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Studies on the Mechanism of Drug Allergy to Sulfanilamides. I.[1] A Colorimetric Method for the Determination of 4-Hydroxylaminobenzenesulfonamide in Biological Materials with Sodium Pentacyanoammineferroate

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A new colorimetric method for the determination of 4-hydroxylaminobenzenesulfonamide was examined. It is said that this compound is a hapten inducing drug allergy due to sulfanilamide and is formed *in vivo* and *in vitro*.

4-Hydroxylaminobenzenesulfonamide produced a red violet complex having λ max at 515 mμ in aqueous solution, when it was reacted with sodium pentacyanoammineferroate. Therefore, the estimation of 4-hydroxylaminobenzenesulfonamide using this reagent was attempted in biological materials.

The effects of variation of times, pH and reagent concentration on the color development have been determined. This method was highly selective for the compound among the large amount of sulfanilamide and its metabolites. All the procedure was carried out in phosphate buffer solution at room temperature. The precision of the method which was obtained by performing 20 analyses on urine, was 50.02 ± 1.76 μg (mean $\pm$SD).

In 1957, Schwarz and Speck[3] demonstrated in guinea-pig experiments that a particular oxidation product, 4-hydroxylaminobenzenesulfonamide(4-HABSA) might be an eczematogen formed by the action of ultraviolet light upon sulfanilamide(SA). This product actually occured in small concentrations by *in vitro* irradiation of SA.[4] Moreover, Murata[5] has confirmed the sensitivity of 4-HABSA on being applied to the guinea-pig skin one or more times. Therefore, it seems likely that 4-HABSA may be a key substance inducing drug allergy to SA, and the relation of 4-HABSA to sensitivity of SA deserves further study.

On the other hand, Thauer, *et al.*[6] showed that rat liver microsomes catalyzed the N^4-hydroxylation of SA. In these studies, 4-HABSA was isolated from the reaction mixture and identified by thin-layer chromatography. Rosenthal and Bauer[7] reported that the hydroxylamine derivative demonstrated in the urine of the rat, rabbit, dog and man after oral administration of SA.

However, no papers deal with quantitative findings on the production of 4-HABSA in animal tissues. It thus becomes of importance to determine whether any of SA is oxidized to 4-HABSA in its passage through the body, especially in liver or skin tissues.

The authors are particularly interested in the distribution, metabolism and protein binding of 4-HABSA because of its possible physiological significance.

An analytical method to determine 4-HABSA is known which is based on diazo reaction.[7] Since this reacts both SA and 4-HABSA, the color reaction which has been used for estimation

1) This work was presented at the 91st Annual Meeting of the Pharmaceutical of Japan, Fukuoka, April 1971.
2) Location: *a) Tamagawa-cho, Takamiya, Fukuoka; b) 5-1 Oemoto-machi, Kumamoto.*
3) K. Schwarz and M. Speck, *Dermatologica*, **114**, 232 (1957).
4) L. Shinn, E.R. Main, and R.R. Mellon, *Proc. Soc. Exp. Biol., N.Y.* **52**, 736 (1939).
5) M. Murata, *Nippon Hifukagakkai Zasshi*, **79**, 358 (1969).
6) P.K. Thauer, G. Stoffler, and H. Uehleke, *Arch. Exp. Path. u. Phamak.*, **252**, 32 (1965).
7) S.M. Rosenthal and H. Bauer, *Pub. Health. Reports*, **54**, 1880 (1939).

of 4-HABSA are not specific and have shown unreliable for this purpose.[8] In order to determine only 4-HABSA, it is necessary to find other method which would eliminate from the metabolic reaction the large amount of SA and its metabolites present in the biological materials.

Formerly, Schwechten[9] and Anger[10] found that pentacyanoammineferroate(PCAF) reacted with aromatic hydroxylamine compounds and yield deep red-violet complexes.

Recently, Boyland and Nery[11] reported a sensitive method with PCAF for arylhydroxylamine. Irving[12] also described that N-hydroxylaminofluorene reacted with PCAF to form a stable colored complex and the resulting colored complex could be used as a basis for the quantitative determination of the extent of enzymic deacetylation of N-hydroxylacetylaminofluorene. However, 4-HABSA[5] was so unstable that it was not evident whether this method could apply for the determination of 4-HABSA in biological materials or not. Therefore, the reaction conditions and the effects of interfering compounds on the complex formation have been examined by the authors.

Hence, it has been found that 4-HABSA in biological materials satisfactorily determined with sodium pentacyanoammine ferroate(PCAFS) in this study.

