Spectrophotometric determination of hydrazine, hydrazides, and their mixtures with trinitrobenzenesulfonic acid

ARTICLE *in* ANALYTICAL BIOCHEMISTRY · DECEMBER 1988

Impact Factor: 2.22 · DOI: 10.1016/0003-2697(88)90371-5 · Source: PubMed

3 AUTHORS, INCLUDING:

Nemat O Keyhani
University of Florida
79 PUBLICATIONS **1,929** CITATIONS

SEE PROFILE

Yuan C Lee
Johns Hopkins University
357 PUBLICATIONS **13,707** CITATIONS

SEE PROFILE

ANALYTICAL BIOCHEMISTRY 175, 139–144 (1988)

Spectrophotometric Determination of Hydrazine, Hydrazides, and Their Mixtures with Trinitrobenzenesulfonic Acid[1]

XIAO-YANG QI, NEMAT O. KEYHANI, AND YUAN CHUAN LEE[2]

Biology Department, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218

Received July 5, 1988

A method has been developed to measure hydrazine, hydrazides, and their mixtures using a modification of the trinitrobenzenesulfonic acid method [T. Okuyama and K. Satake (1960) *J. Biochem. (Tokyo)* **47**, 654–660]. After incubation of the sample containing hydrazine and hydrazide with trinitrobenzenesulfonate at pH 8.5 at room temperature for 40 min, the reaction mixture was diluted with a Na_2CO_3–$NaHCO_3$ buffer (0.1 M, pH 10.8) rather than with 0.5 M HCl. Different chromogens were produced from the reaction of hydrazine (λ_{max} = 570 nm) and hydrazides (λ_{max} = 385 and 500 nm) with trinitrobenzenesulfonic acid. The method allowed simultaneous determination of hydrazine (5 to 60 nmol) with hydrazide (10 to 120 nmol) in a mixture with a standard deviation of less than 5%. The presence of amino compounds (except for amino sugars) did not interfere with the measurement of hydrazine or hydrazides. Interference by amino sugars in the determination of hydrazine or hydrazides was eliminated by pretreatment of the sample with $NaBH_4$ to reduce the amino sugars to 2-amino-2-deoxyhexitols.

KEY WORDS: trinitrobenzenesulfonic acid; hydrazine; hydrazide.

Hydrazine and hydrazides are frequently encountered in biochemical research. Hydrazinolysis is one of the standard methods for determination of the C-terminal residue of a peptide (1). Hydrazides are useful tuberculostatic drugs (2), and acyl hydrazides are precursors to acyl azides, which are employed in peptide bond formation (3).

Several colorimetric methods have been reported for quantitative determination of hydrazine and hydrazides based on the following principles: (A) Reactions with aldehydo-compounds: *p*-Dimethylaminobenzaldehyde forms yellow hydrazones with hydrazine as well as unsubstituted hydrazides (4), and glutaconaldehyde forms a red color with hydrazine (5). *(B) Reactions with keto-compounds:* Alkaline 1,2-naphthoquinone-4-sulfonate forms orange–red chromophores with hydrazides (6,7) and alkaline 2,3-dichloro-1,4-naphthoquinone has been reported to form a blue color specifically with acyl monohydrazides (8). *(C) Acylation with chromogenic reagents:* Hydrazine and hydrazides react with 2,4-dinitrobenzoyl chloride to form colored products (9). *(D) Formation of fluorophores:* Fluorescamine and *o*-phthalaldehyde form intensely fluorescent compounds when reacted with hydrazine (10). None of these methods, however, are useful for determination of hydrazine and hydrazide in a mixture.

2,4,6-Trinitrobenzenesulfonic acid (TNBS)[3] (11) is a sensitive and reliable reagent for measurement of amino compounds. In 1967, LaRue described a spot test for distinguishing hydrazine from its derivatives and other amino compounds based on their reaction

[1] Supported by NIH Research Grant DK09970. Publication No. 0000 from the McCollum-Pratt Institute, The Johns Hopkins University.

