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ANALYSIS OF PROCARBAZINE AND METABOLITES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Twelve compounds representing procarbazine, seven metabolites, and an internal standard were analyzed by gas chromatography—mass spectrometry on a 3% OV-1 column. Procarbazine and four metabolites were derivatized with acetic anhydride.

A sensitive, specific and quantitative assay was established by selected ion monitoring using a synthetic analogue of the drug as an internal standard. The limits of detection were approximately 1 ng/ml of plasma while the limits of quantitation were 10 ng/ml of plasma.

Studies on the degradation of procarbazine · HCl in 0.05 *M* phosphate buffer (pH 7.4) were compared to in vivo studies. At 1 h after incubation of procarbazine · HCl in buffer, the azo and aldehyde metabolites were detected in the highest concentrations representing 27.2% and 20.3% of total drug and metabolites. In the in vivo studies, analyses of rat plasmas indicated that 1 h after an oral dose of procarbazine · HCl, the aldehyde metabolite represented 72% of the total drug and metabolites, and that relatively little of the azo metabolite was present.

INTRODUCTION

Procarbazine · HCl (N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride) is a methylhydrazine derivative which was developed initially as a monoamine oxidase inhibitor [1, 2] and was subsequently shown to be active against a variety of animal tumors. Clinically, it is primarily used as part of the MOPP (mustargen, oncovin, procarbazine, and prednisone) regimen for treatment of Hodgkin's disease and, by itself, is recommended as secondary therapy for treatment of oat cell carcinoma of the lung and primary neoplasms of the brain. Like other cancer chemotherapeutic agents, the clinical use of procarbazine is frequently associated with one or more severe types of toxicity.

However, the pharmacologic basis for either the toxic or therapeutic effect of the drug has not yet been clearly delineated.

Procarbazine · HCl is stable at room temperature for several years in the dry form if it is not exposed to UV light. In solution or on exposure to UV light, the drug is rapidly converted to numerous by-products. Because the drug is rapidly oxidized in solution and also is metabolized in biological systems, it has been very difficult to determine with specificity, the level of procarbazine and its metabolites in biological tissues after a pharmacologic dose of the drug. In addition, since the drug is also known to be rapidly degraded during assay procedures, it is unlikely that accurate data are currently available on the in vivo levels of the drug and its metabolites.

The recent development of a high-performance liquid chromatographic (HPLC) method for the analysis of eight procarbazine metabolites improved previous assay procedures and permitted their detection in vivo from rat plasma [3]. In this study we have developed a very sensitive and quantitative method based on a derivatization procedure which stabilizes the drug and certain metabolites and allows the detection of procarbazine and seven metabolites in biological fluids using a gas chromatograph—mass spectrometer with a data system.

MATERIALS AND METHODS

Materials

N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride (procarbazine · HCl); N-isopropyl-*p*-formylbenzamide methylhydrazone (hydrazone); N-isopropyl-*p*-formylbenzamide (aldehyde); N-isopropyl-α-(2-methyl-2-azoxy)-*p*-toluamide (azoxy), and N-isopropyl-α-hydroxy-4-toluamide (alcohol) were graciously donated by Hoffmann-LaRoche. N-Isopropyl-*p*-toluamide and N-ethyl-*p*-toluamide were synthesized in this laboratory. All other chemicals used in the syntheses and assay procedure were of certified ACS grade or better or were of the highest purity available.

Synthesis of N-isopropyl-p-toluamide and internal standard

N-Isopropyl-*p*-toluamide was synthesized by a modification of the method of Shriner et al. [4]. A 0.5-ml aliquot of *p*-toluoyl chloride was added dropwise to a solution of 0.5 ml of isopropylamine, 5 ml of dry pyridine and 10 ml of dry benzene. The mixture was heated for 30 min at 60—70°C and then added to 100 ml of water. The organic layer was decanted and the water layer was re-extracted with 10 ml of benzene. The combined organic phases were again extracted with a 5% sodium carbonate solution. The organic phase was dried over magnesium sulfate, filtered, and evaporated until white crystals precipitated. The crystals were then recrystallized from a solution of cyclohexane and ethyl acetate.

