Quantitative Analysis and Stability of 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide in Acidic Aqueous Solutions

DAVID S. DRESBACK and JOSEPH F. GALLELLI

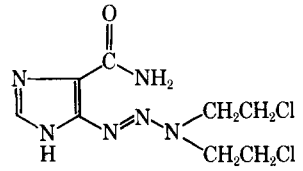
Abstract □ Methods of quantitative analysis by UV spectrophotometric, colorimetric, and ionic chloride determination are reported for 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (NSC-82196) in the presence of its degradation products. With the exception of the ionic chloride method of analysis of samples exposed to light, all three methods showed good agreement for stability studies of NSC-82196 in acidic aqueous solutions exposed to light and dark and stored at 25°. A sterile lyophilized hydrochloride salt of NSC-82196 for parenteral use, reconstituted with water for injection, was found to have a $t_{1/2}$ = 150 min. at 25° and $t_{1/2}$ = 65 hr. at 4°.

Keyphrases □ 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide—quantitative analysis, stability □ TLC—separation □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer □ Ionic chloride determination—analysis

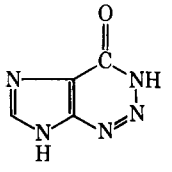
5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (NSC-82196) is a promising antileukemic agent. It has been shown to be superior, in the murine leukemia L-1210 system, to 5(or 4)-[3,3-dimethyl-1-triazeno]imidazole-4(or 5)-carboxamide (NSC-45388), an analog of similar structure presently in clinical trials (1).

A sterile lyophilized hydrochloride salt of NSC-82196, for parenteral use, was prepared by the Pharmaceutical Development Service. Since the drug is extremely unstable in aqueous solutions, adequate assay procedures were needed to determine the potency and stability of the formulated dosage form.

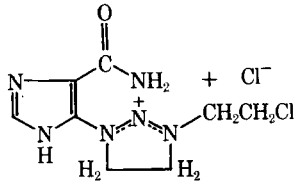
NSC-82196 is an analog belonging to a series of dialkyltriazenoimidazoles that undergoes rapid decomposition in aqueous acidic solutions. The resulting degradation products have been reported to be "transformation" product (NSC-112970) (II) and 2-azahypoxanthine (NSC-22419) (III) (2–4). The formation of NSC-22419 is preceded by the photodegradation of NSC-82196 to an intermediate 5-diazoimidazole-4-carboxamide (IV), which cyclizes by intramolecular coupling to NSC-22419 (3–5).



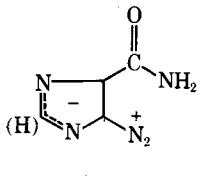
NSC-82196
I



NSC-22419
III



NSC-112970
II



5-diazoimidazole-4-carboxamide
IV

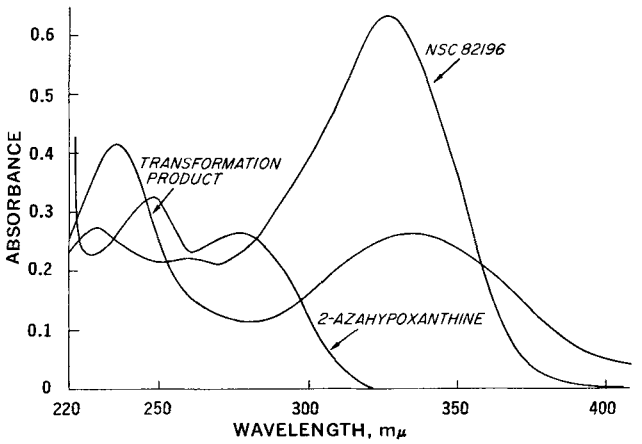


Figure 1—*UV spectra of NSC-82196, NSC-112970, and NSC-22419 in pH 2.31 phosphate buffer.*

Loo and Stasswender (6) reported a colorimetric method of assay suitable for intact NSC-82196. A UV spectrophotometric assay has also been reported which is specific for the degradation products, NSC-112970 and NSC-22419, as well as NSC-82196 (2). Adaptations of these two methods were used in this study to assay intact NSC-82196. Since one chloride ion is liberated for each molecule of transformation product formed, an ionic chloride method of assay was also used to follow the degradation of NSC-82196. Stability studies were performed to correlate all three methods of assay and to outline limitations, if any. The stability of a reconstituted, lyophilized, parenteral dosage form was determined under various conditions of light and dark at 25 and 4°.

