[Chem. Pharm. Bull. 28(3) 755—760 (1980)]

Studies on the Phosphorimetric Determination of Amines with Halonitro Compounds. III.[1] Phosphorimetric Determination of 4-Homosulfanilamide with 2-Chloro-5-nitropyridine

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(Received August 1, 1979)

4-Homosulfanilamide was found to react with 2-chloro-5-nitropyridine to form 5-nitro-2-(*p*-sulfamoylbenzylamino)pyridine, which was strongly phosphorescent in ethanol containing 0.1% (v/v) dimethyl sulfoxide. This observation was used as the basis for a procedure to determine micro amounts of 4-homosulfanilamide, and the method was also applied to the determination of 4-homosulfanilamide added to the rat plasma. A linear relationship between the phosphorescence intensity and the amine concentration was observed in the range of 4×10^{-7}—2×10^{-5} M.

Keywords——phosphorimetry; microdetermination; 4-homosulfanilamide; 2-chloro-5-nitropyridine; 5-nitro-2-(*p*-sulfamoylbenzylamino)pyridine

During the course of a study of the phosphorescence and fluorescence properties of 4-nitroaniline derivatives, it was found that 4-fluoronitrobenzene reacted with 4-homosulfanilamide (4-HS) to give an intensely phosphorescent compound, and this observation was applied to the microdetermination of 4-HS.[3]

Recently, when the phosphorescence and fluorescence properties of the compounds obtained by the reaction of 2-chloro-5-nitropyridine (2-CNP) with amines were examined, an intense phosphorescence was observed from the reaction product of 2-CNP with 4-HS in ethanol. This observation was also utilized in the microdetermination of 4-HS and an application of the method for the determination of 4-HS added to rat plasma was examined.

Experimental

Apparatus——Phosphorescence excitation and emission spectra, and intensity were measured with a Hitachi MPF-4 spectrofluorimeter equipped with a Hitachi phosphoroscope attachment, and the lifetimes were measured with the same apparatus equipped with a Hitachi V-104 synchroscope.

Phosphorimetric measurement was carried out at liquid-nitrogen temperature using a fused quartz microsample tube of 2 mm inner diameter.

Reagent——4-HS: This was obtained as reported previously.[3]

4-HS Stock Standard Solution: 4-HS (93 mg) was dissolved in 500 ml of 5% dimethyl sulfoxide (DMSO)-acetone (Me_2CO) mixture (v/v, %) (1×10^{-3} M). The solution was stable for at least 2 weeks when stored at room temperature protected from light.

4-HS Working Standard Solutions: These were prepared by diluting the stock standard solution with 5% DMSO–Me_2CO mixture to the desired concentrations before use.

2-CNP: This was prepared by reported methods.[4,5]

2-CNP Stock Solution: 2-CNP (4 mg) was dissolved in 500 ml of Me_2CO (5×10^{-3} M). The solution was stable for at least 2 weeks when stored at room temperature protected from light. It was diluted with Me_2CO to the desired concentrations before use.

Ethanol: Reagent-grade EtOH (1000 ml) was distilled after dissolving 10 g of Na metal in it.

DMSO: Reagent-grade DMSO (500 ml) was distilled at 53—55°/4 mmHg.

1) Part II: K. Hirauchi and T. Amano, *Chem. Pharm. Bull.*, **27**, 1120 (1979).
2) Location: *Sagisu, Fukushima-ku, Osaka, 553, Japan.*
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Me_2CO: Reagent-grade Me_2CO was distilled by the usual method.
Hexane, ether, ethyl acetate, chloroform, cyclohexane, and potassium hydroxide were of reagent grade.
Rat: Female Wistar rats (210—250 g) were used.

Standard Procedure——One ml of test solution containing 4-HS (4×10^{-7}—2×10^{-5} M) was pipetted into a test tube, 1.0 ml of 2-CNP solution was added, and the solution was mixed thoroughly.

