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QUANTITATIVE ANALYSIS OF PROCARBAZINE, PROCARBAZINE METABOLITES AND CHEMICAL DEGRADATION PRODUCTS WITH APPLICATION TO PHARMACOKINETIC STUDIES

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SUMMARY

Quantitative analytical methods are described for the analysis of the anticancer drug procarbazine and eight known metabolites including those known to have cytotoxic activity. A direct sample insertion mass spectrometric assay for procarbazine and the urinary excretion product, N-isopropyl-terephthalamic acid, has been developed. This method employs stable isotope labeled variants in a procedure that minimizes analytical errors that may be encountered in the quantitation of the chemically unstable parent drug. A liquid chromatographic method is described for the analysis of seven known procarbazine metabolites. Use of these methods is demonstrated by the analysis of procarbazine metabolism during incubation in a 9000-g rat liver homogenate preparation. Procarbazine disappearance and metabolite appearance are also monitored in rat plasma following intraperitoneal administration of a 150 mg/kg bolus dose. Applications to patient pharmacokinetics is demonstrated using the liquid chromatographic assay to follow the appearance of active procarbazine metabolites on the first and fourteenth day of an oral 250 mg/kg/day course of therapy of a patient being treated for cancer.

INTRODUCTION

Procarbazine, N-isopropyl-α-(2-methylhydrazino)-p-toluamide, Fig. 1, is a clinically useful antineoplastic agent for the treatment of Hodgkin's disease [1] and brain tumors [2,3]. Procarbazine is not an active molecule but may under-

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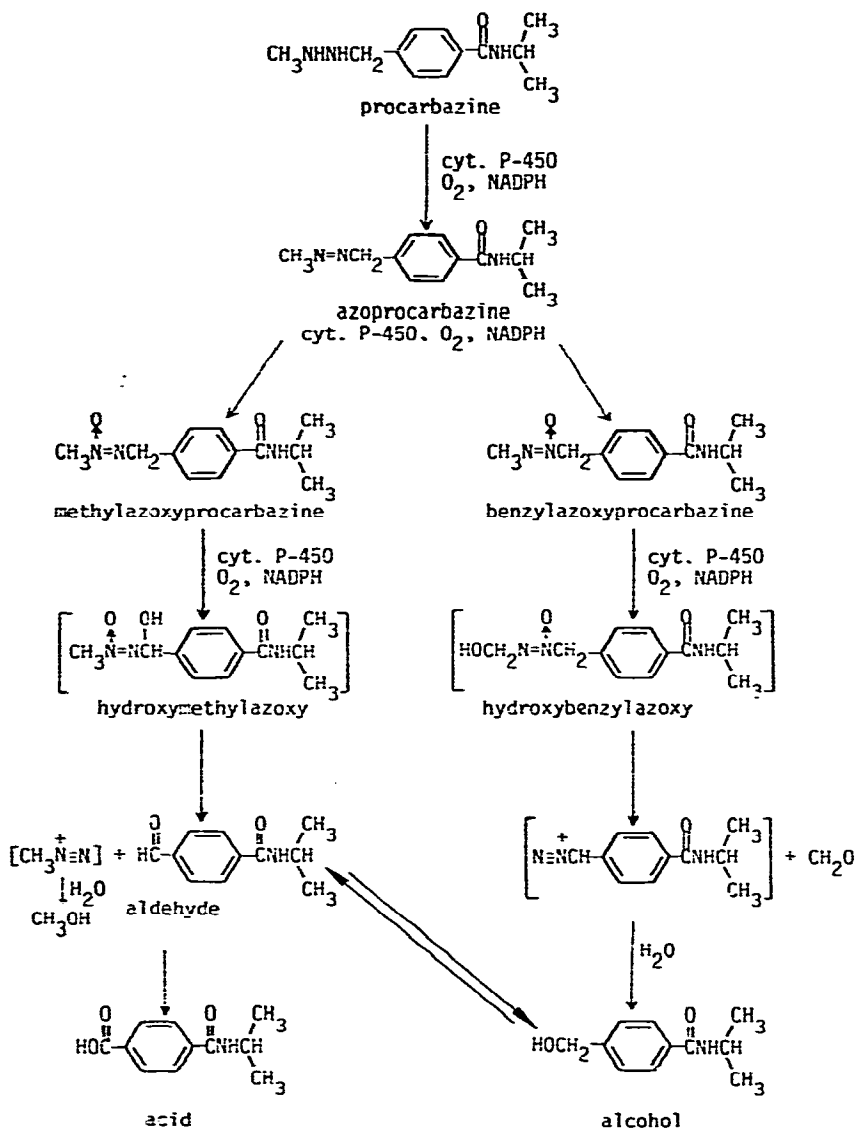
Fig. 1. The proposed pathway for the metabolic activation of procarbazine leading to the formation of alkylating intermediates. The scheme shows the formation of the known active metabolites azo-, methylazoxy- and benzylazoxyprocarbazine, as well as metabolic degradation products.