EXPERIMENTAL

Reagents—The following were used: 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide,[1] transformation product[2] (m.p. 209° dec.), and 2-azahypoxanthine monohydrate[2] [m.p. 208–209° (explosive), darkens at 150°]. 5-Diazoimidazole-4-carboxamide[3] was synthesized according to the method of Shealy

[1] Prepared by Parke, Davis & Co., Detroit, Mich., and obtained from the Drug Development Branch, Cancer Chemotherapy, National Service Center, NCI, Bethesda, Md.; m.p. 208° dec., darkens at 150°, purity determined from IR spectrum and ionic chloride determination.

[2] Prepared by Southern Research Institute, Birmingham, Ala.

[3] *Elemental analysis*—Calcd.: C = 35.04%, H = 2.21%, N = 51.09%; Found: C = 35.01%; H = 2.17%, N = 50.74%. Elemental analysis was performed by Microanalytical Laboratory, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.

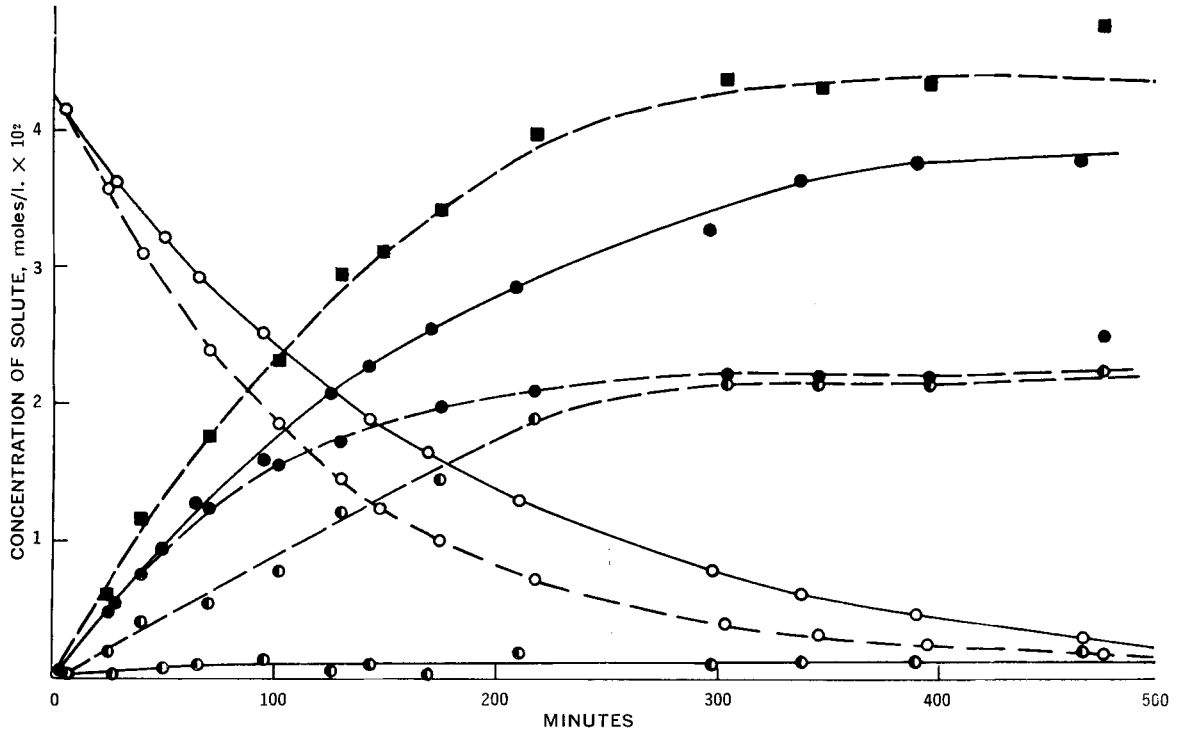


Figure 2—*Verification of stoichiometry of NSC-82196 hydrolysis at pH 2.31 phosphate buffer and 25° in light* (- - -) *and dark* (——). *Key:* ○, *NSC-82196;* ●, *NSC-112970;* ◐, *NSC-22419; and* ■, *NSC-112970 plus NSC-22419.*

et al. (5) [m.p. 199° (explosive), darkens at 149–150°]. Sterile lyophilized hydrochloride salt of NSC-82196 for injection, 100 mg./vial;[4] *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Bratton-Marshall reagent), 3% in distilled water; 0.2 *M* phosphate buffer, pH 2.31; gelatin reagent;[5] and silica gel GF thin-layer plates[6] were also used. All other chemicals were of reagent grade.

Apparatus—The following were used: Cary model 11 recording spectrophotometer; UV lamp (longwave),[7] maximum intensity at 366 mμ; Aminco chloride titrator, model 4–4433;[8] IBM 1620 computer;[9] Beckman zeromatic pH meter; Desaga TLC apparatus;[10] Beckman IR-5A spectrophotometer; constant-temperature water bath (±0.1°); and Thomas-Hoover capillary melting-point apparatus.