N-Ethyl-*p*-toluamide, the internal standard, was prepared from ethylamine and *p*-toluoyl chloride. A commercial 70% aqueous solution of ethylamine was extracted with benzene. The benzene extract was dried over magnesium sulfate and reacted with *p*-toluoyl chloride. Once the dried organic ethylamine solution was obtained, the synthesis of N-ethyl-*p*-toluamide proceeded similarly

to the synthesis of N-isopropyl-*p*-toluamide. For both synthesized compounds, purity was determined by the observance of a single gas chromatographic peak which was characterized by mass spectral analysis.

Extraction and derivatization procedures

Samples to be assayed for drug and metabolites were taken up in 1 ml 0.05 *M* phosphate buffer (pH 7.4) and extracted with 4 ml of toluene by vortex mixing for 1 min. Extraction from buffer was immediately followed by decanting the toluene layer directly into vials containing 0.3 ml of acetic anhydride. However, plasma extracts were centrifuged for 5 min at 2000 *g* to remove remaining solid from the organic phase before decanting. The acetic anhydride reactions were allowed to proceed for 20 min and then air dried. The dried vials were stored at —78°C until the residue was taken up in ethyl acetate for injection into the gas chromatograph.

Gas chromatographic—mass spectrometric assay

A Finnigan 4000 gas chromatograph—mass spectrometer with a 6100 computer data system was utilized for all assays. A 6-ft. glass column with 3% OV-1 on 100—120 mesh Supelcoport packing was employed for all analyses. The following gas chromatographic—mass spectrometric (GC—MS) parameters and conditions were established: injection port temperature, 260°C; oven temperature, 140—270°C at 10°C/min; separator temperature, 265°C; transfer line temperature, 265°C; ionizer temperature, 250°C (electron impact, EI) and 200°C [chemical ionization (CI)—methane and CI—isobutane]; emission current, 4.9 mA; electron multiplier voltage, 1770 V; carrier gas flow-rate, 30 ml/min. Total ion current spectra were obtained in EI, CI—methane, and CI—isobutane. Where possible, peak identification was verified with standards either donated by Hoffmann-LaRoche or synthesized in this laboratory. Once the peak separation, retention time, and identities had been established, all subsequent quantitative work was performed by single ion monitoring (SIM) using CI with isobutane as the reagent gas.

Preparation of standard curves and recovery curves

Recovery curves from rat plasma were established for four procarbazine metabolites (hydrazone, alcohol, aldehyde, and N-isopropyl-*p*-toluamide). Five concentrations ranging from 10 ng/ml to 1 μg/ml were prepared in plasma for each metabolite using N-ethyl-*p*-toluamide as the internal standard. The plasmas were equilibrated for 1 h, extracted and derivatized for injection into the chromatograph. Standard curves were also prepared for the N-isopropyl-*p*-formylbenzamide methylhydrazone and N-isopropyl-*p*-toluamide by direct injection of these compounds in concentrations ranging from 1 ng to 500 ng into the chromatograph along with the internal standard.

Because not all metabolites were available in pure form, several assumptions had to be incorporated into the assay [5]. The levels of procarbazine, azo, and azoxy were all calculated from the hydrazone recovery curve. The levels of N-isopropyl-*p*-ethylbenzamide were calculated from the standard curve established for N-isopropyl-*p*-toluamide.

TABLE I

CHARACTERIZATION OF PROCARBAZINE AND PRODUCTS ISOLATED BY GC–MS

Peak No.	Compound detected	M.W.	Retention time (min)	EI base peak	CI–Methane M ± 1	CI–Methane base peak	CI–Isobutane M ± 1	CI–Isobutane base peak	Drug or metabolite
1	Methylhydrazine (diacetyl)	130	1.65	88	131	89	131	131	methylhydrazine
2	N-Ethyl-*p*-toluamide	163	3.13	119	164	164	164	164	internal Standard
3	N-Isopropyl-*p*-toluamide	177	3.20	119	178	178	178	178	N-isopropyl-*p*-toluamide
4	N-Isopropyl-*p*-ethylbenzamide	191	4.14	133	192	192	192	192	azo
5	N-Isopropyl-*p*-formylbenzamide	191	4.62	133	192	192	192	192	aldehyde
6	N-Isopropyl-α-(2-methylazo)-*p*-toluamide	219	5.70	90	220	177	220	220	azo
7	N-Isopropyl-α-hydroxy-4-toluamide	193	6.15	135	194	194	194	194	alcohol
8	N-Isopropyl-α-hydroxy-4-toluamide (acetyl)	235	6.80	177	236	57	236	236	alcohol
9	N-Isopropyl-α-(2-methyl-2-azoxy)-*p*-toluamide	235	7.79	90	236	236	236	236	azoxy
10	N-Isopropyl-*p*-formylbenzamide methylhydrazone	219	8.60	161	220	220	220	220	azo
11	N-Isopropyl-*p*-formylbenzamide methylhydrazone (acetyl)	261	11.06	73	262	262	262	262	hydrazone
12	N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide (diacetyl)	305	11.79	191	306	89	306	306	procarbazine