go metabolic conversion to cytotoxic alkylating intermediate(s). Investigations of procarbazine metabolism have identified three products that have antitumor activity in vivo [4] and are present in the plasma of animals after procarbazine treatment [5,6]. These include the initial procarbazine metabolite, N-isopropyl-α-(2-methylazo)-p-toluamide (azoprocarbazine) [7] and the isomeric azoxy compounds formed during azoprocarbazine metabolism, N-isopropyl-α-(2-methyl-ONN-azoxy)-p-toluamide (methylazoxy) and N-isopropyl-α-

(2-methyl-NNO-azoxy)-p-toluamide (benzylazoxy) [5]. Conversions of pro-carbazine to azoprocarbazine and of this metabolite to the azoxy isomers are mediated by cytochrome P-450 [8,9] and are stereoselective [5,8,9]. It has been suggested [5] that the azoxy isomers are converted to proximal active alkylating agent(s) by hydroxylation on the carbons adjacent to the azoxy function. The resulting hydroxy-azoxy intermediates may be chemically con-verted to active alkylating species in a reaction analogous to that proposed for 1,2-dimethylhydrazine [10]. The suggested scheme for metabolic activation of procarbazine is shown in Fig. 1.

In addition to the parent drug and the biologically active intermediates, there are a number of other chemical and metabolic reaction products. Reac-tion of the hydroxymethylazoxy intermediate may give a benzyldiazonium ion species that can react with water to give N-isopropyl-α-hydroxy-p-toluamide (alcohol). This product may also arise from the reduction of N-isopropyl-p-formylbenzamide (aldehyde) that may be formed by reaction of the isomeric hydroxy-azoxy intermediate [5]. Both of these metabolites may be converted to and excreted as N-isopropyl-terephthalamic acid (acid) which is the only identified urinary excretion product of procarbazine [11,12]. Azoprocarbazine may also isomerize chemically to N-isopropyl-p-formylbenzamide methyl-hydrazone [13] (hydrazone, structure not shown). Low-molecular-weight products, formaldehyde [14,15] and carbon dioxide [12,16,17] are also released during procarbazine metabolism.

Procarbazine is also converted in vivo to metabolic products that apparently result from free-radical reactions. These include methane [18,19] and N-iso-propyl-p-toluamide [5] (methyl, structure not shown). Methane is expired while the methyl metabolite may undergo successive metabolic oxidation steps to give alcohol, aldehyde, and acid.

As indicated above, procarbazine is converted to at least eight chemical and metabolic products by reactions involving the methylhydrazine portion of the molecule. No metabolic alteration of the aromatic or N-isopropylcarboxamide groups has been observed. This paper reports mass spectral analytical methods for the analysis of procarbazine and the acid urinary excretion product, and high-performance liquid chromatographic (HPLC) procedures for the quantita-tion of the biologically active azo and azoxy metabolites, as well as the alcohol, aldehyde, hydrazone, and methyl degradation products.