The mixture was heated at 120° for 40 min, and then cooled under running water. Ethanol was added to the 5 ml mark and mixed by inversion. At the same time, a reagent blank and a phosphorescence standard solution were prepared by treating 1.0 ml of 5% DMSO–Me_2CO and 1.0 ml of a working standard solution of 4-HS, respectively, as described above.

The phosphorescence intensity was measured at 508 nm with excitation at 370 nm, setting the intensities of the reagent blank and the standard solution to zero and an arbitrary unit, respectively. The value of 4-HS in the test solution was obtained from the calibration curves described below.

Calibration Curves——Working standard solution of 4-HS (4—20×10^{-7} M or 10—200×10^{-7} M) were treated as described above. The calibration plots thus obtained were straight lines, as shown in Fig. 1.

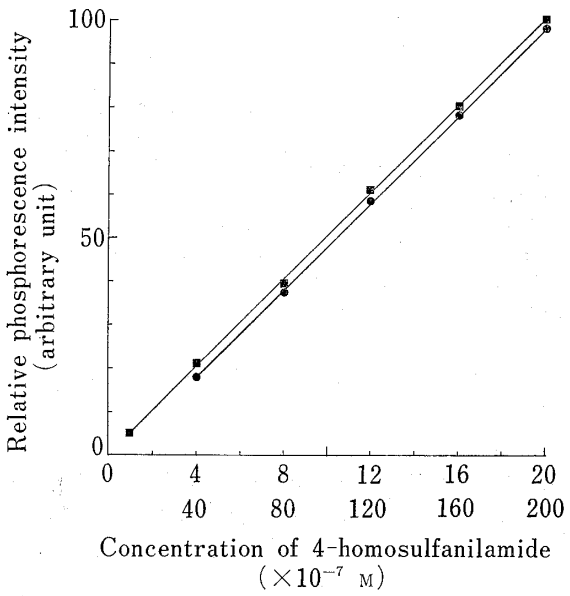

Fig. 1. Calibration Curves for 4-Homosulfanilamide

Mixtures of 4-HS and 2.0×10^{-3} M 2-CNP were heated at 120° for 40 min.
●—●: $(4—20)\times10^{-7}$ M.
■—■: $(10—200)\times10^{-7}$ M.

Application to Rat Plasma Samples——1) Extraction of 4-HS from Rat Plasma: One ml of rat plasma containing 4-HS ($2—20\times10^{-6}$ M) was pipetted into a glass-stoppered centrifuge tube, 7.0 ml of ethyl acetate and 50 μl of 0.05 N potassium hydroxide solution were added, and the mixture was shaken vigorously for 5 min then centrifuged at 3000 rpm for 10 min.

Next, 6.0 ml of the ethyl acetate layer from the tube was transferred into a test tube and evaporated to dryness under reduced pressure. The residue was dissolved with 1.0 ml of 5% DMSO–Me_2CO as a test solution. At the same time, a blank solution was prepared by treating 1.0 ml of rat plasma without 4-HS in the same manner.

2) Determination of 4-HS: Test and blank solutions were treated by the standard procedure described above, and the amount of 4-HS, $X(\times10^{-6}$ M), in the test solution was determined. The amount of 4-HS in 1.0 ml of rat plasma was calculated from the following equation, because the recovery of 4-HS from the test solution was 76.97%.

Amount of 4-HS in 1.0 ml of rat plasma ($\times10^{-6}$ M) =

$$\frac{X(\times10^{-6}\text{ M})\times7}{0.7697\times6}=X(\times10^{-6}\text{ M})\times1.5157$$