[2] To whom all correspondence should be addressed.

[3] Abbreviation used: TNBS, 2,4,6-trinitrobenzenesulfonic acid.

0003-2697/88 $3.00

with TNBS to form different colored compounds (12). More recently, a modification of the TNBS method (13), which has been in use in our laboratory for determination of various amino compounds, was adapted to measure hydrazine and hydrazides (14–16). We now report optimized conditions for maximum color yields for hydrazine and hydrazides as well as a procedure for determining these compounds in a mixture.

MATERIALS AND METHODS

Reagents. TNBS, dihydrazide of adipic acid, and D-galactosamine hydrochloride were obtained from Sigma Chemical Co.; hydrazine, *p*-hydroxybenzoyl hydrazide, and 6-amino-1-hexanol were from Aldrich Chemical Co.; and D-glucosamine hydrochloride was from Pfanstiehl Laboratories, Inc. 2-Amino-D-glucitol hydrochloride was prepared by Dr. J. Scocca. The hydrazide of a synthetic peptide (YSPTSPS) was a gift of Dr. C. Anfinsen. 5-(2′,2′-Dimethoxyethylaminocarbonyl)pentanoyl hydrazide [$(CH_3O)_2CH$-$CH_2NHCO(CH_2)_4CONHNH_2$] was prepared by Dr. T. C. Wong. The buffer solution (pH 10.8) used for dilution of colored solution was prepared by mixing 0.1 M sodium carbonate and 0.1 M sodium bicarbonate in a 9:1 (v/v) ratio. The TNBS reagent and hydrazine or hydrazide standard solutions were made fresh daily.

The TNBS reaction. The same procedure was used for determination of hydrazine and hydrazides as well as for their mixtures. A 0.5-ml sample (containing 5 to 60 nmol of hydrazine or dihydrazides, 10 to 120 nmol of monohydrazides or other amino compounds) in a 13 × 100-mm test tube was mixed with 0.5 ml of 0.2 M sodium borate at pH 8.5. The reaction was initiated by addition of 0.2% (w/v) TNBS. After 40 min at room temperature, the mixture was diluted with 1.5 ml of the pH 10.8 buffer. For routine analyses, the absorbance values at suitable wavelengths (see below) were measured within 20 min using a Spectronic 20 (Bausch & Lomb) colorimeter. When the absorbance was measured after 20 min, excessive TNBS decomposition occurred and the results became less accurate. The absorption spectra (300–700 nm) of the developed color solutions were obtained with a Perkin–Elmer Model 576 spectrophotometer or a Hewlett-Packard Model 8452A diode array spectrophotometer. To prepare a blank for these determinations, water was substituted for the hydrazine/hydrazide solution.

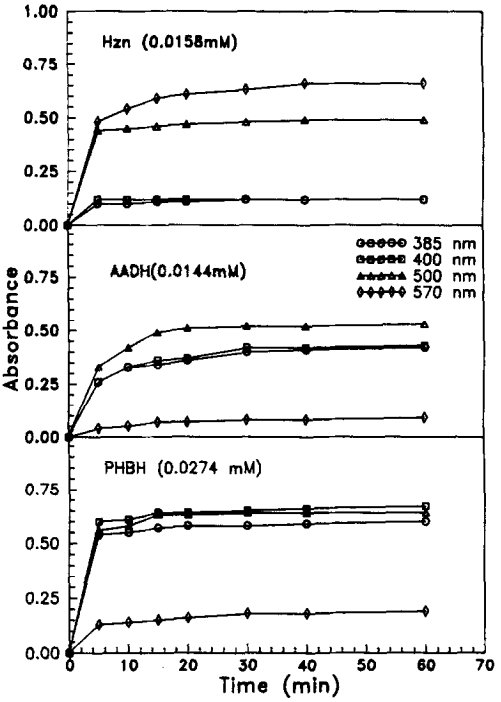

FIG. 1. Time course of the color development by reaction of TNBS with (A) hydrazine (15.8 μM), (B) dihydrazide of adipic acid (14.4 μM), and (C) *p*-hydroxybenzoyl hydrazide (27.4 μM). The TNBS reaction was carried out at pH 8.5 and adjusted to pH 9.9 before measurement of absorbance using a Spectronic 20 colorimeter.