The use of these assay procedures is demonstrated by the analysis of pro-carbazine disappearance and metabolite formation in rat liver homogenate preparations. Applications to pharmacokinetic studies are shown by the analysis of procarbazine disappearance and metabolite appearance in rat plasma following an intraperitoneal (i.p.) dose as well as the monitoring of active metabolites in the plasma of a patient who received orally administered pro-carbazine during a 14-day course of therapy.

EXPERIMENTAL

Instrumentation

Mass spectra were obtained with a Finnigan 3200 mass spectrometer equip-ped with a dual chemical ionization/electron impact source and operated in the

chemical ionization mode. Isobutane was used as a reagent gas at an ion chamber pressure of 0.75 Torr and temperature of 175°C. Samples were introduced by means of a direct sample insertion probe equipped with a ceramic tip. Samples were deposited onto the ceramic rod, introduced into the ion source, and evaporated from the probe tip as it was heated by induction from the ion source. The mass spectrometer was interfaced to a Data General Nova 830 computer capable of operating the instrument in the selected ion monitoring mode.

HPLC analyses were performed utilizing a Chromatronix Model 3500 pump and a Chromatronix Model 220 mixed-wavelength UV detector operating at 254 nm. A Waters Assoc. (Milford, MA, U.S.A.) 30 cm × 3.9 mm I.D. C_{18} μBondapak (10 μm) column was used at ambient temperature.

Reagents and materials

Procarbazine hydrochloride, NSC-77213, was obtained from Dr. W.E. Scott of Hoffmann-LaRoche (Nutley, NJ, U.S.A.). The procarbazine chemical and metabolic products were synthesized as previously described [5]. 4-Methylacetophenone was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used as a standard in HPLC quantitation. N-(1,1,1,3,3,3-Hexadeuteroprop-2-yl)-terephthalamic acid (acid-$^2H_6$) and N-(1,1,1,3,3,3-hexadeuteroprop-2-yl)-α-(2-methylhydrazino)-*p*-toluamide hydrochloride (procarbazine-$^2H_6$ hydrochloride) were prepared using isopropylamine-$^2H_6$, which was synthesized in 27% yield from hydroxylamine hydrochloride (Eastman Kodak, Rochester, NY, U.S.A.) and acetone-$^2H_6$ (gold label, 99.5 atom %, Aldrich) according to the procedure of Colombini et al. [20].

Glass distilled methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and triple distilled water were used as solvents in HPLC analyses.

Mass spectral analysis

Procarbazine and the acid metabolite were analyzed in in vitro enzyme preparations or plasma using mass spectral analysis of extracts without chromatographic separation. A 0.5—1.0 ml sample was acidified to pH 2 by addition of 5—7 drops of 1 N hydrochloric acid. Known amounts of internal standards, procarbazine-$^2H_6$ hydrochloride and acid-$^2H_6$ were prepared as 500 μg/ml stock solutions in ethanol and stored at −30°C. The acidified sample was extracted with 3 × 5 ml of diethyl ether to remove the acid and acid-$^2H_6$. The combined ether extracts were evaporated to dryness with a stream of nitrogen at ambient temperature in preparation for mass spectral analysis. The aqueous phase of the above extraction was made basic, pH 11, by addition of 1 M sodium hydroxide and immediately extracted with 3 × 5 ml of diethyl ether to remove procarbazine and procarbazine-$^2H_6$. The combined ether extracts were evaporated to dryness with a stream of nitrogen at ambient temperature in preparation for mass spectral analysis.