Colorimetric Assay—To a 10-ml. volumetric flask was added 0.2 ml. of freshly prepared 3% Bratton-Marshall reagent followed by 20–110-mcg. portions of NSC-82196. The flask was rapidly brought to volume with pH 2.31 phosphate buffer and mixed in subdued light. Approximately one-half of the solution was removed and placed in the dark. The other half was irradiated with a longwave UV lamp for 30 min. The lamp was positioned approximately 8 cm. from the center of the solution in the 10-ml. volumetric flask. The absorbance of both fractions was measured on a Cary 11 recording spectrophotometer at 520 mμ, using a phosphate buffer as the blank. A blank of the reagents alone, without NSC-82196, was also treated in the same manner, and its absorbance was recorded at 520 mμ.

Ionic Chloride Assay—A sample portion was taken, diluted if necessary, and titrated on an Aminco chloride titrator at one of the three titration speeds. The titrator can accurately determine a minimum of 9 mcg. of chloride ion in a sample. Accuracy was better than 0.1% and reproducibility was ±1%, with the entire determination taking less than 2 min. to perform.

UV Spectrophotometric Assay—Sample solutions from 1 to 2 mg./100 ml. in pH 2.31 phosphate buffer were read on the Cary 11 recording spectrophotometer from 400 to 225 mμ. Absorbances at 326, 278, and 236 mμ were recorded. Calculations were made with an IBM 1620 computer, using simultaneous equations with three equations and three unknowns.

TLC—TLC for qualitative studies was carried out on activated layers of silica gel GF_{254}, 250 μ thick, in two systems: water (saturated with *n*-butanol)–glacial acetic acid (4:1) and *n*-butanol–glacial acetic acid–water (4:3:3). All TLC was done at room temperature. The quantity of the compounds applied on TLC varied from 5 to 60 mcg. The limits of detection for each of the compounds were: NSC-82196, 0.5 mcg.; NSC-112970, 2 mcg.; and NSC-22419, 0.5 mcg. Spots were detected by UV quenching, I_2 vapor, aqueous 0.2% Bratton-Marshall reagent spray, and concentrated H_2SO_4 spray followed by heating.

