time interval, the between-day accuracy for determining quality control solutions of the drug in plasma at concentrations of 2.0, 15 and 40 ng/ml ranged from 97.5 to 98.2% (mean ± S.D., 97.9 ± 0.4%) and the precision was 3.8–6.2% (mean ± S.D., 5.1 ± 1.2%). Stability characteristics of the drug were thoroughly evaluated to establish appropriate conditions to process, store and prepare clinical specimens for chromatographic analysis without inducing significant chemical degradation. The sensitivity achieved with this assay permitted the plasma concentration–time profile of the parent drug to be accurately defined following oral administration of standard doses to brain cancer patients.

Keyword: Procarbazine

1. Introduction

Procarbazine, *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide (Fig. 1), is a cytotoxic chemotherapeutic agent used in the treatment of lymphomas and brain tumors [1]. Its pharmacokinetic behavior remains poorly understood, in both laboratory animals and humans, even though more than three decades have elapsed since the drug was first approved for clinical use. This may be a consequence of difficulties associated with the development of sufficiently sensitive and specific analytical methods to measure the concentration of procarbazine in biological fluids during the time period that the drug was undergoing preclinical and clinical development [2–4]. In common with other alkylating agents, the apparent instability of procarbazine in aqueous solution was also a significant concern for an assay suitable for application to pharmacokinetic studies [2–10].

The antineoplastic activity of procarbazine results from its conversion to highly reactive alkylating species by cytochrome P-450 mediated hepatic oxidative metabolism [11–13]. Supporting medications commonly used in the clinical management of patients with brain tumors, such

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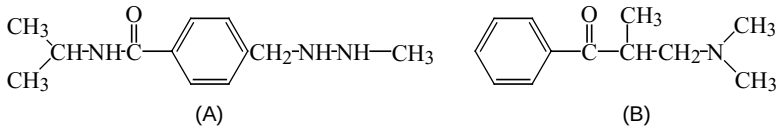

Fig. 1. Chemical structures of procarbazine (A) and the internal standard, 3-dimethylamino-2-methylpropiophenone (B).

as some antiseizure drugs and corticosteroids, have been shown to significantly enhance the systemic clearance of many anticancer agents [14]. In recognition of the potential for such a pharmacokinetic interaction with procarbazine [8,15], a phase I clinical trial was initiated recently to establish the maximum tolerated dose of the drug in patients with advanced glioma, stratified according to whether or not they were concurrently receiving enyzyme inducing antiseizure drugs (EIASDs). Elucidating the influence of various antiseizure drugs on the pharmacokinetic behavior of procarbazine was considered to be an important aspect of this study.

This report describes the development and validation of a highly specific and sensitive assay for determining procarbazine in human plasma based upon high-performance liquid chromatography (HPLC) with detection by electrospray ionization mass spectrometry (ESI–MS). Stability characteristics of the drug were thoroughly evaluated to establish appropriate conditions to process, store and prepare clinical specimens for chromatographic analysis without inducing significant chemical degradation. Application of the analytical method has been demonstrated by defining the plasma concentration–time profile of the parent drug in brain cancer patients following oral administration of a 294 mg/m^2 dose of procarbazine.

2. Experimental

2.1. Reagents and chemicals

A reference sample of procarbazine hydrochloride was generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA). The following chemicals were used as supplied: HPLC grade ammonium acetate, HPLC grade phosphoric acid, 'Optima' grade methanol, HPLC grade methyl *tert*-butyl ether (TBME), ACS plus concentrated hydrochloric acid, HPLC grade 85% phosphoric acid, and ACS reagent dibasic anhydrous potassium phosphate (Fisher Scientific, Fairlawn, NJ, USA); ACS reagent monobasic potassium phosphate acetate (Acros Organics/Fisher Scientific); ACS reagent trichloroacetic acid (>99%), ACS reagent acetic acid (99.7%) and 97% 3-dimethylamino-2-methylpropiophenone hydrochloride (Aldrich Chemicals, Milwaukee, WI, 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). The solution used as the aqueous component of the mobile phase, 25 mM ammonium acetate buffer, pH 5.1, was prepared by combing 17.8 ml of 1.0 M ammonium acetate and 7.2 ml of 1.0 M acetic acid in a 1 l class A volumetric flask and diluting to the mark with deionized distilled water. Frozen, citrated human plasma, obtained from the Blood Transfusion Service, Massachusetts General Hospital (Boston, MA, USA), was thawed at room temperature, mixed on a vortex stirrer, and centrifuged for 5 min at 12,000 × *g* to separate particulate matter and lipids before use.

2.2. Analytical solutions

Glassware was deactivated by treatment with a 1% (v/v) solution of SurfaSil siliconizing fluid (Pierce Chemicals, Rockford, IL, USA) in HPLC grade hexanes (Fisher Scientific) and oven drying at 120 °C. Primary stock solutions of procarbazine hydrochloride in 1 mM phosphoric acid and the internal standard, 3-dimethylamino-2-methylpropiophenone hydrochloride, in water were made at a concentration of approximately 1.0 mg/ml. The compounds were accurately weighed on a Cahn C-34 analytical microbalance (ATI Orion, Beverly, MA, USA) and dissolved within class A borosilicate glass volumetric flasks with Teflon-lined septum screw-tops (Kontes, Vineland, NJ, USA). Working solutions were prepared by diluting the primary stock solutions of procarbazine to 5 μg/ml with 1 mM phosphoric acid and the internal standard to 0.1 μg/ml with water in volumetric flasks. These solutions were stored in a refrigerator (5 °C) and used for up to 1 month. Precautions were taken to prevent prolonged exposure of procarbazine solutions to normal laboratory lighting. The same working solution was used to prepare the calibration solutions and quality control solutions. Calibration solutions were made daily by serially diluting the procarbazine hydrochloride working solution with human donor plasma to concentrations of 50, 35, 20, 10, 5, 2.5, 1.0 and 0.5 ng/ml (194–1.94 nM) in polypropylene tubes. Three quality control solutions containing procarbazine hydrochloride at concentrations of approximately 40, 15 and 2.0 ng/ml in human plasma were prepared along with calibration solutions in the same fashion.

