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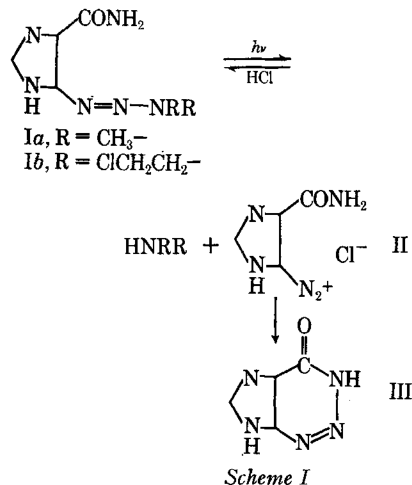
Colorimetric Determination of Dialkyltriazenoimidazoles

By TI LI LOO and ELIZABETH A. STASSWENDER

A colorimetric method has been developed for the determination of dialkyltriazenoimidazoles in plasma and urine.

Recently a series of dialkyltriazenoimidazoles (I) has been synthesized as potential antitumor agents (1). Several of these have shown promise as useful drugs in cancer chemotherapy, notably 5-(dimethyltriazeno)-imidazole-4-carboxamide (I*a*) (DIC, NSC-45388) and 5-[di(β-chloroethyl)triazeno]-imidazole-4-carboxamide (I*b*) (NSC-82196) (2,3). The former is presently under clinical trial. Concurrently, pharmacologic studies on these new agents in man and animals are also in progress in our laboratories. For such studies a simple, sensitive, and specific method for the quantitative determination of the dialkyltriazenoimidazoles is desirable. The method described below appears to meet this demand.

The dialkyltriazenoimidazoles undergo photodecomposition in dilute acid to 5-diazoimidazole-4-carboxamide (II) and a corresponding dialkylamine according to Scheme I.



Scheme I

Received February 6, 1967, from the University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, TX 77025
Accepted for publication April 3, 1967.
This work was supported by contract PH 43-66-1156 with the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.
The authors thank the Cancer Chemotherapy National Service Center, National Cancer Institute, for the drugs NSC-45388 and NSC-82196.

In aqueous solutions II readily cyclizes by intramolecular coupling to 2-azahypoxanthine (III), imidazo-[4,5-*d*]-*v*-triazin-4(3*H*)-one (1). However, in the presence of an aromatic amine such as *N*-(1-naphthyl)ethylenediamine (Bratton-Marshall reagent) (4), coupling takes place with the formation of an intensely colored azo-dye. Under rigidly controlled experimental conditions with an excess of the coupling amine, the competing intramolecular coupling to III could be held to a constant minimum; thus the intensity of the color adequately measures the concentration of I. Because the photodecomposition is a general reaction of I, the colorimetric method is expected to be applicable to all dialkyltriazenoimidazoles, although the present work concerns mainly I*a* and I*b*.

EXPERIMENTAL

Apparatus

A standard colorimeter (for example, Bausch & Lomb Spectronic 20) is required for measurement of absorbance of 0.4 ml. to 2 ml. of solution.

The light source is a long-wave (maximal radiation at 366 mμ) ultraviolet lamp (Blak-Ray lamp, model XX-15, supplied by Ultraviolet Products, Inc., San Gabriel, Calif., equipped with two General Electric F15 T8-BLB 15-w. bulbs).

A wooden rack holds the test tubes (Pyrex 75 × 10 mm.) containing drug solutions rigidly vertical and at a fixed distance of 2 cm. from the light bulbs. The lamp is positioned so that an entire row of test tubes may receive the same maximal exposure to ultraviolet radiation.

Reagents

Bratton-Marshall Reagent—Aqueous solutions of *N*-(1-naphthyl)ethylenediamine dihydrochloride, 0.2% for aqueous and plasma assays, but 1% for urine assays.

DIC Standards—A stock solution of DIC, 10.0 mg./ml. in 0.1 *N* hydrochloric acid is prepared and stored in the dark under refrigeration. It is stable for at least 1 month. Dilutions are made of stock with 0.1 *N* hydrochloric acid, plasma, or urine.

Trichloroacetic Acid—A 10% solution of trichloroacetic acid in 6 *N* sulfuric acid.