Kinetic Studies—The degradation of NSC-82196 was monitored by the UV, colorimetric, and ionic chloride methods of assay. Various portions of NSC-82196 (depending upon the assay method used) were dissolved in pH 2.31 phosphate buffer[11] in 50-ml. volumetric flasks. The solutions were brought to volume at the temperature of the bath. The flasks were then immersed to 90% of their length in the thermostatically controlled bath at 25 ± 0.1° and exposed to the light and dark. The light samples were exposed to two fluorescent bulbs,[12] placed 15 cm. from the center of the volumetric flasks in the bath. At appropriate time intervals, portions were withdrawn for assay.

Stability of Parenteral Product—Amber glass vials of a lyophilized product were each reconstituted with 20 ml. of water for injection and stored in light and dark at 25° and in dark at 4°. All vials were reconstituted with water for injection at 25°. Sample portions

[4] John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, N. J.
[5] American Instrument Co., Silver Spring, Md.
[6] Analtech, Inc., Wilmington, Del.
[7] Burton model 1910, obtained from Cavitron Corp., Van-Nuys, Calif., equipped with two Burton 1911 bulbs.
[8] Obtained from American Instrument Co., Inc., Silver Spring, Md.
[9] Courtesy of Laboratory of Physiology, National Cancer Institute, NIH, Bethesda, Md.
[10] Distributed by Brinkmann Instruments, Inc., Westbury, Long Island, N. Y.

[11] A 0.2 *M*, pH 2.31 phosphate buffer was chosen for the kinetic and stability studies because the parenteral formulation used in clinical trials, when reconstituted with water for injection, is pH 2.3. Also, samples below pH 2.5 were found to be in an optimum stability range as seen with the data on the effect of pH on stability reported by the University of Michigan (2). Since the authors were trying to mimic conditions of the actual formulation, pH 2.3 was chosen for the study.
[12] Westinghouse, 15W No. F15T8ID.

Table I—Results of UV Analyses of Three Mixtures of NSC-82196, NSC-112970, and NSC-22419 in pH 2.31 Phosphate Buffer

Mixtures	Added, mg./100 ml.	Found, mg./100 ml.	Error, %
NSC-82196	1.7380	1.7318	0.8
NSC-112970	1.0450	1.0678	2.2
NSC-22419	1.1360	1.0877	4.2
NSC-82196	0.6440	0.6574	2.0
NSC-112970	0.9280	0.9250	0.3
NSC-22419	0.8180	0.7711	5.7
NSC-82196	0.9111	0.9010	1.1
NSC-112970	0.7651	0.7530	1.6
NSC-22419	0.9121	0.9311	2.1

were withdrawn at appropriate time intervals and assayed by the UV method.

RESULTS AND DISCUSSION

TLC Studies—TLC confirmed the presence of NSC-22419 and NSC-112970 as degradation products of NSC-82196 in acidic aqueous media. The decomposition products had R_f values identical to authentic NSC-22419 and NSC-112970. However, only trace amounts of NSC-22419 were found in samples degraded in the dark, while large amounts of NSC-22419 were found in samples degraded with longwave UV light. No attempt was made to confirm the presence of the photodecomposition product, 5-diazoimidazole-4-carboxamide, because of its small buildup and concentration.

UV Method of Assay—The UV method of assay was specific for NSC-82196 and its decomposition products NSC-22419 and NSC-112970. Table I shows the results of three separate trials assaying mixtures of all three authentic materials. Figure 1 shows the UV spectrum of all three components in pH 2.31 phosphate buffer.