The residue containing acid/acid-$^2H_6$ and procarbazine/procarbazine-$^2H_6$ may be analyzed separately or simultaneously by redissolving the residue in 100 μl of ethanol or methylene chloride and placing an aliquot of this solution onto the ceramic tip of the direct sample insertion probe. Ion current produced during the evaporation of the sample into the ion source is monitored at *m/z*

222.2 and 228.2 for procarbazine and procarbazine-$^2H_6$ protonated molecular ions and at m/z 208.2 and 214.2 for acid and acid-$^2H_6$ protonated molecular ions, respectively. Sample evaporation times ranged between 60 and 120 sec.

Procarbazine and the acid metabolite were quantified by comparison of the ion current ratio for the unknown and standard compounds and calculation of the concentration of the amount of procarbazine or acid in the sample volume by reference to the respective standard curve.

High-performance liquid chromatographic analysis

The metabolic and chemical reaction products, azoprocarbazine, methylazoxyprocarbazine, benzylazoxyprocarbazine, alcohol, aldehyde, hydrazone, and methyl are analyzed by HPLC using 4-methylacetophenone as an internal standard. A 0.5—1.0 ml sample was extracted with 5 × 3 ml of cold diethyl ether. The combined ether extract was evaporated to dryness with a stream of nitrogen at ambient temperature. The residue was redissolved in 250—500 μl of methanol and 10 μl (6.7 μg) of a stock solution of 4-methylacetophenone in methanol (67 mg per 100 ml) was added. After thorough mixing using a vortex mixer (Vanlab, San Francisco, CA, U.S.A.) and centrifugation for 1 min at 12,000 g using an Eppendorf Micro centrifuge, approximately 15 μl of the sample were injected onto the reversed-phase liquid chromatographic C_{18} μBondapak column. Separation was achieved using a water—methanol (20 : 80) solvent at a flow-rate of 2.0 ml/min. The quantities of the above procarbazine products present in the sample aliquot were determined from the ratio of the respective peak area to the internal standard 4-methylacetophenone by reference to the appropriate standard curve.

In vitro procarbazine metabolism kinetics

Procarbazine disappearance and metabolite appearance were monitored during incubation in rat liver 9000-g supernate. The supernate was prepared using Fisher C-344 male rats according to the procedure of Fouts [21]. The incubation mixture containing 10 ml supernate, 1.5 ml of 18.7 mM procarbazine (final concentration 2.0 μM), 2.0 ml of 0.1 M MgSO$_4$ (final concentration 14.3 mM), and 0.5 ml of 14.0 mM NADPH added every 20 min (final concentration 0.5 mM) was incubated with shaking at 37°C for 40 min. Two 1.0-ml aliquots of the incubation mixture were removed at 1, 10, 25, and 40 min. A 1.0-ml aliquot was analyzed for procarbazine and acid using the mass spectral assay and 1.0 ml was analyzed for metabolites using the HPLC assay.

In vivo procarbazine and metabolite kinetics in rat plasma after an i.p. dose

Fisher C-344 male rats weighing an average of 200 g received a 0.3-ml i.p. injection of procarbazine at a dose of 150 mg/kg body weight. The drug solution was prepared by dissolving 100 mg of procarbazine hydrochloride in 1 ml of 0.9% sodium chloride solution. At 10, 30, 60, and 90 min after administration of drug, two rats were anesthetized with diethyl ether and the blood removed by cannulation of the femoral artery. Blood was placed in a conical centrifuge tube and centrifuged at 1000 g for 10 min at 5°C. Equal aliquots of plasma from each of the two animals were pooled to represent a single postinjection time point. A 0.5- or 1.0-ml plasma aliquot was analyzed using the mass spectral and HPLC assays.