Experimental[13]

Preparation of Materials——Sulfanilamide, commercially available, was recrystallized from water. mp 166—167°. N^4-Acetylsulfanilamide was prepared by acetylation of SA.[14] mp 215—216°. N^1,N^4-diacetylsulfanilamide was synthesized using the procedure of Crossley, *et al.*[15] mp 258—259°. 4-HABSA; The method for the preparation of 4-HABSA differed from the methods using by Bratton, *et al.*[16] and Bauer,

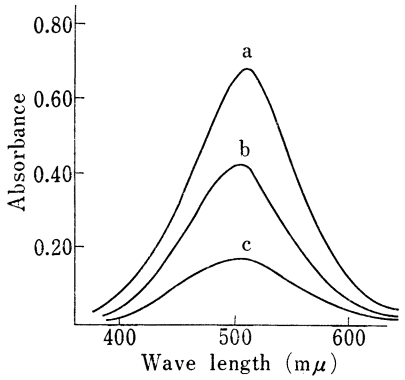

Fig. 1. Absorption Spectra of the Reaction Mixture

a: 50 μg/ml of 4-HABSA solution was treated as in the standard procedure. b: 25 μg/ml, c: 10 μg/ml

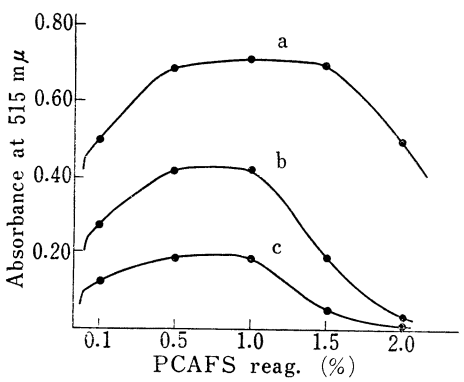

Fig. 2. Effect of the Concentration of PCAFS reag. on the Color Development

4-HABSA solutions were treated as in the standard procedure with the concentrations of PCAFS reag. a: 50 μg/ml, b: 25 μg/ml, c: 10 μg/ml

8) W.V. Thorpe and R.T. Williams, *Biochem. J.*, **1941**, 52.
9) H.W. Schwechten, *Ber.*, **65**, 1734 (1932).
10) V. Anger, *Mikrochim. Acta*, **1962**, 94.
11) E. Boyland and R. Nery, *Analyst*, **89**, 95 (1964).
12) C.C. Irving, *Cancer Res.*, **26**, 1390 (1966).
13) Absorption spectra and absorbances were measured by Shimazu MSP-50L spectrophotometer and Hitachi EPU-2 spectrophotometer, respectively.
14) E. Miller, H.J. Rock, and M.L. Moore, *J. Am. Chem. Soc.*, **61**, 1198 (1939).
15) M.L. Crossley, E.H. Northey, and M.E. Hultquist, *J. Am. Chem. Soc.*, **61**, 2950 (1939).
16) A.C. Bratton, H.J. White, and E.K. Marshall, *J. Proc. Soc. Exptl. Biol. Med.*, **42**, 847 (1939).

et al.[17] In the present work, 4-HABSA was synthesized by the procedure previously described,[18] using L-ascorbic acid as reducing agent. mp 141—142°.

Standard Solution of 4-HABSA——It was prepared by dissolving 4-HABSA in phosphate buffer (pH 7.0) just before use.

Reagents——PCAFS Reagent: JIS first grade PACFS was dissolved in 1% EtOH and filtered before use. All other reagents used were JIS Reagent Grade unless otherwise noted. Buffer solution: As buffer solutions, the following three were used; Glycine hydrochloric acid buffer for pH 1—4, 1/30M KH_2PO_4—1/30M $NaHPO_4$ buffer for pH 5—8, and NH_4OH–NH_4Cl buffer for pH 9—10, respectively.

Estimation of Absorption Maximum——To 5 ml of sample solutions containing 10, 25, and 50 μg of 4-HABSA per ml, 0.1 ml of 1.0% PCAFS reagent was added, respectively well mixed, and after 10 min absorption were estimated. The absorption maximum was observed at 515 mμ as shown in Fig. 1.

Effects of Concentration of PCAFS on the Color Development——To 5 ml of 4-HABSA solution containing 10, 25, and 50 μg of 4-HABSA per ml, 0.1 ml of 0.1—2.0% of PCAFS reagent, were added. The color developed was estimated colorimetrically at 515 mμ. The results are shown in Fig. 2.
After addition of the reagent, the highest color intensity was obtained by using 1% PCAFS solution. Thus, the 1% PCAFS solution was used in the following procedures.