2.3. Sample preparation

Plasma (150 μl) and an equal volume of trichloroacetic acid (10%, w/v) were vigorously mixed in a 1.5 ml polypropylene microcentrifuge tube by vortexing. The mixture was permitted to stand for about 1 min before centrifuging for 5 min at 10,000 × *g*. Thereafter, 200 μl of the clear supernatant was removed from the protein pellet, transferred into another microcentrifuge tube, and vigorously mixed

with 1 ml of TBME on a vortex stirrer for 30 s. The mixture was centrifuged (10,000 × *g*, 2 min), upon which the upper organic phase was taken up with a pipet and discarded, resulting in the removal of approximately 90% of the trichloroacetic acid. Residual TBME was allowed to evaporate by permitting the uncapped tube to stand for up to 30 min within a chemical fume hood. In another microcentrifuge tube, an aliquot of the aqueous phase (150 μl) was mixed with 5 μl of the internal standard working solution, then transferred into a silanized borosilicate glass insert, which was sealed in an amber autosampler vial (12 mm × 32 mm) using a snap closure with a Teflon/silicone liner. The vial was promptly placed in an autosampler with a temperature controlled sample compartment set to 5 °C. The volume of solution injected into the chromatograph was 100 μl.

2.4. LC/MS analysis

Analyses were performed using an Agilent 1100 Series LC/MSD_SL system (Agilent Technologies, Palo Alto, CA, USA) consisting of an isocratic pump, a thermostatted autosampler, and a single-quadrupole SL model mass selective detector (MSD) equipped with an atmospheric pressure ionization–electrospray (API–ES) interface. Chromatography was performed at ambient temperature on a 15 cm × 4.6 mm i.d. Luna C-18 (5 μm particle size) HPLC column preceded by a Luna C-18 guard column (Phenomenex, Torrance, CA, USA) and a 0.5 μm inline filter (Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase was composed of methanol-25 mM ammonium acetate buffer, pH 5.1 (22:78, v/v) and delivered at a flow rate 1.0 ml/min. The solution was degassed in an ultrasonic bath for 15 min before use. Flow from the analytical column was directed into the electrospray ionization chamber of the MSD without splitting. The operating parameters of the API–ES interface were as follows: nebulizer pressure, 40 psi; drying-gas (N_2) flow, 12 l/min; drying-gas temperature, 350 °C; capillary voltage, 1100 V; fragmentor voltage, 120 V. The MSD was operated in the positive ionization mode with selected-ion monitoring (SIM) at *m*/*z* 192.1 and 222.2 with a dwell time of 559 ms (mass–resolution, low). The run time for each chromatogram was 13.0 min and the time between successive injections was approximately 14.5 min. Chromatograms were integrated to provide peak areas using the data analysis functions of the Agilent ChemStation software (rev. A.08.03).

2.5. Absolute recovery and matrix ion suppression

To assess the absolute recovery of procarbazine from plasma, a vehicle was prepared by precipitating drug-free human plasma with an equal volume of trichloroacetic acid (10%, w/v) and extracting the resulting supernatant with TBME, as described in the above. Reference solutions were then made by spiking this vehicle with procarbazine hydrochloride working solutions to afford concentrations equivalent to those of the three quality control samples upon processing in this same manner, assuming quantitative recovery of the drug (i.e. 20, 7.5 and, 1.0 ng/ml). Determining the magnitude of matrix ion suppression is as a standard practice during the development of LC/MS bioanalytical methods in our laboratories, especially when achieving maximum sensitivity is an important consideration. Solutions to assess matrix associated ion suppression of the analyte were prepared, as described in the above, using 3 mM trichloroacetic acid as the non-matrix vehicle. This solution has a pH of 2.5, which is equivalent to that of a plasma sample carried through the sample preparation procedure. Internal standard working solution was added to these solutions, as described in the above, prior to chromatography. Five aliquots of each of the three plasma quality control solutions were prepared and analyzed together with the corresponding absolute recovery and ion suppression reference solutions. Absolute recovery was calculated by comparing the mean procarbazine:internal standard peak area ratio for the quality control samples to that of the corresponding reference solutions. Percent ion suppression for each added drug concentration was calculated as $100 \times (\bar{A}_r - \bar{A}_p)/\bar{A}_r$, where $\bar{A}_p$ and $\bar{A}_r$ are the mean procarbazine peak areas for five replicate determinations of the plasma matrix and non-matrix reference solutions, respectively.

2.6. Within-day and between-day accuracy and precision

Within-day accuracy and precision of the assay were determined by preparing five aliquots of each of the three plasma quality control solutions and assaying them together with a set of plasma calibration standards during a single day. Calibration curves were constructed by plotting the drug:internal standard chromatographic peak area ratio as a function of the nominal procarbazine hydrochloride concentration of the plasma standards. 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. The concentration of procarbazine in the quality control plasma samples was calculated using the parameters for the best-fit line of the calibration curve. The R.S.D. of the mean observed concentration of procarbazine hydrochloride for the five replicate determinations of each quality control solution provided the measure of precision. Accuracy was calculated as the percentage of the mean observed concentration relative to the added concentration of procarbazine hydrochloride in each quality control solution.

Between-day accuracy and precision were evaluated by analyzing the backcalculated concentrations of the three quality control samples and the eight calibration solutions of procarbazine hydrochloride in human plasma that were independently prepared and assayed in single determinations on 11 separate days over a period of 5 weeks. The R.S.D. of the mean predicted concentration for the independently assayed samples provided the measure of precision. The accuracy of the assay was expressed as the percentage of the

mean predicted drug concentration relative to the known concentration of the corresponding solutions.