Preparation of Phosphorescent Compound——5-Nitro-2-(*p*-sulfamoylbenzylamino)pyridine (I)[6]: First, 2-CNP (0.22 g) was added to a solution of 0.13 g of 4-HS in 1.5 ml of DMSO. The mixture was then heated in an oil bath at 120° for 4 hr. The reaction mixture was poured into 80 ml of cold water, then the precipitate was collected by filtration, and recrystallized from methanol to provide yellow needles: mp 186—187°, yield, 0.09 g (42%). *Anal.* Calcd for $C_{12}H_{12}N_4O_4S$: C, 46.75; H, 3.92; N, 18.17; S, 10.40. Found: C, 46.85; H, 3.95; N, 18.10; S, 10.36. IR ν_{max}^{Nujol} cm^{-1}: 3370, 3320, 3248 (NH_2), 1502, 1347 (NO_2), 1322, 1152 (SO_2). NMR (δ in DMSO-d_6) ppm: 4.71 (2H, doublet, $J=6.0$ Hz, $-NHCH_2-$), 6.65 (1H, doublet, $J=9.0$ Hz, pyridine ring proton at the 3 position), 7.26 (2H, singlet, $-SO_2NH_2$), 7.49 (2H, doublet, $J=8.5$ Hz, benzene ring protons at the 3 and 5 positions), 7.80 (2H, doublet, $J=8.5$ Hz, benzene ring protons at the 2 and 6 positions), 8.14 (1H, quartet, $J_1=9.0$, $J_2=3.0$ Hz, pyridine ring proton at the 4 position), 8.56 (1H, triplet, $J_1=J_2=6.0$ Hz, $-NHCH_2-$), 8.89 (1H, doublet, $J=3.0$ Hz, pyridine ring proton at the 6 position).

6) The melting point was determined with a Yanagimoto micro melting point apparatus and is uncorrected. The infrared (IR) spectrum was taken in Nujol with a JASCO DS 403G machine, and the nuclear magnetic resonance (NMR) spectrum in DMSO-d_6 solution with a Varian A-60 machine using tetramethylsilane as an internal reference, chemical shifts being shown as δ.

Results and Discussion

Conditions for the Standard Procedure

Phosphorescent Compound——The phosphorescence of the final reaction mixture showed an excitation maximum at 370 nm and emission maxima at 484 and 508 nm (Fig. 2). The phosphorescent compound produced in the present procedure was prepared in crystalline form and was determined to be I based on the elemental analysis data and infrared and nuclear magnetic resonance spectra.

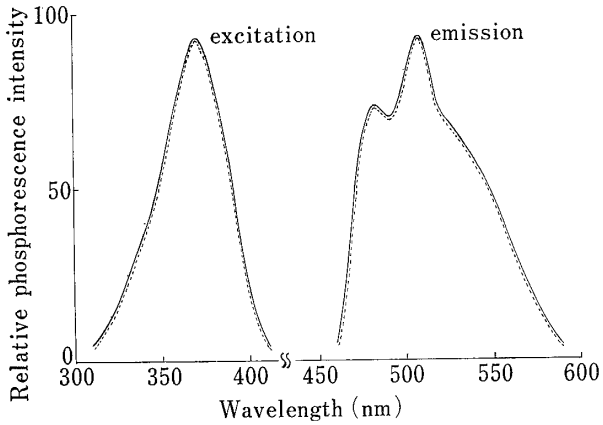

Fig. 2. Phosphorescence Excitation and Emission Spectra of the Reaction Mixture and I (uncorrected)

——: reaction mixture. A solution of 1.0 ml of 1.0×10^{-5} M 4-HS was treated according to the standard procedure. Mean lifetime: 0.22 sec.
------: I dissolved in the same solvent system as the reaction mixture at a concentration of 2.0×10^{-6} M. Mean lifetime: 0.23 sec.

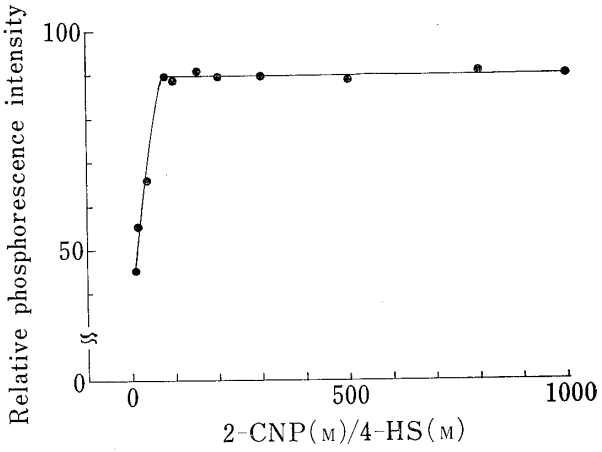

Fig. 3. Effect of 2-CNP Concentration on the Phosphorescence Development

A fixed concentration (1.0×10^{-6} M) of 4-HS was treated according to the standard procedure with various concentrations of 2-CNP.