RESULTS AND DISCUSSION

Time course of the color formation. Optimum reaction time was determined by carrying out the TNBS reaction mixture for different lengths of time and mixing it with the pH 10.8 buffer. As shown in Fig. 1, the

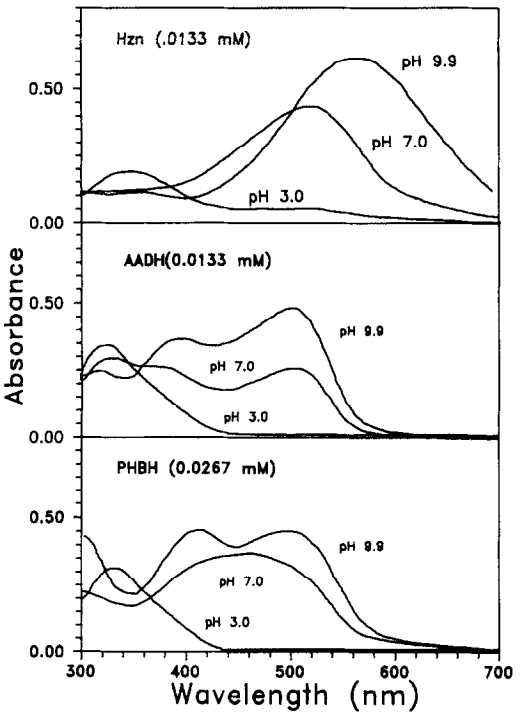

FIG. 2. Absorption spectra of the TNBS reaction products of hydrazine (Hzn, 13.3 μM), dihydrazide of adipic acid (AADH, 13.3 μM), and *p*-hydroxybenzoyl hydrazide (PHBH, 26.7 μM) after dilution with buffers of different pH values (e.g., citric acid–Na_2HPO_4 at pH 3.0 and 7.0 and Na_2CO_3–$NaHCO_3$ at pH 10.8). The measured pH of each solution is indicated in the figure.

TNBS reactions with hydrazine and hydrazides were complete within 40 min.

Absorption spectra and pH. The spectra of chromogens generated by the TNBS reaction varied greatly with pH, which was adjusted by adding different buffers to the TNBS reaction mixture. The highest absorbance values were obtained by addition of the pH 10.8 carbonate buffer for all compounds. The spectra of the chromogens from hydrazine, the dihydrazide of adipic acid, and *p*-hydroxybenzoyl hydrazide, after adjustment to pH 3.0, 7.0, and 9.9 by dilution with the appropriate buffer, are shown in Fig. 2. At the final pH of 9.9 (obtained by mixing with pH 10.8 buffer), hydrazine had a single absorption maximum at 570 nm. The aliphatic hydrazides, 5-(2',2'-dimethoxyethylaminocarbonyl)pentanoyl hydrazide and dihydrazide of adipic acid, exhibited absorption maxima at 385 and 500 nm. The aryl hydrazide, *p*-hydroxybenzoyl hydrazide, also had two absorption maxima, but at different wavelengths (400 and 500 nm). All of the amino acids, amino sugars, and amino alcohols tested gave maxima at wavelengths lower than 480 nm. The millimolar absorbances of a number of selected compounds at different wavelengths are shown in Table 1. The absorbance values obtained using a spectrophotometer and a colorimeter were somewhat different, presumably due to the wider bandwidth of the colorimeter.