2.7. Stability studies

Stability of the stock solution of procarbazine hydrochloride in 1 mM phosphoric acid during storage in a refrigerator was monitored for a period of 45 days by reversed-phase HPLC using a 1050 series photodiode array UV-Vis detector (Hewlett-Packard, Pato Alto, CA). An aliquot of the stock solution was diluted 10-times with 1 mM phosphoric acid, loaded (injection volume, 5 μl) onto a Phenomenex Luna C-18 column (150 mm × 4.6 mm i.d. 5 μm particle size), and eluted isocratically with a mobile phase composed of methanol-25 mM ammonium acetate (35:65, v/v) delivered at 1.0 ml/min. The area of the chromatographic peak for procarbazine monitored at its λ_{max} of 233 nm was determined.

The stability of procarbazine in the final solution resulting from the preparation of plasma samples for analysis was determined at room temperature and at 5 °C. Thirty aliquots of plasma with an added procarbazine hydrochloride concentration of 50 ng/ml were processed for analysis batch-wise. The final solutions were pooled in a test tube and thoroughly mixed before pipetting into individual autosampler vials. The vials were placed in the sample compartment of the autosample, that had been pre-equilibrated to a set point of 5 or 25 °C, and injected into the chromatograph once every 30 min for 15 h.

The stability of procarbazine in human plasma was studied at −80, 8 and 37 °C. Kinetic runs were conducted at initial procarbazine hydrochloride concentrations of approximately 50, 500, and 1000 ng/ml. The experiments were carried out by initially spiking 6 ml of plasma, pre-equilibrated to the desired temperature, or chilled in an ice water bath for studying the stability of frozen samples, in a screw-top borosilicate glass test tube, with 60 μl of a procarbazine hydrochloride working solution (5, 50 or 100 μg/ml in 1 mM phosphoric acid). The tubes were capped and placed in a refrigerator or thermostatted water bath for the experiments at 8 and 37 °C, respectively. An aliquot was removed from each solution for analysis shortly after spiking with the drug and then approximately once an hour for 8 h, with a final sample obtained after continuous incubation for 24 h. To assess stability at −80 °C, eight aliquots (150 μl) of each spiked plasma solution were immediately pipetted into individual polypropylene microcentrifuge tubes, flash frozen, and placed in an ultra-low temperature freezer. An aliquot of each solution was removed for analysis every 3 or 4 days for a period of 29 days. The samples were permitted to thaw at room temperature. Solutions with initial drug concentrations that exceeded the upper range of the standard curve were quantitatively diluted with blank human plasma before processing for chromatographic analysis.

Under all conditions evaluated, the temperature was measured at every sample time using a Fluke 51 K thermocouple (Everett, WA, USA). An Orion 920A pH meter equipped with a ROSS semi-micro combination electrode (Orion Research, Beverly, MA, USA) was used to measure the pH of solutions. Kinetic data was analyzed by fitting the assayed concentration (ESI–MS detection) or chromatographic peak area (UV detection) of procarbazine at each observation time to the integrated equation for first-order decay by non-linear regression, with a weighing factor of $1/y$, using the WinNonlin version 1.1 software package (Scientific Consulting, Inc., Apex, NC, USA). The half-life and time for 5% decomposition were calculated from the estimated value of the apparent first-order rate constant (k_{obs}) according to standard equations [16]. The apparent absence of a trend toward decreasing concentration was substantiated by examining the Pearson and Spearman correlations of the observed concentration–time data pairs. In experiments for which no significant degradation was evident, the mean ± S.D. relative recovery of the drug was calculated as 100 × (concentration found/concentration added) for all determinations made during the observation period.

2.8. Pharmacokinetic studies

Plasma specimens were acquired from patients with refractory malignant gliomas during a phase I clinical trial of procarbazine given orally once a day for 5 days every 4 weeks. The primary objectives of the study were to determine the maximum tolerated dose for this administration schedule of procarbazine in two groups of patients stratified according to the concurrent use of EIASDs and to characterize the pharmacokinetic behavior of procarbazine. The protocol for the study was reviewed and approved by the National Cancer Institute (Bethesda, MD, USA) and the Institutional Review Boards of each participating institution. All patients were fully counseled by the treating physician and signed an institutionally approved informed consent document prior to initiating therapy. Blood specimens (7 ml) were acquired from a peripheral arm vein before dosing and at 7-time points, ranging from 20 min to 4 h after taking the first dose of the drug. Blood was collected in Vacutainer Brand plasma tubes with freeze-dried sodium heparin (Becton-Dickinson, Franklin Lakes, NJ, USA). The sample tubes were immediately placed on ice and centrifuged (1500 × *g*, 10 min, 5 °C) within 15 min after collection. The plasma was separated from the blood and stored in polypropylene cryotubes at −80 °C until assayed.

Study samples were thawed at ambient temperature, mixed by vortexing, and promptly replaced in the freezer after removing an aliquot for analysis. Each sample was assayed in duplicate on separate days together with a series of calibration standards and quality control samples. Values of the slope and *y*-intercept for the best-fit line of the calibration curve were used to calculate the analyte concentration in the study samples. Specimens exceeding the upper range of the standard curve were reassayed upon dilution with drug-free human plasma. The average of the two determinations of each study sample was calculated.

Samples were reassayed in cases where the individual determinations differed from their average by more than 10%. Plasma concentration–time profiles of the drug were fit to the equation for first-order absorption with biexponential disposition and an absorption lag time by non-linear regression using WinNonlin with a weighting factor of $1/y^2$ [17]. Values of the parameters corresponding to the equation that best described the plasma profile were used to calculate pharmacokinetic variables according to standard equations.

