Mechanism for the High Bias in the USP Colorimetric Assay for Diazotizable Substances in Hydrochlorothiazide

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Abstract □ The USP XX colorimetric assay for the determination of diazotizable substances in hydrochlorothiazide was studied. Colorimetric assay results of hydrochlorothiazide bulk powder and captopril-hydrochlorothiazide combination tablets were found to have a high bias when compared with HPLC. A kinetic study of the diazotization step in the colorimetric assay and extrapolation of the free amine content, *i.e.*, 4-amino-6-chloro-1,3-benzenedisulfonamide, to time zero provided results which correlated favorably with those obtained by HPLC. The high bias of the colorimetric assay was shown to result from the formation of 4-nitroso derivative of hydrochlorothiazide, which is formed by acid hydrolysis during the diazotization step. Bendroflumethiazide and flumethiazide also showed increasing free amine content when the time of diazotization was increased.

Keyphrases □ Hydrochlorothiazide—bias of USP XX colorimetric assay for diazotizable substances, free amine content □ Colorimetry—high bias in USP XX assay for diazotizable substances, free amine, hydrochlorothiazide

One of the standard compendial assays performed on benzothiadiazines is the colorimetric test for diazotizable substances (free amine). Here, free amine is a general term used to designate any primary aromatic amine present in the material undergoing testing. In this report, free amine specifically refers to the primary aromatic amide decomposition product of hydrochlorothiazide, *i.e.*, 4-amino-6-chloro-1,3-benzenedisulfonamide. In the compendial assays, any free amine is diazotized with nitrous acid, and the resulting diazonium ion reacts with a color reagent such as disodium 4,5-dihydroxynaphthalene-2,7-disulfonate (disodium chromotropate) or *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton-Marshall reaction). There is little precedent in the literature regarding problems with this colorimetric assay. In 1970, Turner and co-workers (1) reported difficulty in reproducing the procedure reported in the British Pharmacopoeia (2) for the determination of free amine in bendroflumethazide. They suggested lengthening the time of diazotization to 5 min rather than the recommended 1 min. This modification was incorporated into the procedures of the British and U.S. Pharmacopeias. Currently, the British Pharmacopoeia suggests use of a TLC assay (3), but the U.S. Pharmacopeia suggests the continued use of the colorimetric assay.

HPLC methods have been developed for the determination of free amine in hydrochlorothiazide bulk powder and captopril-hydrochlorothiazide combination tablets (4) and in nadolol-bendroflumethiazide combination tablet formulations (5). The active ingredients have also been assayed by HPLC methods. When compared with HPLC, the colorimetric assay gave results which consistently showed a high bias. Consequently, a kinetic study of the diazotization and extrapolation to time zero is reported, along with a discussion of the source of the anomoly produced in the diazotization step. A mechanism for the production of this artifactual value is also given.

EXPERIMENTAL SECTION

Materials—Hydrochlorothiazide[1] bulk powder and captopril-hydrochlorothiazide combination tablets[2] were used as received. 4-Amino-6-chloro-1,3-benzenedisulfonamide[3] and ACS reagent-grade disodium 4,5-dihydroxynaphthalene-2,7-disulfonate[4], phosphoric acid[4], hydrochloric acid[4], sodium nitrite[4], and ammonium sulfamate[4] were obtained commercially and used without further purification. The *N*-nitroso derivative of hydrochlorothiazide was prepared by the method of Gold and Mirvish (6), mp 155–156°C (dec.) [lit. (6) mp 155–156°C (dec.)].

High-Performance Liquid Chromatography—A prepacked, reverse-phase, medium-polarity column[5] (250 × 4.6 mm i.d.) containing 5-μm particles was used. The mobile phase was double-distilled water–methanol–phosphoric acid (80:20:0.05) at a flow rate of 2.0 mL/min, with detection at 210 nm. Details of the conditions used have been described previously (5).

Kinetic Studies—The USP procedure (7) for the determination of diazotizable substances in hydrochlorothiazide was followed. In a typical study, four weighings of hydrochlorothiazide powder (100 mg each), each with its own blank and standard, were carried through the procedure up to the addition of the sodium nitrite and hydrochloric acid (diazotization). The diazotization was then allowed to proceed for 5, 10, 15, and 30 min, respectively, at ambient temperature. Then, each solution was carried through to the end of the USP procedure.

The kinetic study was repeated exactly as described above with the 4-nitroso derivative of hydrochlorothiazide instead of hydrochlorothiazide. A third experiment was carried out by omitting the addition of sodium nitrite in the diazotization step while carrying the 4-nitroso derivative of hydrochlorothiazide through the procedure.