Determination of hydrazine and hydrazides. The standard curves for hydrazine and hydrazide, obtained at final pH of 9.9 and measured with a Spectronic 20 colorimeter, are shown in Fig. 3. The Lambert–Beer's law was obeyed in the concentration range 2 to 20 μM for hydrazine and dihydrazides and 5 to 40 μM for hydrazides. The slope of each line, after least-square regression analysis, gave millimolar absorbance values.

If a solution contains either hydrazine or a hydrazide alone, the measurement at a single wavelength is satisfactory for quantitative determination. When hydrazine and hydrazides are present together, as is often found in hydrazinolysis reaction mixtures, measurement of absorbance at two wavelengths allows determinations of both components, since their maxima are sufficiently separated from one another.

Quantitative determination of mixtures of hydrazine and hydrazides. Based on Lambert–Beer's law, the concentration of each component in a mixture of hydrazine and hydrazide can be expressed in terms of the predetermined millimolar absorbances of the components and the measured absorbance of the mixture at two different wavelengths as shown:

$$C_x = \frac{E_2^y \times A_1 - E_1^y \times A_2}{Q}$$

$$C_y = \frac{E_1^x \times A_2 - E_2^x \times A_1}{Q},$$

TABLE 1

MILLIMOLAR ABSORBANCE OF DIFFERENT CHROMOGENS AT DIFFERENT WAVELENGTHS

Compound	Wavelength (nm)						
	355	385	400	425	480	500	570
Hydrazine[a]	10.2	8.43	8.80	10.6	22.7	32.3	44.9
Dihydrazide of adipic acid[a]	17.9	28.6	27.4	23.2	32.7	39.8	5.18
p-Hydoxybenzoyl hydrazide	10.5	18.9	20.1	17.0	17.9	20.8	4.52
5-(2',2'-Dimethoxyethyl-aminocarbonyl)pentanoyl hydrazide	8.99	14.4	13.7	12.8	17.3	19.6	2.69
Hydrazide of a synthetic peptide[b]	8.85	13.7	13.0	12.3	17.3	19.5	2.65
D-Glucosamine hydrochloride	4.00	1.55	1.75	4.10	10.4	6.65	0.00
D-Galactosamine hydrochloride	4.14	1.62	1.76	4.38	12.2	8.48	0.00
D-Glucosaminitol hydrochloride	5.33	4.15	4.24	5.39	2.15	1.02	0.00
6-Amino-1-hexanol	9.55	7.61	9.82	12.2	3.82	1.06	0.00
Glycine	11.3	7.24	8.48	10.5	3.10	1.00	0.00

[a] Contains two NH_2-groups but absorbance was calculated on the basis of single NH_2-groups.
[b] Having a sequence of YSPTSPS.

where C_x and C_y are the concentrations (in mM) of compounds X (e.g., hydrazine) and Y (e.g., hydrazide); A_1 and A_2 are the measured absorbance values at wavelengths 1 and 2; E_1^x, E_1^y, E_2^x, and E_2^y are the millimolar absorbance values of X and Y at wavelengths 1 and 2, respectively, and $Q = E_1^x \times E_2^y - E_1^y \times E_2^x$. The concentration of the dihydrazide is expressed in terms of hydrazide equivalents in all calculations.

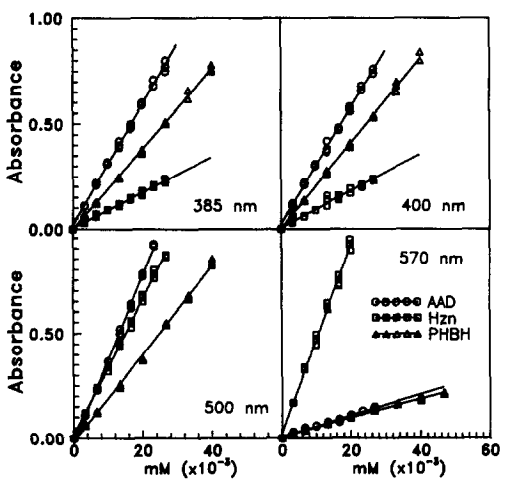

FIG. 3. Standard curves of various compounds reacted with TNBS measured at different wavelengths. (□) Hydrazine (Hzn), (○) dihydrazide of adipic acid (AADH), (△) *p*-hydroxybenzoyl hydrazide (PHBH). The final pH of the colored solution was 9.9.