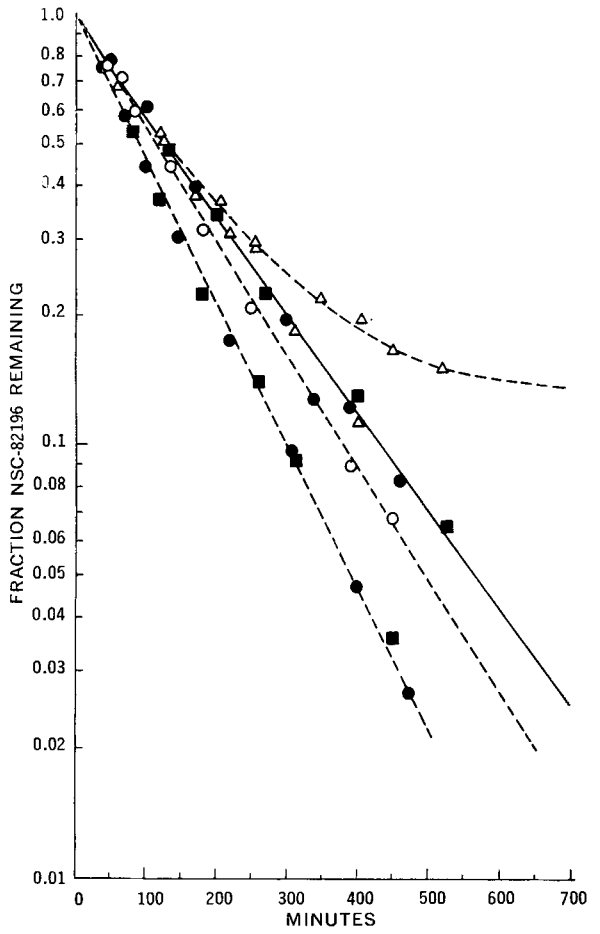


Figure 3—*Semilogarithmic plot of fraction NSC-82196 remaining* versus *time at 25.0°, pH 2.31 phosphate buffer, in light and dark. Key: dark, ——; light, - - -; UV, ●; colorimetric corrected, ■; colorimetric uncorrected, ○; and chloride, △.*

Table II—a Values (at pH 2.31) for NSC-82196, NSC-112970, and NSC-22419 at Three Wavelengths Used in the UV Assay

Mixtures	326 mμ	278 mμ	236 mμ
NSC-82196	635.2	230.3	252.0
NSC-112970	250.2	112.2	412.5
NSC-22419	000.0	263.8	255.0

The $\lambda_{max.}$ used for the calculations were 278 mμ (NSC-22419), 236 mμ (NSC-112970), and 326 mμ (NSC-82196).[13] The a values at pH 2.31, used to calculate the final formulas using simultaneous equations, are seen in Table II. All calculations were based on three components only.

Colorimetric Assay—Intact NSC-82196 follows Beer's law over the concentration range 1–12 mcg./ml. The a value was found to be 937.5 ± 1.4%. At low concentrations, less than 3 mcg./ml., the $\lambda_{max.}$ of the solution was seen to shift from 520 to 525–526 mμ. Samples of intact NSC-82196 developed for 30 min. in the dark

