Colorimetric Method for the Determination of 1,3-Bis(2-chloroethyl)-1-nitrosourea

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The ready liberation of nitrous acid by 1,3-bis(2-chloroethyl)-1-nitrosourea in hydrochloric acid solution has been utilized successfully in the development of a sensitive colorimetric method for its quantitative estimation in biological fluids.

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), a novel type of cancer chemotherapeutic agent, has shown marked activity against experimental neoplasms, especially intracerebral mouse leukemia (1). Its possible usefulness in meningeal leukemia and brain tumors has been advocated. Clinical trial of this agent is currently in progress (2). In addition, BCNU is a potent inhibitor of several microorganisms (3). This paper describes a sensitive method for the quantitative determination of BCNU in biological fluids.

Being a derivative of a nitrosamide, BCNU decomposes in an excess of strong hydrochloric acid into nitrous acid and most likely 1,3-bis(2-chloroethyl)urea, the reversal of its mode of formation (4). By varying the experimental conditions, the authors have been able to make the liberation of nitrous acid quantitative. The nitrous acid thus formed is determined conveniently colorimetrically by the familiar Bratton-Marshall (5) method for sulfonamides, except in the present method sulfanilamide is used as a reagent. Theoretically, the method is expected to apply equally well to any compounds that give nitrous acid upon treatment with hydrochloric acid. These include nitrosamines, nitrosamides, and nitrosoureas; and, in fact, satisfactory results were obtained with 1-methyl-1-nitrosourea.

BCNU is much more soluble in lipid solvents than in water. Using an ether-to-water ratio of 2:1, the extraction of BCNU is quantitative. Plasma solutions can be extracted directly with ether without prior precipitation of protein.

EXPERIMENTAL

Apparatus

Any colorimeter that can measure adequately the absorbance of light of 540 mμ by 1.6 ml. of solution can be used. A Cary spectrophotometer model 14 in conjunction with quartz cells of 2-ml. capacity and a 1.0-cm. light path was used in this investigation. A constant-temperature bath is required to maintain the temperature at 50° during digestion with hydrochloric acid.

Reagents

Sulfanilamide Reagent.—A solution of 5 Gm. of sulfanilamide in 1 L. of 2 *N* HCl is used for BCNU standards and aqueous solutions of BCNU that do not need extraction. For biological fluids requiring extraction, 1 vol. of the above is diluted with water to 3 vol.

Bratton-Marshall Reagent.—Dissolve 0.3 Gm. of *N*-(1-naphthyl)ethylenediamine dihydrochloride in 100 ml. of water.

BCNU Standards.—A stock solution of 1.0 mg./ml. of BCNU (supplied by Merck Sharp and Dohme Research Laboratories, Rahway, N. J., through the courtesy of the Cancer Chemotherapy National Service Center, National Cancer Institute) in water is prepared by dissolving a carefully weighed sample in a minimal volume of ethanol and diluting with water. Dilutions are made from fresh stock prepared the same day. Because of rapid deterioration,[1] all BCNU solutions are kept under refrigeration in red flasks.

PROCEDURES

Standardization Curves.—In a 16 × 150 mm. test tube are placed 1.0 ml. of a BCNU sample (concentration ranging from 0.5 to 5.0 mcg./ml.) and 0.5 ml. of the sulfanilamide reagent. The test tube is immersed in a water bath at 50° to a depth of 6–8 cm. for 45 min. with occasional shaking. After incubation, it is removed and chilled in an ice bath. To the cold solution is added 0.1 ml. of Bratton-Marshall reagent. After 10 min. at room temperature, the absorbance at 540 mμ is measured against a water blank and plotted against concentration.

Recovery of BCNU from Biological Fluids.—A 2.0-ml. sample of urine, plasma, or cerebrospinal fluid is extracted with 4.0 ml. of ether in a stoppered tube. A 2.0-ml. aliquot of the ether extract is pipeted into a test tube and cooled in ice. (The ether-to-aqueous phase ratio may be greater than 2. Also, the volume of the aliquot removed for analysis may be varied. In either case, an appropriate factor must then be introduced to obtain the correct concentration of BCNU.) Nitrogen is allowed to blow gently over the surface of the ether through a capillary at a flow rate of no more than 3 L./min. Care must be taken to regulate the flow rate of nitrogen, or the results show high variability. On completion of evaporation, the residue is dissolved in 1.5 ml. of the *diluted* sulfanilamide reagent, and the determination is carried out as above. The absorbance is measured against a blank consisting of a sample of biological fluid containing no drug, that has undergone the entire extraction and evaporation procedures. For the analysis of plasma, the final solution after the addition of Bratton-Marshall reagent should be filtered through a fritted disk of medium porosity if turbid. If the determination cannot be completed immediately, the tube with the BCNU residue must remain in the

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[1] By the present method, the half-life of BCNU in plasma or urine is found to be about 15 min. at room temperature. The full details of these studies will be reported in another paper.