3. Results

3.1. ESI–MS detection

A preliminary series of experiments indicated that procarbazine was very responsive to ESI–MS with positive ion detection. With appropriate selection of the fragmentor voltage, the protonated molecule at *m/z* 222.2 (calcd. exact mass for $C_{12}H_{20}N_3O$, 222.1608) appeared as the base peak in the ESI mass spectrum of the drug (Fig. 2), without significant fragmentation to lower mass ions. Less intense peaks attributable to adduct ions, including the sodium adduct at *m/z* 244.1 (calcd. exact mass, 244.1428) and an ion consistent with the $[2M+H]^+$ species at *m/z* 443.3 (calcd. exact mass, 443.3138), were also present in the mass spectrum. After establishing the final conditions for the chromatographic analysis of procarbazine in human plasma, all operational parameters of the detector interface and mass spectrometer were systematically optimized to maximize the response for the procarbazine $[M+H]^+$ ion with detection in the selected ion monitoring (SIM) mode, since a single-quadrupole mass spectrometer was being used. The parameters examined included the nebulizer pressure, drying gas flow rate and temperature, capillary voltage, SIM ion, mass resolution, and fragmentor voltage. A solution of 26 ng/ml procarbazine hydrochloride in 1 mM phosphoric acid with an injection volume of 100 μl was used for the optimization. Using the optimal detection parameters, the limit of detection of procarbazine was 25 pg on-column, expressed as the hydrochloride salt, which afforded a signal-to-noise ratio of 5.0 on average. The base peak in the mass spectrum of the compound selected for use as the internal standard in the assay, 3-dimethylamino-2-methyl-propiophenone ($C_{12}H_{17}NO$, M_r 191.3), was also the protonated molecule at *m/z* 192.1 (not shown).

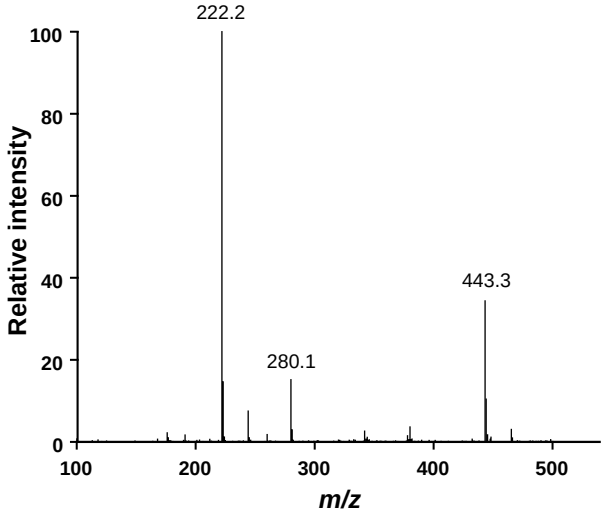

Fig. 2. Positive ion ESI mass spectrum acquired at the apex of the chromatographic peak for procarbazine hydrochloride (50 ng on-column) by continuous scanning over a mass range of 100–500 amu. Aside from operating the mass spectrometer in the scanning mode, the interface and detector were operated at the settings that provided optimal response of the procarbazine $[M+H]^+$ ion at *m/z* 222.2.

3.2. Sample preparation and liquid chromatography

The preparation of plasma samples for liquid chromatography simply involved removing protein by precipitation induced by adding trichloroacetic acid to a final concentration of 0.31 M and extracting the protein-free supernatant with TBME to remove excess acid. After removing the organic phase, the internal standard was added to the aqueous phase and the sample was loaded onto a conventional bore HPLC column packed with the Luna C-18 stationary phase. The separation was performed isocratically using methanol-25 mM ammonium acetate buffer, pH 5.1 (22:78, v/v) as the mobile phase at a flow rate of 1 ml/min. The detector was programmed to concurrently monitor positive ions at *m/z* 192.1 and 222.2 originating from the column effluent. Under these conditions, both procarbazine and the internal standard eluted as sharp chromatographic peaks with excellent symmetry. Procarbazine had an average retention time of 6.3 ± 0.1 min and a peak width at half-height of 0.19 ± 0.01 min during the analysis of a reference solution on 11 days over a period of 5 weeks on a single HPLC column. Average values of the retention time and peak width of the internal standard were 9.9 ± 0.3 and 0.27 ± 0.01 min, respectively.

Chromatograms of plasma samples obtained from a cancer patient before and after oral administration of a 200 mg/m^2 dose of procarbazine are shown in Fig. 3. The chromatogram of pretreatment plasma (Fig. 3A) showed no peaks that would interfere with the detection of either procarbazine or the internal standard. Panels B and C of Fig. 3 show chromatograms of plasma obtained from the same patient after administration of the drug with measured concentrations of procarbazine hydrochloride near the upper and lower range of the calibration curve. The specificity of the assay was initially examined by analyzing plasma from four anonymous human donors and five cancer patients who were not receiving procarbazine. Potentially interfering chromatographic peaks were not observed in any of these samples or in pretreatment plasma samples obtained from 33 patients that were analyzed during the application of this assay to a clinical pharmacokinetic study of procarbazine