In vivo procarbazine metabolite kinetics in human plasma after an oral dose

A female patient undergoing chemotherapy for treatment of a malignant brain tumor received an oral dose of 250 mg procarbazine hydrochloride per kg body weight/day during a 14-day course of therapy. Plasma was assayed for circulating metabolites on the first and fourteenth day of therapy. At 20, 40, 60, 90, 120, 190, 250, and 360 min after administration of drug, a 3.0-ml aliquot of blood was removed and centrifuged at 12,000 g for 1 min. A 1.0-ml aliquot of plasma was removed and extracted with 3 × 4 ml of diethyl ether. The combined ether extract was evaporated with a nitrogen stream at ambient temperature and the residue was redissolved in 20 μl of methanol and 5 μl (3.4 g) of the 4-methylacetophenone standard stock solution were added. This solution was again centrifuged at 12,000 g for 1 min and 15—20 μl of the solution removed for analysis of procarbazine metabolites using the HPLC assay. Plasma concentrations for procarbazine and the acid metabolite were not obtained during this preliminary study.

RESULTS AND DISCUSSION

Procarbazine hydrochloride is reasonably stable in acidic solution, but the free hydrazine group is rapidly oxidized to azoprocarbazine when the solution is made basic. The rapid degradation reaction complicates the accurate analysis of this drug as significant decomposition may occur in the process of extracting small amounts of procarbazine from plasma into organic solvents. For this reason, we have selected to use the mass spectral analysis method in which an isotopic variant, procarbazine-^{2}H$_6$, is added to the sample aliquot. This establishes a ratio between intact procarbazine and the standard that is not altered by subsequent partial losses due to chemical oxidation or extraction. This procedure has an advantage over previously reported assays for procarbazine as a pure substance or in drug dosage forms [22] in that these methods are not suitable for analysis of small amounts of drug in plasma. Quantitative methods based on HPLC analysis of radiolabeled drug and metabolites are not readily applicable to patient pharmacokinetic studies [9]. A gas chromatography—mass spectrometry assay for procarbazine and metabolites has been reported [23]; however, results obtained using this assay method are not consistent with this report and others [5, 7—9, 13].

Standard curves for the analysis of procarbazine and the acid metabolite were found to be linear for sample/standard ion current ratios of 0.2—20. Recovery of procarbazine from human plasma averaged 95% and acid recovery averaged 103% over a range of plasma concentrations. The standard deviation of the procarbazine assay, determined from multiple analyses of known plasma doped samples, was 10% and the sensitivity limit was 5 nmol/ml plasma. Accuracy and sensitivity limits for the acid metabolite were comparable to those of procarbazine. The acid is assayed by the mass spectral method as a matter of convenience. The compound could be analyzed with HPLC, but the large difference in polarity between the acid and other procarbazine metabolites (Fig. 1) would have required a separate HPLC assay for the acid or the use of gradient elution for the simultaneous analysis of acid with the other metabolites.

Data obtained with this assay procedure are included in Figs. 2 and 3. Fig. 2 shows the partial disappearance of procarbazine during incubation in a 9000-*g* rat liver homogenate preparation. The acid is found to be a significant metabolite that is formed in the latter stages of the incubation. Fig. 3 shows procarbazine clearance from rat plasma following a 150 mg/kg i.p. bolus dose. Procarbazine plasma concentration decreases from 0.48 n*M* to 0.03 n*M*, four half-lives, within 90 min of administration. The acid appearance in plasma reaches a steady-state concentration after 20 min and is maintained at 0.10–0.18 n*M*.