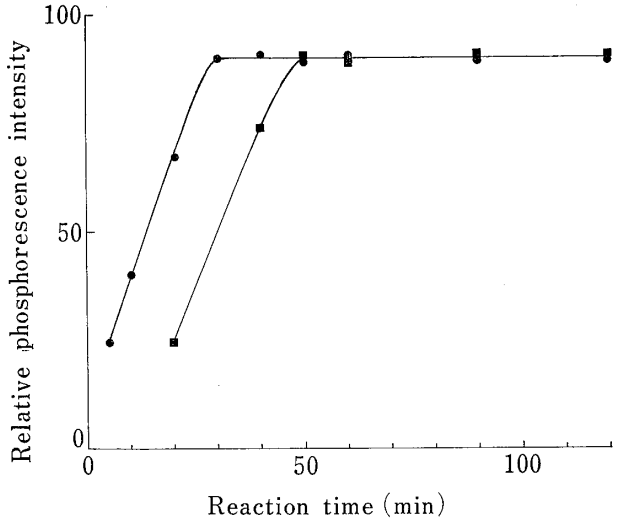

Fig. 4. Effect of the Reaction Temperature and Time on the Phosphorescence Development

Portions (1.0 ml) of 1.0×10^{-5} M 4-HS solutions were treated according to the standard procedure.
●—●: 120°.
■—■: 100°.

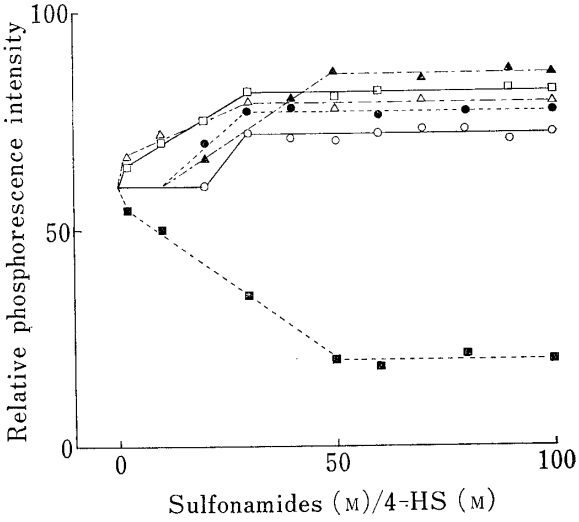

Fig. 5. Effect of Sulfonamides on the Reaction of 4-HS with 2-CNP

Portions (1.0 ml) of 1.0×10^{-5} M 4-HS solutions were treated according to the standard procedure with various concentrations of sulfonamides.
□——□: sulfanilamide, ■------■: sulfisoxazole,
△——△: sulfadimethoxine, ▲------▲: sulfamerazine,
○——○: sulfamethoxazole, ●------●: sulfamonomethoxine.

Fig. 2 shows the phosphorescence excitation and emission spectra of I dissolved in ethanol containing 0.1% (v/v) DMSO.

These spectra coincided with those of the final reaction mixture, indicating that I was the sole phosphorescent compound formed in the procedure.

Effect of 2-CNP Concentration——The concentration of 2-CNP affected the phosphorescence intensity. Fig. 3 shows that the concentration of 2-CNP should be maintained at more than 80 times that of 4-HS (molar ratio) to obtain a constant intensity.

Correlation between Reaction Temperature and Time——The effects of the reaction temperature and time on the phosphorescence development are shown in Fig. 4. The time required for the optimum phosphorescence development was 50 min at 100° and 30 min at 120°.