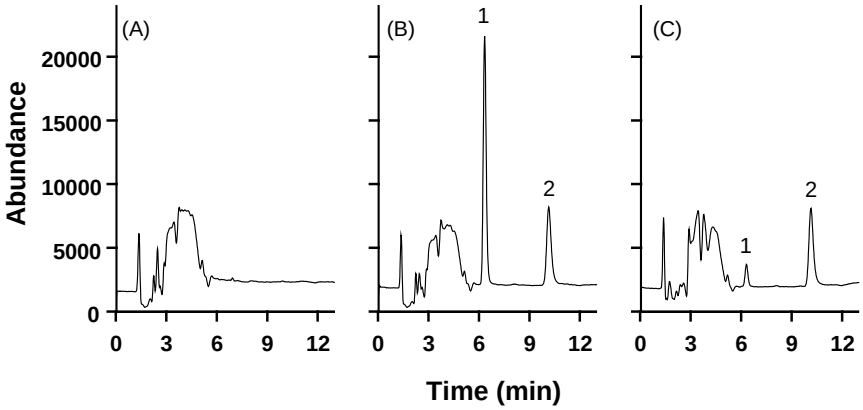

Fig. 3. Time-selected ion profiles depicting the abundance of positive ions at *m*/*z* 222.2 from 0 to 7.5 min and *m*/*z* 192.1 from 7.5 to 13 min during the LC/ESI–MS analysis of plasma samples. (A) Chromatogram of pretreatment plasma obtained from a brain cancer patient assayed without addition of internal standard. Chromatograms of plasma samples obtained 40 min (B) and 4 h (C) after oral administration of 200 mg/m^2 procarbazine to the same patient. Procarbazine (1) elutes near 6.3 min and the internal standard (2) is the peak at 9.9 min. The sample in panel B was diluted 10-fold with drug-free plasma prior to analysis. The area of the chromatographic peak in panel B corresponds to a procarbazine hydrochloride concentration of 39.3 ng/ml in plasma and the concentration calculated from the peak in panel C is 3.5 ng/ml.

in patients with brain cancer. These patients were concurrently receiving a host of supporting medications, including various antiseizure drugs, glucocoricoids, antiemetics, and analgesics. In addition, there was no evidence of late eluting peaks during uninterrupted sequential analysis of numerous plasma extracts with a run time of 13 min for each sample.

3.3. Assay performance

The procarbazine:internal standard chromatographic peak area ratio increased linearly over a concentration range of 0.5–50 ng/ml procarbazine hydrochloride in human plasma. Mean values (±S.D.) of the regression parameters for 11 standard curves that were independently prepared and analyzed over a period of 5 weeks were: slope, 0.0622 ± 0.0058; *y*-intercept, −0.0100 ± 0.0041; correlation coefficient, 0.9997 ± 0.0005. The lowest concentration of procarbazine hydrochloride included in the standard curve, 0.5 ng/ml, was determined with a mean accuracy of 105.2% and a R.S.D. of 3.60% during this period. Mean values of the accuracy calculated for each of the seven other calibration solutions of procarbazine hydrochloride in plasma at concentrations of 1.0–51.6 ng/ml that defined the standard curve ranged from 98.0 to 101.6% and the precision ranged from 1.6 to 3.3%.

Results of studies to assess the within- and between-day accuracy and precision of the method and absolute recovery of the drug from plasma are summarized in Table 1. The three quality control solutions containing procarbazine hydrochloride in human plasma at concentrations of 2.0, 15 and 40 ng/ml were used for these experiments. The within-day accuracy of the assay ranged from 102.5 to 104.1% of the known drug concentration with a grand mean (±S.D.) of 103.4 ± 0.8%. The grand mean between-day accuracy for the three quality control solutions was 97.9 ± 0.4% (range, 97.5–98.3%). The range of values for the within- and between-day precision were 2.5–3.5 and 3.8–6.2%, respectively. The mean absolute recovery of procarbazine from plasma was 102.9 ± 1.0% at these concentrations. Matrix associated ion suppression of the response of the drug to ESI–MS detection was relatively minor and independent of the drug concentration, with values that ranged from 3.0 to 5.6% for the quality control solutions. The analytical method is considered to be accurate and reproducible for determining procarbazine in human plasma at

Table 1
Absolute recovery of procarbazine from human plasma and the accuracy and precision for its quantitation in plasma specimens

Actual concentration (ng/ml)	Absolute recovery[a] (%)	Within-day (%)[b]		Between-day (%)[c]	
		Accuracy	Precision	Accuracy	Precision
40.0	101.8 ± 5.6	102.5	3.50	98.1	5.12
15.0	103.8 ± 4.4	104.1	3.44	98.2	6.21
2.0	103.0 ± 5.0	103.5	2.49	97.5	3.75
Grand mean	102.9 ± 1.0	103.4 (0.8)[d]	3.14 (0.57)	97.9 (0.4)	5.03 (1.23)

[a] Values are mean ± S.D. of five replicate determinations.
[b] Results from five sets of quality control samples assayed on a single day.
[c] Results from 11 sets of quality control samples independently prepared and assayed on separate days.
[d] Numbers in parentheses, S.D.

concentrations ranging from 0.5 to 50 ng/ml expressed as the hydrochloride salt. In addition, the ability to dilute plasma samples with drug concentrations exceeding the upper range of the calibration curve with human donor plasma to a level within the quantifiable range prior to analysis has been examined. Plasma with an added drug concentration of 511 ng/ml was measured with an accuracy of 101.8% and a precision of 7.45% ($n = 9$) upon 20-fold dilution. Similarly, the accuracy and precision for measuring procarbazine hydrochloride at a concentration of 1021 ng/ml in plasma with a 40-fold dilution were 99.5 and 5.01%, respectively.

3.4. Stability of procarbazine

The stock solution of procarbazine hydrochloride in 1 mM phosphoric acid (pH 3.1) proved to be stable for at least 6 weeks when stored in a refrigerator with an average temperature 7.5 ± 1.5 °C. The solution was sampled every 2–5 days over a period of 45 days and subject to reversed-phase HPLC with UV detection at 233 nm. The chromatograms showed no evidence of peaks consistent with degradation products and the area of the chromatographic peak for procarbazine was extremely consistent, as indicated by an R.S.D. of 1.62% for the 13 determinations made during this time interval.

The stability of procarbazine in the final solution resulting from the preparation of plasma samples for chromatographic analysis was examined at 5 °C and at room temperature (27 ± 1 °C) to assess the feasibility of overnight analysis using an autosampler. The time for 5% degradation of the drug in samples maintained at room temperature was 3.9 h. In contrast, there was no discernable loss of drug over a time interval of 15 h when the temperature of the sample compartment of the autosampler was decreased to 5 °C. On the basis of this finding, plasma samples were chilled over wet-ice immediately upon completing their preparation for analysis and stored in a refrigerator until placed within the refrigerated sample compartment of the automated injector.