To test the validity of this method, artificial mixtures containing different amounts of hydrazine and a hydrazide (up to a fivefold excess of one component over the other) were prepared, and the concentration of each component was determined. The results indicate that the largest error in the concentration determination of hydrazine, dihydrazide of adipic acid, *p*-hydroxybenzoyl hydrazide, and 5-(2',2'-dimethoxyethylaminocarbonyl)-pentanoyl hydrazide was 5.7, 6.5, 2.9, and 8.6%, respectively. In addition, a blind test using artificial mixtures of unknown composition was performed and its results are tabulated in Table 2. The errors in this analysis were less than 5%.

Interference by amino compounds. A variety of amino compounds, with the exception of amino sugars, did not affect the determination of hydrazine and/or hydrazides by this

TABLE 2

ANALYSIS OF A MIXTURE OF HYDRAZINE AND HYDRAZIDE[a]

Sample	Wavelengths (nm)	Concentration (μM) Expt. A[b]	Expt. B	Calc. A	Calc. B	Error (%) A	Error (%) B
1	380/570	4.4	11.4	4.2	11.1	+4.8	+2.7
	500/570	4.4	11.2			+4.8	+0.9
2	380/570	2.1	14.7	2.1	14.8	0	−0.7
	500/570	2.1	14.8			0	0
3	380/570	9.8	1.9	9.5	1.9	+3.2	0
	500/570	9.8	1.9			+3.2	0

[a] The data in this table represent a blind test in which the composition of the sample mixtures was unknown prior to the analysis.

[b] Compound designation: A, hydrazine; B, dihydrazide of adipic acid.

method when absorbance was measured at 500 and 570 nm (see Table 1). 6-Amino-1-hexanol, 2-amino-2-deoxy-D-glucitol, and glycine yielded maxima at 355 and 425 nm, but no appreciable absorbance at 500 and 570 nm. The absorbance at 570 nm by the reaction product of D-glucosamine or D-galactosamine was negligible; thus, the presence of glucosamine (or galactosamine) does not interfere with determination of hydrazine. Although the colored reaction products, D-glucosamine and D-galactosamine, showed a peak at 475 rather than 500 nm, their absorbance at 500 nm was still about 33 and 43% that of the monohydrazides, respectively. Since 2-amino-2-deoxyalditols have no appreciable absorbance at 500 nm, interference by amino sugars as described above, caused by absorption at 500 nm, can be eliminated by reduction (see below).

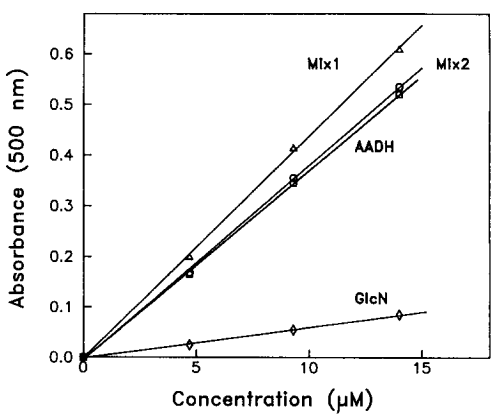

FIG. 4. Elimination of the interference of D-glucosamine in the determination of dihydrazide of adipic acid (by $NaBH_4$ reduction). (□) Dihydrazide of adipic acid (AADH), (◇) D-glucosamine hydrochloride (GlcN), (△) mixture of dihydrazide of adipic acid and D-glucosamine hydrochloride in a 1:1 (v/v) ratio without the treatment of $NaBH_4$ (Mix1), (○) the same mixture (△) after reduction with $NaBH_4$ (Mix2).