Degradation study in buffer

In order to study the degradation of drug in buffer, 2 mg of procarbazine · HCl were dissolved in 100 ml of 0.05 *M* phosphate buffer (pH 7.4) and allowed to incubate with constant stirring for 24 h at room temperature. One-milliliter aliquots were removed at timed intervals for analysis.

Animal sample preparation and extraction

Normal Sprague-Dawley male rats weighing 180—200 g were housed in plastic cages and fed Purina lab chow and water ad libitum before and during experimentation. The rats were given a single 30 mg/kg oral dose of procarbazine · HCl in deionized water. One hour after administration, the rats were decapitated and trunk blood was collected in heparinized vials. The blood was centrifuged at 2000 *g* for 5 min to obtain plasma and 1-ml aliquots were placed in tubes containing the internal standard, N-ethyl-*p*-toluamide. Each tube was extracted with 4 ml of toluene by mixing for 1 min in a vortex mixer.

RESULTS

The GC assay of a mixture of procarbazine and metabolites as displayed by total ion current (TIC) using N-ethyl-*p*-toluamide as the internal standard is given in Fig. 1. Good separation of all peaks was achieved on the Supelcoport

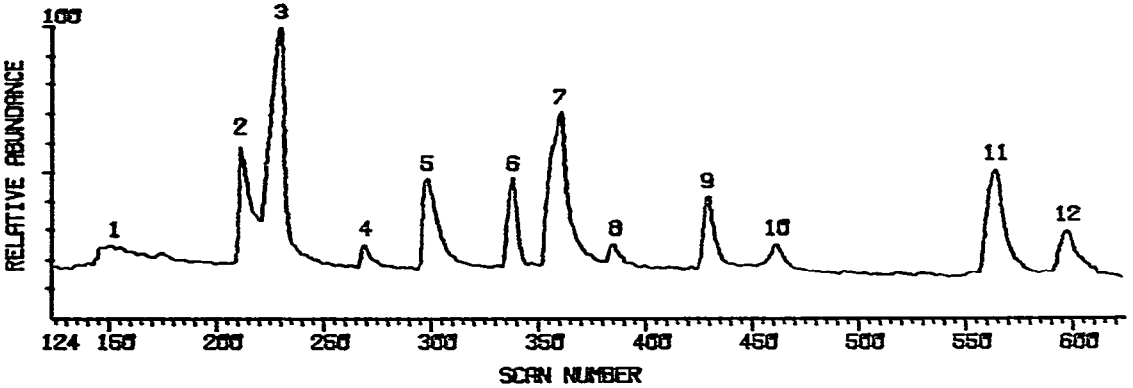

Fig. 1. TIC plot of a mixture of procarbazine, metabolites, and degradation products after derivatization with acetic anhydride. Peak numbers correspond to those identified in Table I.

OV-1 column. Maximum sensitivity for quantitation was achieved by SIM using CI—isobutane by focusing on the base peak for procarbazine and for each metabolite. The data system was programmed to sequentially focus on three sets of ions so that all twelve compounds assayed could be monitored from a single injection. The time interval from elution of the first peak, methylhydrazine, to the elution of the final peak, procarbazine, was 10.4 min. All peaks were initially identified from their EI and CI mass spectra and from their characteristic retention times as compared to that of standard compounds (Table I). Although twelve distinct peaks were eluted from the column, these represented internal standard, drug and only seven metabolites. Direct injection of these products resulted in a sensitivity limit in the picogram range and was linear from 1 ng to at least 500 ng.