No significant difference was observed in the phosphorescence intensity of the reaction mixtures. Therefore, a reaction time of 40 min and a temperature of 120° were selected for the procedure in order to reduce the experimental time required.

Effect of Foreign Substances——The effects of several sulfonamides on the phosphorescence development in the procedure were examined by adding them to the test solution. As shown in Fig. 5, sulfanilamide, sulfadimethoxine, and sulfisoxazole interfered with the phosphorescence development at the same concentration level as 4-HS, but sulfamonomethoxine, sulfamerazine, and sulfamethoxazole did not interfere with the phosphorescence development up to concentration levels of 10, 10, and 20 times that of 4-HS (molar ratio), respectively.

Table I. Effect of Solvent on the Extraction of 4-HS from Rat Plasma[a)]

Solvent	Relative phosphorescence intensity
Hexane	19
Ether	21
Ethyl Acetate	100
Chloroform	35
Cyclohexane	30

a) Portions (1.0 ml) of rat plasma containing 1.0×10^{-5} M 4-HS were treated according to the standard procedure.

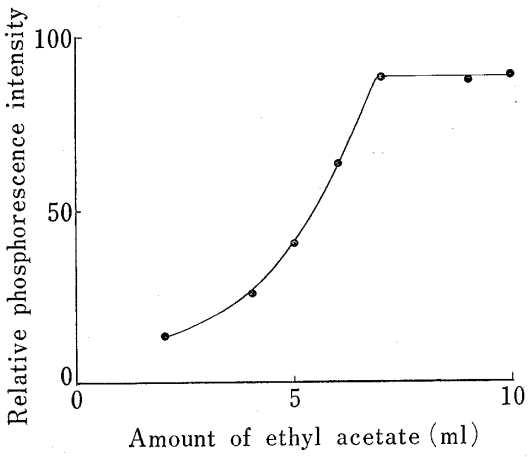

Fig. 6. Effect of Amount of Ethyl Acetate on the Extraction of 4-HS from Rat Plasma

Portions (1.0 ml) of rat plasma containing 1.0×10^{-5} M 4-HS were treated according to the standard procedure.

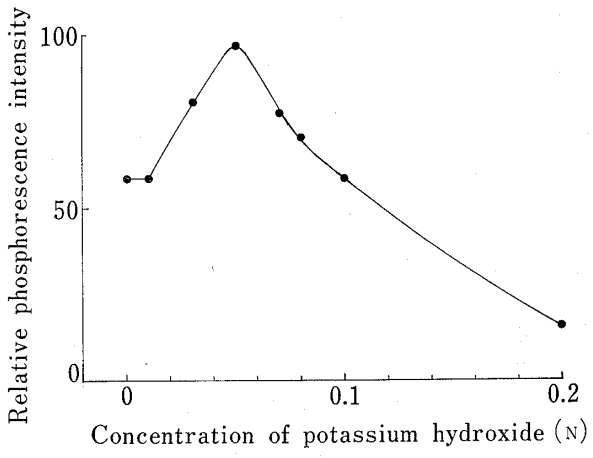

Fig. 7. Effect of the Concentration of Potassium Hydroxide on the Extraction of 4-HS from Rat Plasma

Portions (1.0 ml) of rat plasma containing 1.0×10^{-5} M 4-HS were treated according to the standard procedure with various concentrations of potassium hydroxide.

Conditions for the Determination of 4-HS in Rat Plasma

Effect of Solvent on the Extraction of 4-HS——Five organic solvents were examined for the extraction of 4-HS. As shown in Table I, the maximum phosphorescence intensity was obtained using ethyl acetate. Thus, ethyl acetate was selected as the extraction solvent.

Effect of Amount of Ethyl Acetate on the Extraction of 4-HS——Fig. 6 shows the effect of amount of ethyl acetate on the extraction of 4-HS.

The phosphorescence intensity was constant in the range from 7 to 10 ml. Thus, 7 ml of ethyl acetate was used for the extraction.