Effects of the Reaction Temperature and the Reaction Time on the Color Development——To 5 ml of 4-HABSA solution containing 10, 25, and 50 μg of 4-HABSA per ml, 0.1 ml of 1% PCAFS solution was added, and then the reaction mixture was allowed to stand at 5—90° for 10 min. Correlation between the reaction temperature and the color development was shown in Fig. 3. The results indicated the stable color intensity at the temperature between 20—70°. From the results described above, the reaction was treated at the room temperature for convenience. The color developed under the conditions mentioned above was stable at the time longer than 10 min and when left standing at room temperature, the stability was maintained even over 720 min long, as shown in Fig. 4.

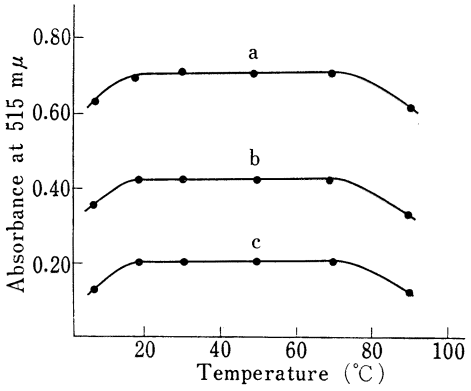

Fig. 3. Effect of the Reaction Temperature on the Color Development

a: 50 μg/ml of 4-HABSA solutions was treated as in the standard procedure. b: 25 μg/ml, c: 10 μg/ml

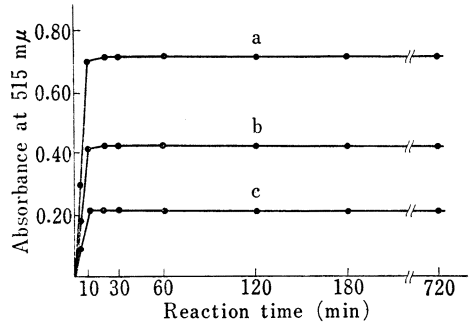

Fig. 4. Stability of the Color Development

a: 50 μg/ml of 4-HABSA was treated as in the standard procedure and left standing at room temperature. b: 25 μg/ml, c: 10 μg/ml

Effects of the pH on the Color Development——This was studied with respect to effect of pH on the color development. 4-HABSA solutions adjusting to pH 1—10 with buffer solution described before were used by the standard procedure as shown in Fig. 5. It was observed that the pH had relatively a large effect on the color development, although N-phenylhydroxylamine and N-(2-naphthyl)-hydroxylamine complexes showed maximum stability between pH 6.0 and pH 10,[11] and Feigl described that the color complex was independent to the pH.[19] Even when borate or acetate buffers were used, this tendency did not change.

Calibration Curve of 4-HABSA with PCAFS——The standard 4-HABSA solution was prepared by dissolving 4-HABSA in phosphate buffer pH 7.0. To 50 ml of the 4-HABSA solutions, 0.1 ml of 1.0% PCAFS reagent was added and well mixed. The reaction mixture was estimated colorimetrically at 10—30 min after addition of the reagent. The results gave linear relationship as shown in Fig. 6.

17) H. Bauer and S.M. Rosenthal, *J. Am. Chem. Soc.*, **66**, 611 (1944).
18) I. Yamamoto, R. Eyanagi, K. Fujita, M. Fukunaga, Y. Tatsukawa, and K. Yoshida, *The Annual Report of the Daiichi College of Pharmaceutical Sciences*, **1**, 9 (1971).
19) F. Feigl, "Spot Tests in Organic Analysis," 7th ed., Elsevier Publishing Co., Amsterdam, 1966, p. 285.

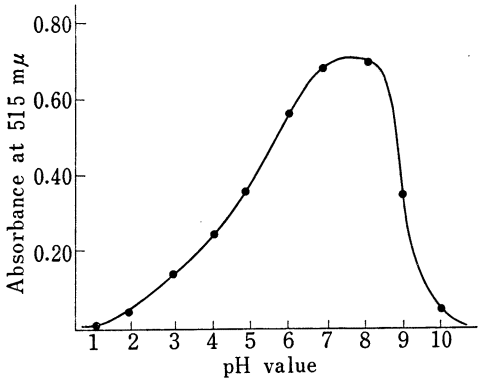

Fig. 5. Effect of pH on the Color Development

50 μg/ml of 4-HABSA solutions were treated as in the standard procedure.