PROCEDURES

Determination in Aqueous Solutions—A mixture of 2.0 ml. of DIC solution, ranging from 0.1 mcg./

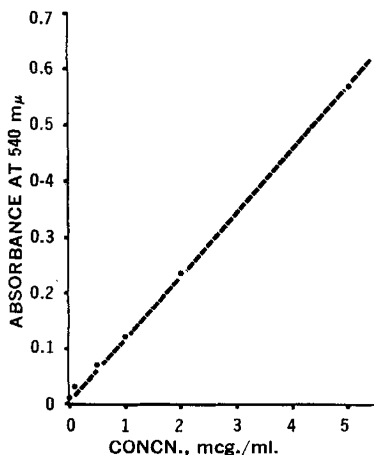


Fig. 1—Linearity between absorbance and concentration of DIC, 5-(dimethyltriazeno)-imidazole-4-carboxamide, NSC-45388.

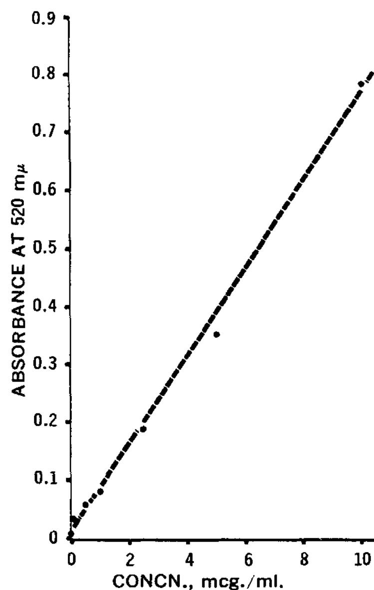


Fig. 2—Linearity between absorbance and concentration of NSC-82196, 5-[di(β-chloroethyl)triazeno-imidazole-4-carboxamide.

ml. to 5.0 mcg./ml., and 0.1 ml. of 0.2% Bratton-Marshall reagent is thoroughly shaken and placed in front of the ultraviolet light. After a 30-min. exposure, the absorbance (540 mμ for DIC and 520 mμ for NSC-82196) is measured against water. A blank is similarly run with 2.0 ml. of 0.1 *N* hydrochloric acid.

Plasma Recovery—A 3.0-ml. plasma solution of the drug is thoroughly mixed with 1.5 ml. of trichloroacetic acid. The protein precipitate is removed by centrifugation at about 2,000 r.p.m. (top speed of International clinical centrifuge, model CL) for 20 min. An aliquot of 2.0 ml. of the supernatant is withdrawn and assayed as for aqueous solutions. A blank containing no drug is likewise run. As a rule, in every experiment standards are established by adding given amounts of the drug to the pretreatment plasma collected from the subject and assayed in accordance with the above procedure. Whenever possible, three concentrations are arbitrarily chosen and run in triplicates.

The above procedure is slightly modified as follows for smaller amounts of plasma. To 1.0 ml. of a plasma solution is added 0.5 ml. of trichloroacetic acid solution. The mixture is thoroughly shaken and centrifuged. An aliquot of 0.4 ml. of the supernatant is removed and mixed with 0.02 ml. of 0.2% Bratton-Marshall reagent and assayed as before.

Urinary Recovery—All urine samples are diluted before assay because, first of all, the presence of natural pigments in urine makes direct determination impractical. Second, in actual clinical pharmacological work, the rapid excretion of the drug causes very high initial urinary concentration. For these reasons, the standard curve is constructed with urine solutions ranging from 250 mcg./ml. to 2000 mcg./ml. These are then appropriately diluted to 5.0 mcg./ml. with 0.1 *N* hydrochloric acid. Except that the Bratton-Marshall reagent used is 1% instead of 0.2%, the assay procedure is the same as described for aqueous solutions. The observed absorbance multiplied by the dilution factor gives the true value corresponding to each original concentration. Again, as in plasma recovery, urine standards are run with pretreatment urine in every experiment.