Results of the studies to assess the stability of procarbazine hydrochloride in human plasma at temperatures of −80, 8 and 37 °C are summarized in Table 2. There was no evidence of drug degradation when solutions with initial concentrations of 51, 510 and 1022 ng/ml in plasma were stored at −80 ± 1 °C over a period of 29 days. Similarly, solutions of procarbazine in plasma at these same initial concentrations that were maintained at 8 ± 1 °C in a refrigerator did not show a trend toward decreasing concentration when monitored periodically over 10 h. The drug degraded slowly in plasma incubated at 37.5 °C, with a mean half-life of 47.6 ± 9.0 h, corresponding to a time for 5% degradation of 3.4 h. Estimated values of the first-order rate constant for the loss procarbazine were similar at each of the three initial drug concentrations.

3.5. Application of the assay to a clinical pharmacokinetic study

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of procarbazine in brain cancer patients. Plasma concentration–time profiles of procarbazine for the first daily oral dose of 294 mg/m^2 in a patient who was not concurrently receiving an EIASD and another patient who was being treated with phenytoin are shown in Fig. 4. The plasma profiles were very similar for both patients, characterized by an absorption lag time of 19 min, upon which the concentration of procarbazine in plasma increased rapidly to a peak level of 546 ng/ml at 37 min in the −EIASD patient and 669 ng/ml at 34 min in the +EIASD patient. Plasma levels of the drug decayed in a biexponential manner, with a terminal phase half-life of 86 min in the −EIASD patient and 54 min in the +EIASD patient. In both cases, plasma levels remained well above the 0.5 ng/ml lower limit of quantitation of the assay at the end of the scheduled 4 h sampling interval. The area under the plasma concentration–time curves

Table 2
Stability of procarbazine in human plasma

Temperature (°C)	Initial concentration (ng/ml)	Time interval	Number of sample times	k_{obs} (h^{-1})[a]	Mean concentration found/added (%)
−80 ± 1	51	29 days	8[b]	–	95.9 (9.5)[c]
	511		9	–	95.8 (13.7)
	1021		9	–	98.8 (13.9)
8 ± 1	51	10 h	9	–	96.9 (5.0)
	511		9	–	101.8 (7.5)
	1021		9	–	99.5 (5.0)
37.5 ± 0.1	51	10 h	9	0.013	
	511		9	0.019	
	1021		9	0.014	
Mean				0.015 (0.003)[d]	

[a] Apparent first-order rate constant.
[b] Single determinations at each sample time.
[c] Numbers in parentheses, R.S.D.
[d] Number in parentheses, S.D.

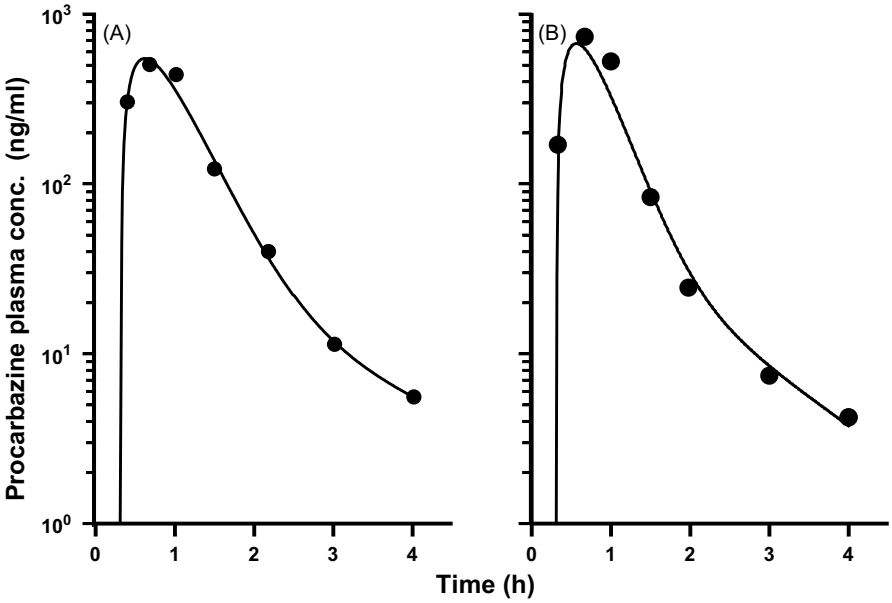

Fig. 4. Plasma concentration–time profiles of procarbazine for the first oral dose of 294 mg/m^2 given to a brain cancer patient who (A) was not receiving an enzyme inducing antiseizure drug and (B) another patient who was taking phenytoin.

from time zero to infinity were 502 and 491 ng h/ml in the −EIASD and +EIASD patients, respectively. These preliminary findings suggest that the concurrent use of EIASDs did not significantly influence the pharmacokinetics of procarbazine.