Elimination of amino sugar interference by reduction. One milliliter of a mixture containing 14 μmol each of the dihydrazide of adipic acid and D-glucosamine hydrochloride was treated with an 11-fold excess (over the amino sugar) of solid sodium borohydride. The reaction was carried out overnight at 4°C. After addition of 10 μl of glacial acetic acid to stop the reaction, the mixture was left for 1 h at room temperature and was evaporated at 40°C. The boric acid produced as a result of the acidification was removed by repeated evaporation of the reaction mixture with addition of 1 ml each of methanol and the final product was brought up in 1 ml of water and subjected to the TNBS assay. The

absorbance of the chromogens resulting from the mixture of dihydrazide and reduced D-glucosamine was very similar to that of the dihydrazide in the absence of amino sugar (Fig. 4).

CONCLUSION

Hydrazine or hydrazide can be determined by the TNBS method by measuring the absorbance of the resulting chromogens at 570 or 500 nm, respectively. When hydrazine or hydrazide is present in a mixture, absorbance measurements at 500 and 570 nm result in the accurate determination of both compounds. With the exception of amino sugars, amino compounds of other types do not appear to interfere in the hydrazide determination using this method. In addition, the interference of amino sugars during determination of the hydrazide content of a mixture can be eliminated by converting the amino sugars to amino alcohols prior to the TNBS reaction.

REFERENCES

1. Yosizawa, Z., Sato, T., and Schmid, K. (1966) *Biochim. Biophys. Acta* **121,** 417–420.
2. Murti, K. (1975) *in* Antibiotics (Corcoran, J. W., and Hahn, F. E., Eds.), Vol. 3, pp. 623–652, Springer-Verlag, New York.
3. Bodanszky, M., Klausner, Y. S., and Ondetti, M. A. (1976) *in* Peptide Synthesis, 2nd ed., pp. 56–57, Wiley, New York.
4. Schmidt, E. W. (1984) Hydrazine and Its Derivatives, p. 435, Wiley, New York.
5. Asmus, E., Ganzke, J., and Schwartz, W. (1971) *Fresenius' Z. Anal. Chem.* **253,** 102–105.
6. Schmidt, E. W. (1984) Hydrazine and Its Derivatives, p. 434, Wiley, New York.
7. Pratt, E. L. (1953) *Anal. Chem.* **25,** 814–816.
8. Plaizier, J. A., Van Damme, J. G., and De Neve, R. E. (1976) *Anal. Chem.* **48,** 1536–1538.
9. Obtemperanskaya, S. I., Tikhonov, Yu N., Moroz, N. S., Shinskaya, T. A., Egorova, A. G., Likhosherstova, V. N., Chalenko, E. G., and Tsypkina, I. Ya. (1970) *Prob. Anal. Khim.* **1,** 220–228 (cited in *Chem. Abst.* **75,** (1971), 58446q).
10. Schmidt, E. W. (1984) Hydrazine and Its Derivatives, p. 434, Wiley, New York.
11. Okuyama, T., and Satake, K. (1960) *J. Biochem. (Tokyo)* **47,** 654–660.
12. LaRue, T. A. (1967) *Talanta* **14,** 1344–1346.
13. McKelvy, J. F., and Lee, Y. C. (1969) *Arch. Biochem. Biophys.* **132,** 99–110.
14. Lee, Y. C. (1978) *Carbohydr. Res.* **67,** 509–514.
15. Kawaguchi, K., Kuhlenschmidt, M., Roseman, S., and Lee, Y. C. (1980) *Arch. Biochem. Biophys.* **205,** 388–395.
16. Kawaguchi, K., Kuhlenschmidt, M., Roseman, S., and Lee, Y. C. (1981) *J. Biol. Chem.* **256,** 2230–2234.