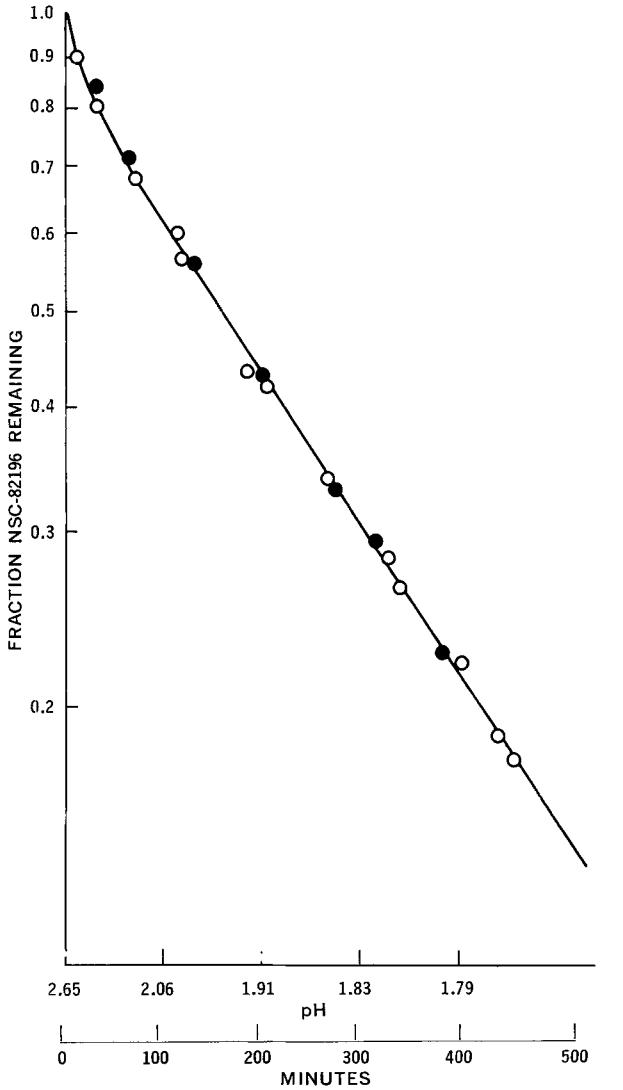


Figure 4—*Semilogarithmic plot of fraction NSC-82196 remaining* versus *time for reconstituted parenteral product at 25° in light* (○) *and dark* (●).

[13] The wavelength of 209 mμ for NSC-22419 recommended by the University of Michigan group (2) for quantitation could not be used in this laboratory due to limitations of the Cary 11 recording spectrophotometer.

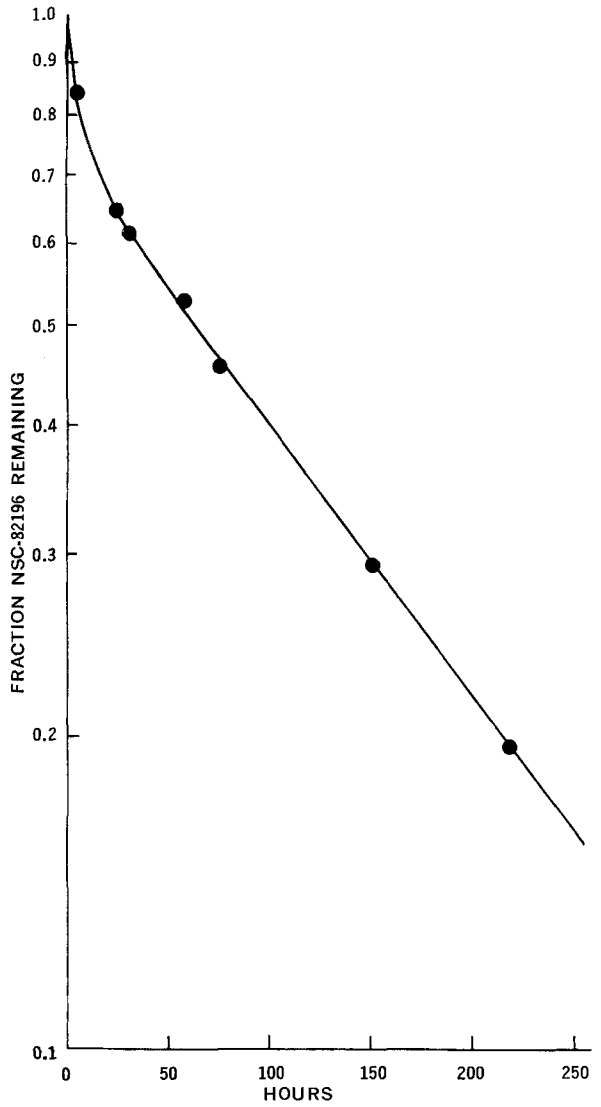


Figure 5—*Semilogarithmic plot of fraction NSC-82196 remaining* versus *time for reconstituted parenteral product at 4° in dark.*

demonstrated zero absorbance, thus indicating that no 5-diazoimidazole-4-carboxamide was formed in the absence of light. If diazoimidazole carboxamide was found, it would be indicative of what was already present at the time the sample was taken. Therefore, subtraction of the sample's absorbance from the irradiated sample's absorbance is necessary to obtain the corrected absorbance of the remaining intact NSC-82196 in the sample. This procedure is necessary since in a stability study with rigorous lighting conditions, a buildup of 5-diazoimidazole-4-carboxamide is expected.