TABLE II

DEGRADATION OF PROCARBAZINE · HCl IN 0.05 *M* PHOSPHATE BUFFER (pH 7.4)

Values represent percent of total procarbazine and metabolites analyzed with time in a 1-ml aliquot of a 100 ml 0.05 *M* phosphate buffer (pH 7.4) solution containing 2 mg of procarbazine · HCl.

Time	Procarbazine	Hydrazone	Azo	Azoxy	Alcohol	Aldehyde	N-Isopropyl-*p*-toluamide	Methyl-hydrazine
0 min	58.0	19.8	5.6	N.D.*	N.D.	12.3	4.2	N.D.
5 min	54.5	16.6	9.4	N.D.	N.D.	14.3	5.2	N.D.
45 min	12.0	13.7	26.8	N.D.	14.9	16.5	16.3	N.D.
1 h	8.0	13.6	27.2	N.D.	14.3	20.3	16.4	N.D.
2 h	3.1	11.2	31.2	N.D.	13.4	26.5	14.6	N.D.
4 h	0.6	8.1	25.9	N.D.	10.9	40.3	14.2	N.D.
8 h	N.D.	5.8	23.2	N.D.	6.6	54.0	13.7	N.D.
24 h	N.D.	8.8	14.6	N.D.	6.8	60.4	9.3	N.D.

*N.D. = Not detected.

TABLE III

PER CENT OF PROCARBAZINE AND METABOLITES IN PLASMA

Values obtained from normal male Sprague-Dawley rats decapitated 1 h after a single oral dose (30 mg/kg) of procarbazine · HCl in deionized water.

	Procarbazine	Hydrazone	Azo	Azoxy	Alcohol	Aldehyde	N-Isopropyl-*p*-toluamide	Methyl-hydrazine	Total (μg/ml)
Rat 1	0.2	2.3	4.4	3.3	15.8	72.0	2.1	N.D.*	6.0
Rat 2	0.4	4.5	5.9	2.8	13.4	68.0	4.7	N.D.	7.5

*N.D. = Not detected.

Direct injection of N-isopropyl-α-(2-methylazo)-*p*-toluamide (peak 6) into the gas chromatograph resulted in the elution of two additional peaks (peaks 4 and 10). These two peaks apparently resulted from on-column isomerization and degradation of the N-isopropyl-α-(2-methylazo)-*p*-toluamide. Therefore, the total azo metabolite was estimated by including these two peaks in the azo pool. Since the alcohol metabolite was only partially derivatized (peaks 7 and 8) these two peaks were also added together to determine total alcohol metabolites. All other peaks represent compounds that were stable under the conditions of the assay procedure.

For the purpose of comparison with the in vivo studies, the levels of drug and degradation products were determined after a 1-h incubation period of procarbazine in 0.05 *M* phosphate buffer (pH 7.4) (Table II). The major degradation products were the azo and aldehyde. Small quantities of unchanged procarbazine were also present as were hydrazone, alcohol, and N-isopropyl-*p*-toluamide. Levels of azoxy and methylhydrazine were not detected.

Normal male Sprague-Dawley rats were given a single oral 30 mg/kg dose of procarbazine · HCl and sacrificed 1 h later (Table III). Of the total drug found in plasma, the aldehyde appeared as the major metabolite constituting 72.0% of the total extractable drug and metabolites. No other single metabolite constituted more than 16% of the total. Only 0.2% of unchanged procarbazine was present in the plasma 1 h after administration of the drug.

In order to gain insight into the accuracy and reproducibility of the assay procedure from the initial extraction to the final quantitation, several aliquots of the same plasma sample from a rat given a 30 mg/kg single oral dose of procarbazine were assayed. In Table IV, the data suggest that for the low concentrations being measured, there is acceptable reproducibility from plasma. This indicates that the assay procedure itself is a viable means of determining levels of procarbazine and metabolites from biological fluids.