An extraction procedure[6] was used to prepare the captopril–hydrochlorothiazide combination tablets for free amine analysis. An acidified aqueous tablet extract was washed with chloroform to remove the interfering captopril, and the resulting aqueous phase was carried through the kinetic study as described above.

RESULTS AND DISCUSSION

The free amine content of hydrochlorothiazide bulk powder was determined by the procedure described in USP XX (6). The current USP procedure calls for a 5-min diazotization. A kinetic study of the diazotization step yielded increasing free amine content with increasing reaction time (Fig. 1). Extrapolation of the free amine content to time zero, *i.e.*, *y*-intercept, with linear regression analysis gave results of 0.0004% (r^2 = 0.977) and 0.06% (r^2 = 0.994) for two batches of hydrochlorothiazide. In Table I the data used to calculate these results are shown. Linear regression analysis also gave slopes of 0.0191 ± 0.0021 (11.0%) and 0.0174 ± 0.0019 (10.9%), respectively, for the two batches of hydrochlorothiazide.

In Fig. 1, a similar phenomenon which occurred when captopril-hydrochlorothiazide tablets were assayed for free amine content by the USP procedure is also shown. Once again, a kinetic study of the diazotization step yielded increasing free amine content with increasing reaction time. Extrapolation of the free amine content to zero time, *i.e.*, *y*-intercept with linear regression analysis, gave a result of 0.11% (r^2 = 0.999) for captopril-hydrochlorothiazide tablets which contained 15 mg of hydrochlorothiazide per tablet and 0.13% (r^2 = 0.999) for tablets which contained 25 mg of hydrochlorothiazide per tablet. In Table I, the data used to calculate these results are

[1] Merck.
[2] E. R. Squibb & Sons, Inc.
[3] Fluka AG.
[4] Fisher Scientific Co.
[5] Phenyl; Waters Associates or E. S. Industries.
[6] R. Clay and B. Patel, unpublished results.

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Table I—Free Amine Content of Hydrochlorothiazide Bulk Powder and Captopril-Hydrochlorothiazide Combination Tablets *Versus* Time of Diazotization

	Reaction Time, min[a]	Free Amine Content, %[a]	Reaction Time, min[b]	Free Amine Content, %[b]
Captopril-hydrochlorothiazide Combination tablets	5	0.18	5	0.19
	10	0.26	10	0.26
	15	0.32	15	0.32
	30	0.54	30	0.50
	Lot NX008		Lot NX018	
Hydrochlorothiazide Bulk powder	5	0.11	5	0.16
	10	0.21	10	0.23
	15	0.24	15	0.30
	30	0.59	30	0.59

[a] The tablets contained 15 mg of hydrochlorothiazide. [b] The tablets contained 25 mg of hydrochlorothiazide.

shown. Linear regression analysis also gave slopes of 0.0143 ± 0.0015 (10.5%) and 0.0123 ± 0.0013 (10.6%), respectively, for the combination tablets.

The *RSD* of the slopes was calculated to be 19.5%. This value is surprisingly high in light of the fact that each individual set of data points provided excellent correlation coefficients. One possible factor is that the temperature of the reaction was not strictly controlled, *i.e.*, it was performed at room temperature. An increase in room temperature would be paralleled by an increase in the slope of the line, and a decrease in room temperature would be paralleled by a decrease in the slope of the line.

The extrapolated values are compared with the values obtained from the HPLC assay (Table II). In Table II are also included the values determined by the USP procedure, which has a 5-min diazotization. A high bias exists in the USP method. Since the HPLC assay has a limit of detection of 0.1% and the results of the kinetic study are close to that limit, it is difficult to make a quantitative comparison. Qualitatively, the HPLC assay and the kinetic assay gave results that were substantially lower than those of the USP procedure.

It is tempting to attribute the increase in free amine content to the acid-catalyzed hydrolysis of hydrochlorothiazide. This, however, is not the case. When hydrochlorothiazide was allowed to stand in dilute hydrochloric acid (1:10), no increase in free amine content was observed even after 1 h. The kinetics and rate of decomposition of hydrochlorothiazide in acidic media have been studied by other investigators. For example, Yamana *et al.* (8) have published a report covering the pH-rate profile of hydrochlorothiazide. Mollica *et al.* (9) have published an extensive report on the hydrolysis of benzothiadiazines. They reported the rate constant for hydrolysis of hydrochlorothiazide at pH 1.47 and 45°C to be 1.46×10^{-3} h^{-1}. By using the Arrhenius relationship, the rate constant at 25°C should be $\sim 1.5 \times 10^{-4}$ h^{-1}. This translates into a half-life of 4620 h. Thus, under the acid treatment employed in the colorimetric procedure, a negligible amount of hydrochlorothiazide is hydrolyzed to free amine.