4. Discussion

Procarbazine has been a clinically important anticancer drug since 1969. Nevertheless, notably few analytical methods for measuring the compound in biological fluids have been described in the literature, with the majority reported more than two decades ago. The drug has been determined by UV absorption and chemical ionization MS after extraction from plasma into a non-polar organic solvent without a chromatographic separation [2,4]. Aside from concerns associated with poor specificity and the extent of drug degradation during the extraction procedure, which required alkalization of the sample, these methods were not particularly sensitive. The lower limit of quantitation was 10 μg/ml for the spectrophotometric assay and the limit of detection of the MS assay was reported as 1.3 μg/ml (5 μM). In the MS assay with direct sample introduction, isobutane was used as the reagent gas and quantitation was performed by measuring the response of the $[M + H]^+$ ion at *m*/*z* 222.2 relative to the intensity of the corresponding ion for hexadeuteroprocarbazine at *m*/*z* 228.2, which was added to plasma samples at a constant amount prior to extraction. A substantial improvement in selectivity and sensitivity were achieved when isobutane chemical ionization MS detection was preceded by gas chromatographic separation of the diaectyl derivative of procarbazine, readily prepared by treating toluene extracts of plasma with acetic anhydride [3]. Reported values of the lower limits of detection and quantitation for this assay were 1 and 10 ng/ml, respectively. The identification of procarbazine in plasma by normal- and reversed-phase HPLC with UV detection has been described [18,19]. Although the compound was found to be 20-times more responsive to oxidative electrochemical detection than UV detection at 254 nm during reversed-phase HPLC, it appears unlikely that procarbazine could be detected in plasma at concentrations lower than approximately 1 μg/ml in this manner [19]. The extent of information to support the validation of each of these methods is extremely limited, at best. None were considered to be particularly suitable for pharmacokinetic drug-level monitoring.

More recently, thermospray ionization LC/MS was used successfully for the qualitative identification of procarbazine metabolites and degradation products in cultures of human leukemia cells and cell-free aqueous solutions [10,20]. However, to the best of our knowledge, the utilization of LC/MS techniques for the determination of procarbazine or quantitative analysis of its metabolites has not been previously reported. Accordingly, we evaluated the use of ESI–MS detection following reversed-phase HPLC as the basis of an assay for procarbazine in human plasma that would be suitable for clinical pharmacokinetic studies. Preliminary experiments revealed that procarbazine was very responsive to ESI–MS with positive ion detection. The base peak in the ESI mass spectrum of the drug was the $[M + H]^+$ ion at *m*/*z* 222.2 and the extent of fragmentation to lower mass ions was relatively minor. The limit of detection for a pure reference sample of procarbazine hydrochloride was near 25 pg using a single-quadrupole mass spectrometer operated in the SIM mode for maximum sensitivity.

The method by which plasma samples were prepared for chromatographic analysis was largely dictated by the physicochemical properties of procarbazine. In several previously reported analytical methods, plasma samples were adjusted to a strongly alkaline pH to facilitate extraction of the neutral form of the drug into a non-polar organic solvent, since procarbazine is a weakly basic molecule with a pK_a of 6.8 [2,4]. Isolating the drug from plasma in this manner is undesirable because it degrades rapidly in alkaline aqueous solution [2,21]. Perchloric acid and trichloroacetic acid have been used in the preparation of biological matrices for the analysis of procarbazine and its metabolites [6,18]. Procarbazine is not only soluble in acidic aqueous solutions, as a consequence of being fully protonated, but also relatively stable against autoxidation [6]. Although both acids are very efficient protein precipitating reagents [22], trichloroacetic acid is compatible with ESI–MS detection due to its volatility. In the present application, the supernatant resulting from the treatment of plasma with trichloracetic acid, at a concentration required to completely precipitate proteins, was far too acidic to introduce directly onto an HPLC column with a silica-based bonded stationary phase. This problem was conveniently resolved by washing the supernatant once with TBME, which removed approximately 90% of the added trichloroacetic acid, as indicated by HPLC with UV detection, thereby increasing the pH of the aqueous phase to pH 2.5 without significant loss of procarbazine. It was necessary to add the compound selected for use as an internal standard for the assay, 3-dimethylamino-2-methylpropiophenone hydrochloride, to the sample after performing the TBME wash because it was extracted to a significant extent from the aqueous phase. Efforts to identify a compound for use as an internal standard that could be added directly to the plasma sample, which required it to be adequately retained under the chromatographic conditions employed without being extracted to a significant extent into TBME, were unsuccessful. Nevertheless, use of an internal standard introduced at some point to the sample solution is absolutely necessary for quantitative analysis with MS to compensate for the relatively high degree of variability in response typically associated with this method of detection.

The primary considerations for establishing chromatographic conditions were compatibility with ESI–MS detection and the ability to adequately separate the drug from polar constituents present in the final sample solution that could significantly diminish response to ESI–MS detection [23,24]. A standard bore HPLC column packed with the Luna C-18 stationary phase was selected for the separation of procarbazine prior to ESI–MS detection because the interface in the particular instrument used in this study was designed to accommodate mobile phase flow rates of 0.5–1.5 ml/min without flow splitting. The aqueous component of the mobile phase was restricted to solutions of the volatile buffers ammonium acetate and ammonium formate at concentrations of 10 or 25 mM. Methanol and acetonitrile were evaluated as organic modifiers. The retention time of procarbazine increased in proportion to the pH of the buffer solution in the mobile phase, within the range pH 3.02–6.63, in eluents with the same buffer concentration and volume percentage of methanol. The drug was not resolved from low-wavelength UV absorbing, acid soluble endogenous compounds in plasma and residual trichloroacetic acid when the mobile phase buffer was less than pH 4.7. Differences between the chromatographic behavior of procarbazine and the internal standard (3-dimethylamino-2-methylpropiophenone) also factored prominently in establishing the composition of the mobile phase. The internal standard was more strongly retained than procarbazine, with the difference between their retention times becoming markedly greater as the pH of the mobile phase buffer increased from pH 5.0 to 6.7. In addition, the capacity factor of the internal standard was considerably greater in mobile phases containing acetonitrile as compared to methanol, when the percentage of the organic modifier was adjusted to elute procarbazine at the same time. The final composition of the mobile phase selected for use in the assay was methanol-25 mM ammonium acetate buffer, pH 5.1 (22:78, v/v). Under these conditions, procarbazine and the internal standard were more strongly retained on the HPLC column than polar matrix components, eluting as sharp, symmetrical peaks in less than 10 min.