An HPLC assay was developed for the simultaneous assay of azoprocarbazine, methylazoxy, benzylazoxy, alcohol, aldehyde, hydrazone, and methyl metabolites. A representative chromatogram of a mixture of these compounds and the 4-methylbenzophenone standard is shown in Fig. 4a. Good separation is obtained for all compounds except for the methylazoxy and hydrazone derivatives under conditions in which the eight compounds are eluted within 70 min. Table I shows the ratio of elution times for each metabolite relative to the standard. Normal elution times for 4-methylacetophenone averaged 60 min using 20% water in methanol solvent at a flow-rate of 2.0 ml/min. Standard curve slopes and *y*-intercepts are reported as are the standard deviations of
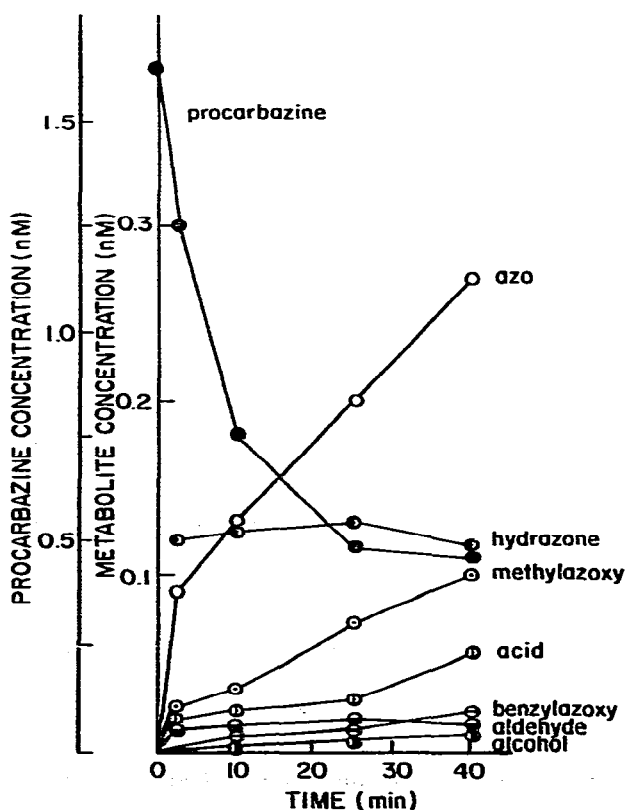

Fig. 2. Procarbazine disappearance and metabolite appearance monitored during a 40-min incubation of a 1.62 n*M* procarbazine solution in a 9000-*g* rat liver supernate preparation.
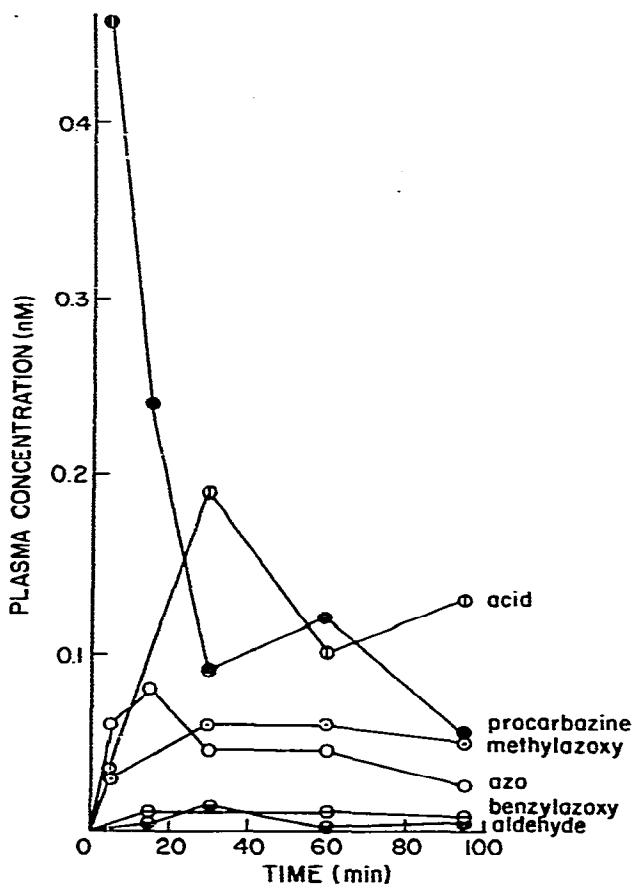
Fig. 3. Procarbazine disappearance and metabolite appearance in rat plasma following administration of a 150 mg/kg i.p. bolus dose of procarbazine.