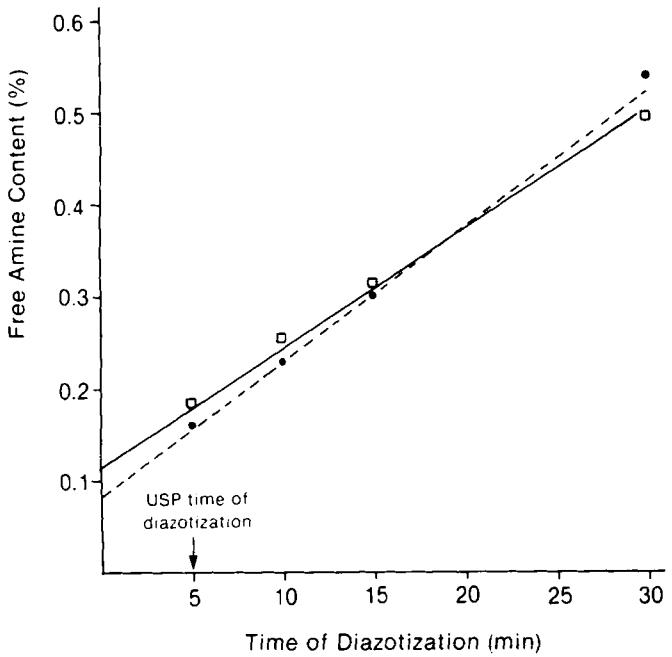


Figure 1—*Free amine content of hydrochlorothiazide bulk powder* (●) *and captopril-hydrochlorothiazide combination tablets* (□) *as a function of time of diazotization.*

Table II—Comparison of HPLC and Kinetic Assays for the Determination of Free Amine Content in Hydrochlorothiazide Bulk Powder and Captopril-Hydrochlorothiazide Combination Tablets

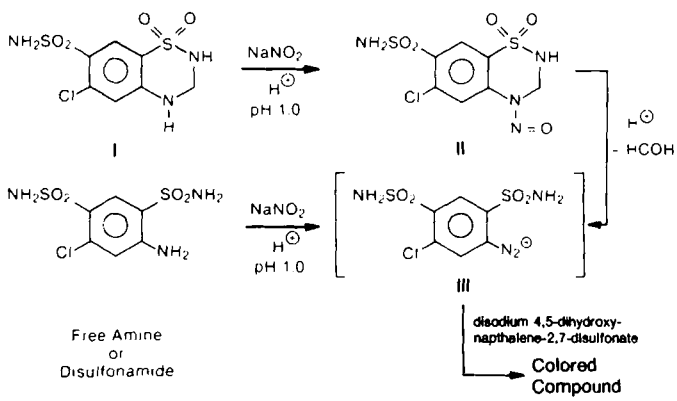
Formulation	Free Amine Content, % HPLC	Colorimetry USP	Colorimetry Kinetic
Captopril-hydrochlorothiazide Combination tablets	≤0.1[a]	0.17[a]	0.11[a]
	≤0.1[b]	0.23[b]	0.13[b]
Hydrochlorothiazide bulk powder	≤0.1[c]	0.11[c]	0.0004[c]
	≤0.1[d]	0.13[d]	0.06[d]

[a] The tablets contained 15 mg of hydrochlorothiazide. [b] Tablets contained 25 mg of hydrochlorothiazide. [c] Lot NX008. [d] NX018.

One of the standard syntheses of *N*-nitrosamines is nitrous acid addition to a secondary amine in which the nitrous acid is generated *in situ* by the addition of sodium nitrite to an aqueous acidic solution of the amine. Depending on the secondary amine, this nitrosation can be very slow (*e.g.*, dimethylamine) or quite rapid (*e.g.*, *N*-methylaniline) (10). In fact, for secondary aromatic amines, the reaction mixture is usually cooled to 0°C to allow nitrosation to take place at a reasonable rate. Since hydrochlorothiazide is a secondary aromatic amine, it would not be surprising that some nitrosamine would be generated in the diazotization reaction. This was found to be the case.