The lower limit of quantitation of this assay, 0.5 ng/ml (1.95 nM) of procarbazine hydrochloride in plasma using a sample volume of only 150 μl, is far superior to any other analytical method for determining the drug in biological fluids that has been previously reported. The drug was quantitatively recovered from plasma when spiked samples with concentrations of 2–40 ng/ml were processed for chromatographic analysis in this manner. The extent of sample clean up achieved upon preparing plasma specimens for chromatographic analysis simply by acid-induced protein precipitation is substantially less than achieved with techniques in which the analyte is actually isolated from the original matrix, such as liquid–liquid or solid phase extraction. Nevertheless, the response of procarbazine to ESI–MS detection following HPLC was not significantly affected by the presence of residual trichloroacetic acid or polar endogenous substances in the final sample solution.

It has been reported that procarbazine undergoes rapid, spontaneous degradation in water and cell culture medium containing 10% fetal calf serum, both in the presence and absence of human leukemia cells that did not have any demonstrable cytochrome P-450 activity [10]. Azoprocarbazine was identified as the initial degradation product and it underwent further decomposition to its hydrazone tautomer and trace amounts of methylazoxyprocarbazine and benzylazoxyprocarbazine. Consequently, minimizing the loss of drug due to chemical degradation during the collection, storage and preparation of plasma specimens for analysis represented an extremely important consideration for

the quantitative analysis of procarbazine in biological fluids. Experiments were, therefore, undertaken to thoroughly assess the stability of procarbazine in stock solutions, human plasma, and the conditions to which it was exposed during the preparation of plasma specimens for LC/MS analysis.

Stock solutions of procarbazine were initially prepared in 1 mM phosphoric acid (pH 3.1) because it was previously reported that the compound exhibited good stability in acidic aqueous solution [6]. We found that these solutions could be stored with refrigeration for more than 1 month without any evidence of degradation. Procarbazine was sufficiently stable in the acidic aqueous solution afforded upon precipitating plasma proteins with trichloroacetic acid such that the potential for degradative loss during sample preparation was inconsequential. However, because the time for 5% degradation of procarbazine in the final sample solution was 3.9 h at room temperature, it was necessary to use a refrigerated autosampler to permit unattended overnight analysis of fully prepared samples.

Solutions of procarbazine in human plasma were stable for at least 1 month when stored frozen at −80 °C. Stability of the drug in plasma when maintained frozen at higher temperatures was not evaluated during the course of this study because biological samples are stored in our facilities in ultra-low temperature freezers as a matter of routine. In addition, the drug did not degrade to a detectable extent when frozen specimens were permitted to thaw at room temperature, even after three or more freeze-thaw cycles. Procarbazine degraded in a first-order manner with a half-life of approximately 48 h in human plasma and 4.4 h in 0.1 M potassium phosphate buffer, pH 7.4, when incubated at 37.5 °C. These observations imply that plasma protein may be responsible for stabilizing the drug to metal ion catalyzed autoxidation [10]. With a time for 5% degradation of 3.4 h in plasma at 37 °C, the extent of drug loss during the collection of blood specimens and their subsequent centrifugation to harvest plasma, as typically performed in clinical pharmacokinetic studies, would be negligible if these processes were completed to allow the sample to be stored frozen in a reasonably timely manner. The results of these studies have served to unequivocally demonstrate that the chemical stability of procarbazine is not problematic and that no extraordinary measures are required for the collection and subsequent handling of plasma specimens to insure the accuracy of pharmacokinetic data.

The sensitivity achieved with this LC/MS assay was necessary to accurately define the plasma concentration–time profile of the drug following oral administration of tolerated doses of procarbazine to brain cancer patients. Preliminary data suggests that the concurrent use of EIASDs did not have a marked influence on the plasma pharmacokinetics of procarbazine in these patients. This was an unexpected finding, as one of the major cytochrome P-450 isozymes involved in the bioactivation of the drug is induced by phenobarbital [25]. In addition, the growth inhibitory activity of procarbazine against murine L1210 leukemia in vivo was shown to be significantly enhanced when mice were pretreated with phenobarbital or diphenylhydantoin, but not methylprednisolone [15]. Additional data on the pharmacokinetic behavior of procarbazine is being obtained during an ongoing phase I clinical trial. Substantiation of these preliminary findings would imply that the significantly greater incidence of hypersensitivity reactions to procarbazine in brain cancer patients receiving EIASDs [26] may result from increased systemic exposure to a toxic metabolite that is not the immediate product of procarbazine biotransformation. In view of this possibility, the ability to measure the major oxidative metabolites of procarbazine assumed considerable interest. Unfortunately, it was found that the assay for the parent drug could not be used directly for determining these compounds due to substantial differences in their chromatographic behavior and chemical stability. Methods that are being developed and validated to assay procarbazine metabolites will be reported in a subsequent communication.

In summary, a procedure involving isocratic reversed-phase HPLC with ESI–MS detection has been developed for the determination of procarbazine in human plasma. ESI–MS with SIM at *m/z* 222.2 proved to be a highly specific and very sensitive method of detection for the quantitative analysis of procarbazine in plasma. The assay has been thoroughly validated and shown to be accurate and reproducible for measuring procarbazine hydrochloride at concentrations ranging from 0.5 to 50 ng/ml in plasma, with a sample volume of only 150 μl. The ability to determine procarbazine with this degree of sensitivity was required to accurately define the plasma concentration–time profile of the parent drug in patients receiving clinically relevant doses. Procarbazine exhibits sufficient stability in human plasma to permit specimens obtained from clinical trials to be processed for storage at −80 °C for subsequent analysis without any special precautions. In addition, a relatively large number of study samples can be prepared for analysis in a batch-wise manner because the drug is adequately stable under all conditions to which it is exposed during the procedures. Applicability of the assay for routine pharmacokinetic drug level monitoring has been established by its excellent performance during the analysis of approximately 500 plasma samples from a phase I clinical trial of the drug in brain cancer patients.

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