TABLE I

HPLC ASSAY PARAMETERS OF PROCARBAZINE METABOLITES OBTAINED USING A C_{18} μBONDAPAK REVERSED-PHASE COLUMN AND 20% METHANOL IN WATER AS A SOLVENT

Compound	Elution ratio*	Slope**	y-Intercept	Recovery (%)	S.D.*** (%)
Aldehyde	0.27	2.81	−0.14	98	11
Benzylazoxy	0.39	9.31	−0.16	110	6
Methylazoxy	0.50	10.19	−0.40	107	5
Hydrazone	0.55	5.96	−0.18	98	5
Azo	0.67	3.91	0.10	99	2
Methyl	0.75	4.60	−0.09	117	8

*Retention time relative to 4-methylacetophenone = 1.00.
**Obtained from a plot of ratio of (sample/reference amounts added) (sample/reference peak area)⁻¹.
***Standard deviation of amount found from known was determined from nine or more measurements made over a range of concentrations of metabolite in human plasma.
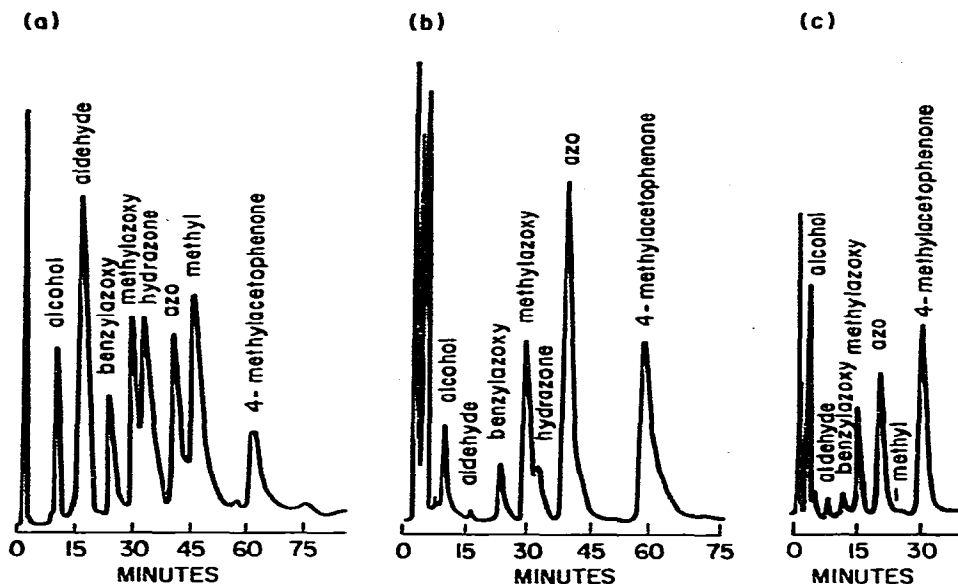
Fig. 4. HPLC chromatograms obtained using a C_{18} μBondapak reversed-phase column to separate (a) a standard mixture of synthetic procarbazine metabolites, (b) metabolites extracted from a 9000-g rat liver supernate incubation mixture, (c) metabolites extracted from rat plasma following administration of a 150 mg/kg i.p. bolus dose.

measured concentrations from known values of each agent added to human plasma. The concentration of each metabolite was calculated from the standard curves and the ratio of peak areas for the agent and internal standard. Peak areas were calculated from the peak height times the peak width at one-half peak height. Recovery of metabolites from plasma using the described ether extraction procedure was near 100%. Sensitivity limits for these HPLC assays were 2 nmol/ml or 100 ng injected onto the column.

This procedure was used to assay the metabolite formation in 9000-g rat liver supernate and the plasma concentration of metabolites in rat. Representative HPLC traces from the analyses are shown in Fig. 4b and c, respectively. The elution time for this assay may be shortened for convenience as in the in vivo plasma assay (Fig. 4c), where the hydrazone product was not detected.