Table I—Concentration and Absorbance (± Standard Error) of NSC-45388 and NSC-82196

Concn., mcg./ml.	NSC-45388	NSC-82196
0	0.012 ± 0.0019	0.012 ± 0.0010
0.1	0.032 ± 0.0003	0.032 ± 0.0010
0.5	0.073 ± 0.0006	0.056 ± 0.0003
1	0.121 ± 0.0012	0.080 ± 0.0015
2	0.237 ± 0.0022	
5	0.569 ± 0.0063	0.353 ± 0.0041
10		0.783 ± 0.0104

RESULTS AND DISCUSSION

The excellent linearity between absorbance and concentration for both NSC-45388 and NSC-82196 in aqueous solutions is shown in Figs. 1 and 2, respectively, each point representing 10 replicates. The standard errors are illustrated in Table I. Obviously, the reproducibility is good. It appears the method is less sensitive with NSC-82196 than with NSC-45388. This is hardly surprising because the ratio of their molecular weights is about 3:2. Theoretically, the wavelength of maximal absorption should be the same for all dialkyltriazenoimidazoles. In practice, however, there was a slight variation from drug to drug and hence the $\lambda_{max.}$ should be determined individually: it was 540 mμ for NSC-45388 and 520 mμ for NSC-82196.

After 30 min. the photodecomposition is complete and further exposure to ultraviolet light is of no

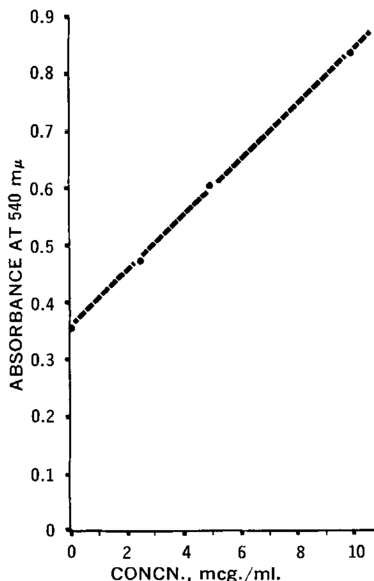


Fig. 3—Plasma standard curve of DIC, NSC-45388.

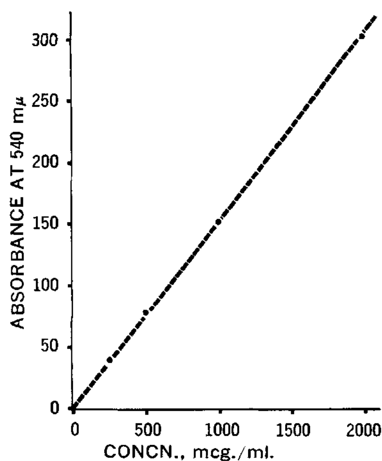


Fig. 4—Urinary standard curve of DIC (high concentration), NSC-45388.

advantage. If the Bratton-Marshall reagent is not added prior to irradiation, no color is produced, probably because the diazo compound has cyclized.

A typical plasma standard curve of DIC is represented by Fig. 3. Bratton-Marshall reagent of 1% is not used in plasma assay because it gives a higher blank than the 0.2% reagent. The results of urinary determination of the same drug are illustrated in Fig. 4. It is readily seen that the method applies well to all these determinations.

No naturally occurring material in either plasma or urine is known to give rise to a diazo compound upon illumination under ultraviolet light. Consequently, this method is specific for dialkyltriazenoimidazoles. Moreover, it is reasonable to expect the method to be applicable equally well to other dialkyltriazenoimidazoles.

The very small absorbance of the aqueous blank is accounted for by the slight brown tint produced by the Bratton-Marshall reagent after exposure to ultraviolet light. However, with plasma and especially with undiluted urine the brown tint becomes very much enhanced to afford high blanks which vary from one subject to another. This is one of the reasons why plasma and urine standards are always prepared and assayed in every experiment. The high blanks are not caused by azo dyes but by the end absorption due to certain brown pigment resulting from the interaction of Bratton-Marshall reagent and some unidentified constituents of plasma and urine after ultraviolet irradiation. Both plasma and urine blanks are assumed to remain constant throughout the experiment. This is a reasonable assumption since an experiment rarely lasts longer than 4–5 hr.

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