Effect of Potassium Hydroxide Concentration on the Extraction of 4-HS——Fig. 7 shows the effect of potassium hydroxide concentration on the extraction. The maximum phosphorescence intensity was obtained using 0.05 N potassium hydroxide and the spectra scarcely changed over the concentration range tested. Thus, 0.05 N potassium hydroxide solution was used for the extraction.

Table II. Recovery of 4-Homosulfanilamide added to Rat Plasma[a]

Sample No.	Added ($\times 10^{-5}$ M) (X)	Found ($\times 10^{-5}$ M) (Y)	Sample No.	Added ($\times 10^{-5}$ M) (X)	Found ($\times 10^{-5}$ M) (Y)
1	0.27	0.29	13	1.60	1.40
2	0.27	0.35	14	1.60	1.56
3	0.29	0.27	15	1.73	1.12
4	0.29	0.35	16	1.73	1.24
5	0.54	0.52	17	2.13	1.71
6	0.54	0.62	18	2.13	1.74
7	0.58	0.44	19	2.30	1.81
8	0.58	0.51	20	2.30	1.60
9	1.07	1.20	21	2.67	2.12
10	1.07	0.87	22	2.67	2.36
11	1.15	0.76	23	2.89	2.27
12	1.15	0.90	24	2.89	2.49

a) Regression equation: $Y=0.7697X+0.083$, $s=0.15$, $r=0.978$.

Table III. Regression Analysis for the Determination of 4-Homosulfanilamide in the Presence of Sulfamerazine[a]

Sample No.	Components in 1 ml of mixed sample solution ($\times 10^{-6}$ M)		Found ($\times 10^{-6}$ M) 4-Homosulfanilamide (Y)
	4-Homosulfanilamide (X)	Sulfamerazine	
1	1.0	10	1.3
2	1.0	10	1.6
3	2.0	20	1.6
4	2.0	20	2.2
5	4.0	40	3.9
6	4.0	40	3.6
7	5.0	50	4.6
8	5.0	50	4.7
9	6.0	60	5.5
10	6.0	60	5.9
11	8.0	80	7.9
12	8.0	80	8.2
13	10.0	100	9.3
14	10.0	100	10.7

a) Regression equation: $Y=0.9782X+0.044$, $s=0.43$, c.v.$=8.50\%$.

Recovery of 4-HS from Rat Plasma——The recovery of 4-HS after adding known amounts to rat plasma is shown in Table II. The calculated relationship between the added (X) and found (Y) values gave a recovery of 76.97%, because the constant term in the regression equation was regarded as zero in the statistical test.

Determination of 4-HS

In Mixed Samples——Regression analysis for the determination of 4-HS in the concentration range of $1—10\times10^{-6}$ M was examined using fourteen mixed sample solutions in which sulfamerazine was present in a 10-fold molar excess over 4-HS. As shown in Table III, the calculated relationship between the theoretical (X) and experimental (Y) values indicated that the present method correctly determined 4-HS with a coefficient of variation of 8.5%.

In Rat Plasma——Regression analysis for the determination of 4-HS in the concentration range of $2.1—20.7\times10^{-6}$ M was examined using six rat plasma samples in which sulfamonomethoxine was present in molar ratios of 1:1 to 10:1 relative to 4-HS. As shown in Table IV, the calculated relationship between the theoretical (X) and experimental (Y) values indicated that the present method correctly determined 4-HS with a coefficient of variation of 9.8%.

Table IV. Regression Analysis for the Determination of 4-Homosulfanilamide in the Presence of Sulfamonomethoxine[a)]

Sample No.	Components in 1 ml of rat plasma ($\times10^{-6}$ M)		Found ($\times10^{-6}$ M)
	4-Homosulfanilamide (X)	Sulfamonomethoxine	4-Homosulfanilamide (Y)
1	2.1	20.8	3.2
2	4.1	20.8	6.4
3	8.3	20.8	7.5
4	12.4	20.8	11.9
5	16.6	20.8	16.7
6	20.7	20.8	20.9

a) Regression equation: $Y=0.9196X+1.260$, $s=1.09$, c.v.$=9.82\%$.