DISCUSSION

Several derivatizing processes were attempted in order to prevent the oxidation of procarbazine to the azo and hydrazone products. Trimethylphenylammonium hydroxide, several silylating reagents, and trifluoroacetic anhydride reacted with only one of the hydrazine nitrogens. Acetic anhydride and N-acetylimidazole were able to derivatize both active sites on the nitrogens of the hydrazine moiety of procarbazine although N-acetylimidazole produced only a partial acetylation reaction. Acetic anhydride gave a complete conversion of procarbazine to its diacetyl form. It also acetylated the one active site on the nitrogen of the hydrazone. It was necessary to react both nitrogens of the hydrazine in order to distinguish the hydrazine from the hydrazone given the potential ability of the monoacetylhydrazine to oxidize to monoacetylhydrazone. The alcohol with an active alcoholic hydrogen was incompletely acetylated and, therefore, both alcohol and acetylated alcohol were detected. Methylhydrazine, a proposed procarbazine metabolite was acetylated on each of its hydrazine nitrogens to yield a diacetyl product.

The GC—MS assay permitted the detection of procarbazine and seven

TABLE IV

STUDIES ON THE REPRODUCIBILITY OF THE ASSAY PROCEDURE FROM PLASMA

Aliquot	Procarbazine	Hydrazone	Azo	Azoxy	Alcohol	Aldehyde	N-Isopropyl-*p*-toluamide	Methyl-hydrazine	Total (μg/ml)
1	0.4	4.3	4.8	3.8	N.D.	84.0	2.3	N.D.*	4.9
2	0.2	2.3	4.4	3.3	15.8	72.0	2.1	N.D.	6.0
3	0.3	1.3	4.3	3.5	16.0	72.0	2.1	N.D.	6.4
$\overline{X}$ ± S.D.**	0.3 ± 0.1	2.6 ± 1.7	4.5 ± 0.3	3.5 ± 0.3	13.2 ± 2.8	76.0 ± 8.0	2.2 ± 0.1		5.8 ± 0.9

*N.D. = Not detected.

**Mean ($\overline{X}$) = Derived from reported values plus one-half the minimal detectable level (where one value is N.D.).

metabolites from biological systems. Although some variation in sensitivity exists between metabolites, the general limit of sensitivity has been determined to be approximately 1 ng/ml of plasma. The limit of quantitation, based upon linearity of standard curves, is 10 ng/ml of plasma. The HPLC method for the detection of procarbazine and its metabolites as described by Burce and Boehlert [6] reported a sensitivity limit of 20 ng and a limit of quanitation of 200 ng per injection. Because the maximal human dose range of procarbazine is six times lower per kg than the relatively low animal dosage (30 mg/kg) used in the present studies, this increase in sensitivity of the GC—MS method may be crucial for clinical studies with this drug.

The retention time of the final peak of the GC—MS assay was 11.8 min. The HPLC assay of Burce and Boehlert [6] which separated procarbazine and three metabolites had a final peak retention time of 14.2 min. The HPLC assay established by Weinkam and Shiba [3] which separated eight procarbazine metabolites required 90 min for the elution of the final peak. Such long retention times could encourage on-column degradation of procarbazine and metabolites.

Eight percent of the total drug and metabolites present in the buffered solution at the end of 1 h existed as unchanged procarbazine, but the percentage of unchanged drug present in the blood of rats given a single dose of procarbazine at an equivalent period of time ranged from only 0.2 to 0.4%. The hydrazone metabolite was present at 13.6% in the buffer study whereas it was approximately one-fourth lower in plasma at an equivalent period of time. The azoxy metabolite did not form by degradation in buffer but was found in low but significant levels in blood. The majority of the drug was present as the azo metabolite in the in vitro buffer study and as the aldehyde metabolite in plasma.

A very slow conversion of the aldehyde to N-isopropylterphthalamic acid has also been suggested [3]. However, under the conditions employed in the assay procedure used in our study, this metabolite was not detected. Methylhydrazine, a proposed metabolite of procarbazine, was not detected in either the buffer or the in vivo studies by the method used in this work.

As was expected, the in vivo breakdown and metabolism of the drug and its metabolites was even more rapid than the degradation of the drug in buffer. It is possible that the more rapid conversion of procarbazine to its metabolites in vivo was due to microsomal metabolism [3, 7—9]. Since the level of the azo metabolite in blood was markedly lower than that noted in buffer at an equivalent period of time, it would seem likely that this metabolite is a major substrate for enzymatic metabolism.

The GC—MS assay described in this study provides a sensitive, rapid analysis of procarbazine and its major metabolites. The data obtained from the animal studies supports the viability of this assay procedure for the determination of procarbazine and metabolites in biological systems.

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