Kinetic Studies—Apparent adherence to stoichiometry during the course of the reaction of NSC-82196 in acidic aqueous solution with time using the UV assay method is seen in Fig. 2. Samples exposed to light showed a significant amount of NSC-22419 formation. Table III shows the rate constants and half-lives obtained by the assay methods used. Figure 3 is a semilogarithmic plot of fraction NSC-82196 remaining *versus* time for all assay methods. With the exception of the ionic chloride method for light-stored samples, all assay methods used indicated that the degradation of NSC-82196 followed apparent first-order kinetics. All chloride calculations were based on the premise that NSC-82196 was degrading solely to NSC-112970; thus the chloride method would be expected to be invalid if anything other than NSC-112970 was being formed.

It can be seen in Fig. 3 that an error can be introduced if one does not account for existing 5-diazoimidazole-4-carboxamide at any given time when using the colorimetric method. The presence of a significant amount of the diazo compound also would be expected to introduce error into the UV results, since the UV calculations

Table III—First-Order Rate Constants and $t_{1/2}$ Values for Degradation of NSC-82196 in Phosphate Buffer at pH 2.31, 25.0°, in Light and Dark

Assay Method Used	Light		Dark	
	k min.$^{-1}$ × 10^3	$t_{1/2}$, min.	k min.$^{-1}$ × 10^3	$t_{1/2}$, min.
Colorimetric corrected	7.90 ± 3.13%	87.8	5.46 ± 4.3%	126.9
Colorimetric noncorrected	6.14 ± 3.12%	112.9	5.46 ± 4.3%	126.9
Chloride	Invalid		5.59 ± 2.8%	123.9
UV	7.75 ± 3.9%	89.5	5.30 ± 5.1%	130.7

allow for three components only in the system. In this study the amount of diazo compound existing at a given time in light samples was small (there was a maximum amount of 10% at $t_{1/2}$) and was calculated to result in less than 3% error in the concentration of NSC-82196 in a solution at $t_{1/2}$ by the UV method of assay.

Stability of Parenteral Product—Figures 4 and 5 are the semilogarithmic plots for the reconstituted parenteral product at 25 and 4°, respectively. No difference in stability of NSC-82196 was noted between those samples stored in light or dark at 25°, presumably due to the drug being stored in the amber glass vials.

The pH was found to change considerably in this unbuffered system. It is likely that the pH lowering causes apparent variance from first-order kinetics in both cases, since the drug in general has been reported to be more stable in acidic aqueous media (2). Also, water at 25° was used to reconstitute the 4° sample; thus a faster degradation rate would be expected in the first few minutes (Fig. 5) until the system actually comes to 4°.

The purpose of this experiment was to mimic conditions the product would actually encounter in clinical use. Therefore, the results are different than would be predicted from a controlled stability study carried out in a buffer system at constant pH. The apparent half-life of the parenteral product is approximately 150 min. at 25° and 65 hr. at 4°

CONCLUSIONS

Good agreement was found between the UV and colorimetric methods of assay. The University of Michigan's UV method of assay was found to be quite specific for NSC-82196 and its decomposition products, NSC-22419 and NSC-112970, but does not include the decomposition product 5-diazoimidazole-4-carboxamide. The University of Michigan group did not conduct their studies of the drug in light. Since the diazo compound is a photodegradation product of the drug, no allowances were made for it in the UV method of assay. However, as mentioned previously, even when there was a buildup of diazo compound to a maximum of 10% at $t_{1/2}$ in the light, this resulted in an error of 3% in the concentration of NSC-82196.