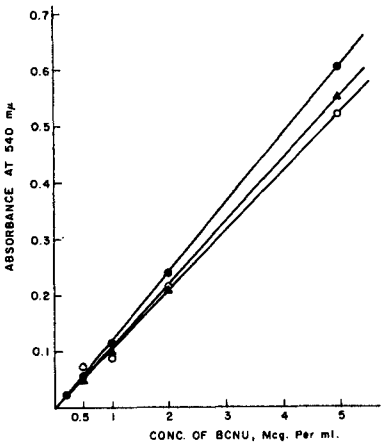

Fig. 1.—Linearity between absorbance and concentration of BCNU. Key: ●, water; △, plasma; ○, urine.

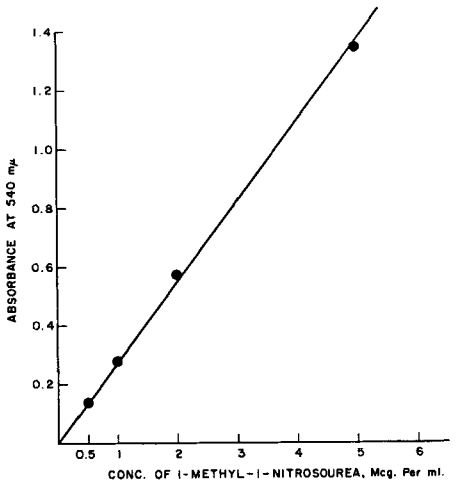

Fig. 2.—Linearity between absorbance and concentration of 1-methyl-1-nitrosourea.

TABLE I.—ABSORBANCE, CONCENTRATION, AND PER CENT RECOVERY OF BCNU

Concn., mcg./ml.	Aqueous[a] Absorbance ± S.E.	Plasma[b] Absorbance ± S.E.	Plasma[b] % Recovery	Urine[b] Absorbance ± S.E.	Urine[b] % Recovery
5	0.605 ± 0.0017	0.551 ± 0.0088	91	0.521 ± 0.0101	86
2	0.240 ± 0.0005	0.211 ± 0.0040	88	0.214 ± 0.0038	89
1	0.115 ± 0.0005	0.098 ± 0.0020	85	0.089 ± 0.0022	78
0.5	0.055 ± 0.0003	0.046 ± 0.0019	93	0.073 ± 0.0025	133

[a] Ten determinations for each concentration. [b] Six determinations for each concentration.

ice bath, and the sulfanilamide reagent must not be added until just before digestion at 50°. Because of the very short half-life of BCNU in both plasma and urine,[1] samples should be frozen as soon as collected and determinations performed as speedily as possible.

Appropriate plasma and urine standards are prepared by diluting a given volume of a 1.0 mg./ml. fresh aqueous solution of BCNU with a relatively large volume of plasma or urine in order to minimize the dilution effect. It is desirable to have fresh urine or plasma standards prepared prior to each series of determinations. These standards are necessary to compensate for the short half-life of BCNU.

RESULTS

As illustrated in Fig. 1, excellent linearity exists between absorbance and concentration of BCNU. The actual absorbance readings together with the standard errors are shown in Table I. For aqueous solutions of 1–5 mcg./ml., the variation in absorbance is less than 1%. For more dilute solutions, the variations are wider, being 2% for 0.5 mcg./ml. and 7% for 0.2 mcg./ml. In the same table is shown also the recovery of added BCNU from human plasma and urine, ranging from 85–93% for plasma and 78–89% for urine (except 133% for the low concentration of 0.5 mcg./ml. of urine). These data clearly indicate that the method is sensitive, and reproducibility is satisfactory for pharmacological applications.

This method is also applicable to 1-methyl-1-nitrosourea as shown in Fig. 2.

DISCUSSION

The effectiveness of nitrosourea derivatives as therapeutic agents is a recent discovery, and no method for their quantitative assay is available. In ethanol or water at pH 7, BCNU exhibits maximal absorption of ultraviolet radiation at 230 mμ; however, the absorption is not sufficiently intense to be useful for the determination of small amounts of the drug. In common with other derivatives of nitrosamine, BCNU gives a positive Liebermann color reaction (6). Because of the necessity of using highly toxic and corrosive reagents, such as phenol and concentrated sulfuric acid, the possible utilization of Liebermann reaction for the colorimetry of BCNU was not explored. As an alkylating agent, BCNU forms a purple quaternary pyridinium salt with Koenig's reagent, γ-(4-nitrobenzyl)-pyridine (7). However, the method described here is almost 10 times more sensitive for BCNU than Epstein's method.

The acid hydrolysis of BCNU is highly dependent on temperature and acidity. The conditions described in this paper are found to give the best results and should be followed closely.

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