The formation of procarbazine metabolites in vitro is shown in Fig. 2. Azoprocarbazine is the major metabolite after 40 min incubation. Methylazoxy is present in a higher concentration than the benzylazoxy isomer, and both compounds may be further metabolized to alcohol, aldehyde, and acid. Hydrazone is present at relatively high concentrations initially and does not increase during incubation. Hydrazone is probably present as an impurity or as a chemically generated artifact in this experiment.

Procarbazine metabolites may also be monitored in rat plasma (Fig. 3). Azoprocarbazine is less abundant in rat plasma than methylazoxyprocarbazine. Benzylazoxy and aldehyde are present in trace amounts. The alcohol was not monitored. Methyl metabolite, although sometimes observed, was not detected in these animals.

The plasma concentrations of procarbazine metabolites in man are shown in Fig. 5. Following a 250 mg/kg oral dose, the three active metabolites, azo, methylazoxy, and benzylazoxy are present in plasma in significant concentrations. Methylazoxyprocarbazine is the major circulating metabolite and the benzylazoxy isomer is present at a concentration approximately equal to its precursor, azoprocarbazine, on the first day of treatment. Since both methylazoxy and benzylazoxy procarbazine require further metabolic activation, the plasma concentration of these compounds may not be simply related to antitumor activity. The turnover, or amount of azoxy isomer formed and then converted to active product, may be more important than the steady-state plasma concentration of these intermediate metabolites. Data contained in Fig. 5 also demonstrate that a significant increase in the plasma concentration and a change in the relative concentration of metabolites occur by the fourteenth day of therapy. Studies are in progress to determine patient pharmacokinetics of circulating procarbazine metabolites using this assay procedure.
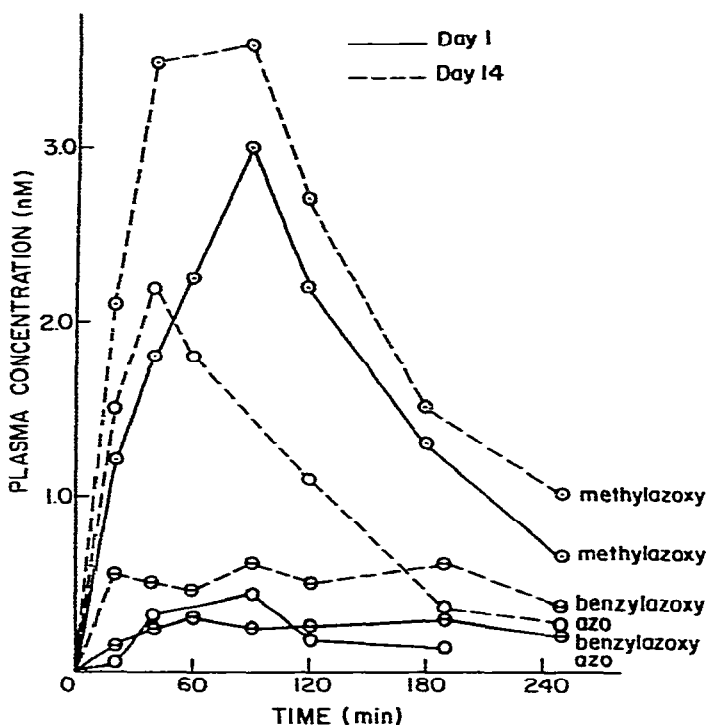

Fig. 5. Plasma concentrations of azo and azoxyprocarbazine metabolites are shown for a period of 250 min following the oral administration of a 250 mg/kg/day dose on days 1 and 14 of a 14-day treatment schedule.

CONCLUSION

Assay methods are described for the analysis of procarbazine and eight metabolic products. These procedures are useful for the analysis of in vitro metabolism and for the quantitation of plasma concentrations in rat and man.

The sensitivity of the HPLC assay is sufficient to permit pharmacokinetic studies of circulating active procarbazine metabolites in patients receiving normal therapeutic oral doses of this anticancer drug. This is the first procedure described that is applicable to clinical studies of procarbazine biodistribution.

ACKNOWLEDGEMENTS

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