Values obtained from the colorimetric method would also be in error if any 5-diazoimidazole-4-carboxamide was present as a degradation product and not accounted for, as seen in Table III. Therefore, correction of the colorimetric method, as reported by Loo and Stasswender (6), is definitely needed in a study with rigorous lighting conditions where the buildup of diazo compound, although small, does occur.

The chloride method was found in agreement with the other two methods of assay when used for stability studies in which NSC-112970 was the only degradation product (samples protected from light). The chloride method is of no value in determining the absolute amount of NSC-82196 remaining in the sample because it is not specific for intact NSC-82196. Also, obvious problems arise if one attempts to use the chloride method to follow the stability of a hydrochloride salt of NSC-82196. Thus the chloride method is not recommended due to these serious limitations. By protecting the solutions from light, it appears that the 5-diazoimidazole-4-carboxamide formation can be inhibited. The degradation of the reconstituted parenteral product did not appear to follow first-order kinetics, possibly due to the pH decrease which accompanies the degradation. The apparent $t_{1/2}$ was 150 min. for amber glass vials

of the reconstituted hydrochloride salt of NSC-82196 stored at 25° in dark or light. The apparent $t_{1/2}$ was 65 hr. for the same vials stored at 4° in the dark.

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TECHNICAL ARTICLES

Automated Dual Extraction Procedure for Analysis of Phenmetrazine Hydrochloride Tablets

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Abstract □ An automated method of analysis, based on dual extraction of phenmetrazine hydrochloride, has been developed for analysis of phenmetrazine hydrochloride tablets. The method requires use of a continuous digestor along with other more familiar modules of an automatic analyzer. A total of 520 individual tablets was analyzed by this method. Of all the tablets analyzed, 99.8% were within ±10% of the indicated dosage and 100% were within ±15% of the indicated dosage.

Keyphrases □ Phenmetrazine HCl tablets—analysis □ Automated extraction procedure—phenmetrazine HCl analysis □ Diagram—automated system, phenmetrazine HCl analysis □ UV spectrophotometry—analysis

Automated analytical methods have been limited generally to dissolution and/or extraction of active ingredients from the formulation, filtration, and dilution to suitable concentration for analytical determinations. Manual methods involving evaporation of the organic solvent, followed by dissolution of the residue in suitable aqueous solvents, have been considered difficult to automate. One semiautomated approach to the solution of this problem has been reported by Feller *et al.* (1) for analysis of ethopabate in poultry feeds. This approach should provide a valuable means of automating pharmaceutical methods of analysis, where such extractions are frequently used.

A variety of manual methods (2–9) has been used for analysis of phenmetrazine hydrochloride.[1] For several years, a manual dual-extraction procedure (9) has been used in the authors' laboratories for analysis of 25-mg. tablets of this compound. The tedious and time-consuming manual procedure was automated by the use of the continuous digestor[2] along with other more familiar modules of the automatic analyzer.[2]

Table I—Recoveries and Precision with the Automated Method

Number	Percent Phenmetrazine Hydrochloride Recovered
1	99.2
2	101.2
3	100.4
4	103.2
5	101.2
6	98.0
7	101.2
8	103.2
9	99.2
10	100.4
Average	100.7
Relative *SD*	±1.7

EXPERIMENTAL

Materials and Methods—The flow diagram of the analytical system, including tubing sizes, is shown in Fig. 1. The Solidprep sampler is programmed to operate at a rate of 13 samples/hr. The sample (tablet) is deposited in a cup placed on the turntable of the sampler. In turn, each sample is dumped into the homogenizer and homogenized with 100 ml. of water; a 0.9-ml./min. sample (segmented with air) is pumped from the sampler. The sample is made alkaline with 1% w/v NaOH and filtered through a continuous filter. About 50% is resampled and extracted with chloroform, and the chloroform extract is washed with water and fed into the continuous digestor. Simultaneously, dilute hydrochloric acid (4 in 100)

[1] Preludin, Geigy Pharmaceuticals, Ardsley, N. Y.

[2] Technicon Corp., Tarrytown, N. Y.