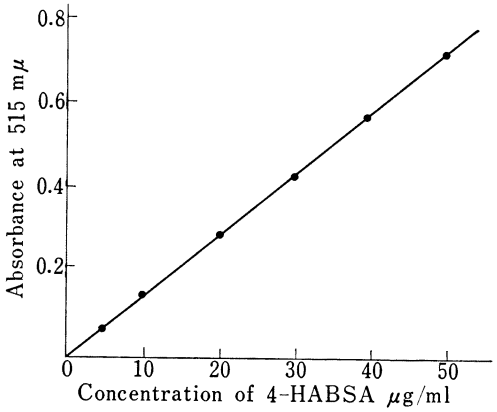

Fig. 6. Calibration Curve for 4-HABSA

Table I. Effect of Sulfanilamide or Its Metabolites added on Absorbance

Substance added to 4-HABSA (35 μg)			Absorbance(515 mμ)
Sulfanilamide		250 μg	0.491
		500	0.482
		1000	0.470
		10000	0.478
N^4-Acetylsulfanilamide		250	0.463
		500	0.471
		1000	0.470
		10000	0.468
N^1,N^4-Diacetylsulfanilamide		250	0.471
		500	0.476
		1000	0.470
		10000	0.472
Control 4-HABSA	35 μg	1	0.486
		2	0.476

Effects of SA and Its Metabolites on the Estimation of 4-HABSA——Other compounds, such as SA and N^4-acetylsulfanilamide and N^1,N^4-diacetylsulfanilamide, may be excreted as metabolites in animal urine[20] So, the effect of SA and its metabolite, which are supposed to be influential on estimating 4-HABSA, was examined. A control solution was prepared by adding 6 ml of phosphate buffer pH 7.0 to 4 ml of 4-HABSA solution. On the other hand, instead of the buffer solution, 6 ml of SA or its metabolite containing 250, 500, 1000, and 10000 μg was added to 4 ml of 4-HABSA solution. When SA or its metabolite could not dissolve in buffer solution, a little EtOH was used as solvent. Those three kinds of samples were estimated and each of absorbance was compared as shown in Table I.

Recoveries of 4-HABSA contained in Phosphate Buffer, Human Urine and Rat Serum——Known amount of 4-HABSA in the phosphate buffer was estimated by the same method as described above. The urine sample was prepared by adjusting to pH 7.0 with phosphate buffer and was used. The results were shown in Table II.

20) R.T. Williams, "Detoxication Mechanisms," Chapman & Hall Ltd., London, 1959, p. 503.

Table II. Recoveries of 4-HABSA from Phosphate Buffer, Human Urine and Rat Serum

Sample	4-HABSA added μg/ml	4-HABSA recovered μg/ml	Recovery %	Average %
Phosphate buffer (pH 7.0)	5.0	5.0	100.0	
	10.0	9.9	99.0	
	20.0	20.0	100.0	
	30.0	28.8	96.0	100.8
	40.0	39.9	99.7	
	50.0	54.7	109.4	
Human urine[a)]	5.0	4.9	98.0	
	10.0	10.0	100.0	
	20.0	19.3	96.5	
	30.0	29.3	97.0	97.0
	40.0	38.2	95.7	
	50.0	47.5	95.0	
Rat serum[a)]	5.0	4.8	96.0	
	10.0	10.0	100.0	
	20.0	20.1	99.1	
	30.0	29.5	98.3	97.9
	40.0	39.6	99.0	
	50.0	47.5	95.0	

a) adjusted to pH 7.0 with phosphate buffer

Discussion

PCAFS reagent rapidly formed a stable colored complex with 4-HABSA as reported by Boyland, *et al.*[11)] and by Irving[12)] for other arylhydroxylamines. The complex had maximum absorbance at 515 mμ. The method for determination of 4-HABSA with PCAFS is comparatively simple and rapid one as shown in the experiments described above. Moreover, the recoveries of 4-HABSA in urine and serum gave good results.

SA and its metabolites did not effect the estimation of 4-HABSA. Therefore, the present method is successfully applied to estimate 4-HABSA without any consideration with existences of SA and its metabolites which might be present urine and other biological materials. Some normal substances other than SA and its metabolites did not influences the color development in a concentration of 25 μg of 4-HABSA per ml. Those examined were 10 μg of cysteine, 25 μg of ascorbic acid and 10 μg of glutathione per ml. Urea also did not effect the estimation of 4-HABSA. The precision of the method was studied with respect to repeatability, which was obtained by performing 20 analyses on human urine with mean 4-HABSA value of 50.02 μg per ml. The standard deviation was 1.76(coefficient of variation: 3.5%).