The nitrosation kinetics of hydrochlorothiazide have been reported by Gold and Mirvish (6). They have reported that the reaction was second order and possessed a rate constant of 0.76 M^{-1} s^{-1}. Hydrochlorothiazide was dissolved in 25% aqueous acetic acid, the pH was adjusted to 1.0 with 70% perchloric acid mixed with an aqueous sodium nitrite solution, and the kinetics were followed by measuring the disappearance of the UV maximum and appearance of the λ_{max} of the nitrosamine at 392 nm. The conditions used by Gold and Mirvish are nearly identical to those present in the diazotization reaction in the procedure described in USP XX, *i.e.*, pH 1.0 at 25°C in aqueous media. We can approximate the amount of hydrochlorothiazide nitrosated under these conditions by using the second-order rate equation: $(1/a - x) - (1/a) = kt$, where a is the initial concentration of hydrochlorothiazide (~0.003 M), x is the concentration of the nitrosamine at time t, and k is 0.76 M^{-1} s^{-1}. For example, in 5 and 30 min, 41 and 81% of the hydrochlorothiazide, respectively, was nitrosated.

In Scheme I is depicted the reactions of both hydrochlorothiazide and any



Scheme I

Table III—Acid-Catalyzed Decomposition of the 4-Nitroso Derivative of Hydrochlorothiazide

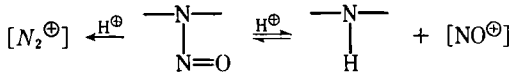
Time, min	Free Amine Content, %[a]	
	Dilute HCl (1:10)	Sodium Nitrite Dilute HCl
5	0.42	1.07
10	0.55	1.08
15	0.63	1.22
30	1.04	2.09

[a] Procedures described in USP XX for diazotizable substances.

free amine present under nitrosation conditions. The addition of the nitroso group in the 4-position (II) greatly increases the acid lability of the compound compared with hydrochlorothiazide (I). Acid-catalyzed hydrolysis of the 4-nitroso derivative of hydrochlorothiazide could lead to the same intermediate diazonium ion that would be produced by the diazotization of any free amine. This diazonium ion is subsequently reacted with disodium 4,5-dihydroxynaphthalene-2,7-disulfonate, and the resulting colored compound is quantitated by reading at 500 nm.

To prove this hypothesis, the 4-nitroso derivative of hydrochlorothiazide was synthesized by previously reported procedures (6), recrystallized, and then assayed for free amine content by the procedure described in USP XX. The free amine content increased with time just as in the determination of free amine in hydrochlorothiazide (Table III). The nitrosamine was also allowed to react in dilute HCl (1:10) for various periods of time, with no sodium nitrite present. Once again, increasing free amine content with increasing time was observed. Obviously, the nitrosamine is undergoing acid-catalyzed decomposition to the diazonium ion (III, Scheme I), which is subsequently reacting with the color reagent in the assay.

The rate of decomposition of the 4-nitroso derivative of hydrochlorothiazide in nitrous acid was found to be about twice that in dilute HCl (Table III.) This might be due to an equilibrium shift. In addition to the decomposition to the diazonium ion, secondary *N*-nitrosamines can undergo denitrosation back to the secondary amine. This denitrosation is an equilibrium process:



In dilute HCl when $NaNO_2$ is present, the concentration of NO^+ present is increased. Thus, the equilibrium is shifted to the left causing the observed increase in the rate of decomposition.

The results of these experiments indicate conclusively that the diazotization step generates an artifactual interference in the colorimetric assay. Although HPLC is an excellent alternative for the determination of the free amine (disulfonamide) content of hydrochlorothiazide bulk powder and captopril–hydrochlorothiazide combination tablets, it has a limit of detection of ~0.1%. To achieve the sensitivity of the procedure described in USP XX, an alternative colorimetric procedure has been developed[7] which will be discussed in a later report.

All other colorimetric USP XX tests for diazotizable substances in benzothiadiazines in which sodium nitrite in acid media is used have been thrown into doubt as a result of these experiments. In fact, bendroflumethiazide and flumethiazide were shown to yield increasing free amine content with increasing time of diazotization. For example, USP free amine assays gave contents of 0.8% for bendroflumethiazide bulk powder, whereas a value of 0.2% was obtained by HPLC (4).

Numerous *N*-nitrosamines are known carcinogens and mutagens. However, the 4-nitroso derivative of hydrochlorothiazide has been found to be neither carcinogenic nor mutagenic[8] and does not pose any danger in handling. Since the other 4-nitrosobenzothiadiazines have not been tested for carcinogenicity and mutagenicity, care should be exercised when performing the assay for diazotizable substances. In the new colorimetric procedure[7], no *N*-nitrosamines are formed.

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[7] Whigan and P. Stears, unpublished results

[8] R. Kupper and D. Nagel, unpublished results.