[Chem. Pharm. Bull. 28(7)1986—1991(1980)]

Studies on the Phosphorimetric Determination of Amines with Halonitro Compounds. IV.[1] Phosphorimetric Determination of Epinephrine with 2-Chloro-5-nitropyridine

KAZUMASA HIRAUCHI and AKIKO FUJISHITA

Shionogi Research Laboratory, Shionogi and Co., Ltd.[2]

(Received December 5, 1979)

A phosphorimetric method for the determination of epinephrine was established. The method is based on measurement of the phosphorescence of the phosphorescent compound formed in the reaction of epinephrine with 2-chloro-5-nitropyridine after isolation of the compound by thin-layer chromatography.

The method can be applied to the determination of epinephrine in the concentration range from 2 to 20 μg/ml, and interfering substances such as norepinephrine, tyrosine, metanephrine, normetanephrine, phenylalanine, 3,4-dihydroxyphenylalanine, and 3-hydroxytyramine are easily removed.

Keywords——phosphorimetry; microdetermination; epinephrine; 2-chloro-5-nitropyridine; 5-nitro-2-{methyl[2-(3,4-dihydroxyphenyl)-2-hydroxyphenyl)-2-hydroxyethyl]-amino}-pyridine

In the previous paper,[1] it was reported that 2-chloro-5-nitropyridine (2-CNP) was a useful reagent for the microdetermination of 4-homosulfanilamide by phosphorimetry, because 2-CNP gave an intensely phosphorescent compound upon reaction with 4-homosulfanilamide. In order to utilize this reagent in the microdeterminations of other amines, epinephrine was selected as an initial test compound, and the possibility of its determination by phosphorimetry, using the reaction of 2-CNP with the amine, was examined.

In the present work, the reaction of 2-CNP with the amine was found to give an intensely phosphorescent compound, and this observation was utilized for the microdetermination of epinephrine.

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. Other apparatus used consisted of silica gel $60F_{254}$ plates, 20×20 cm (E. Merck), a Terumo microsyringe (50 μl), and a chromatographic chamber (9×22×22 cm).

Reagents——Epinephrine, norepinephrine, tyrosine, metanephrine, normetanephrine, phenylalanine, 3,4-dihydroxyphenylalanine, and 3-hydroxytyramine: These were obtained from E. Merck (Germany).

Epinephrine Stock Standard Solution: Epinephrine (20 mg) was dissolved in 500 ml of 0.02 N hydrochloric acid. The solution was stable for at least 2 weeks when stored in a refrigerator.

Epinephrine Working Standard Solutions: These were prepared by diluting the stock standard solution with 0.02 N hydrochloric acid to the desired concentrations before use.

2-CNP: This was prepared by the reported methods.[3,4]

2-CNP Stock Solution: 2-CNP (4 g) was dissolved in 500 ml of acetone. The solution was stable for at least 2 weeks when stored at room temperature protected from light. It was diluted with acetone to desired concentrations before use.

1) Part III: K. Hirauchi and A. Fujishita, *Chem. Pharm. Bull.*, **28**, 755 (1980).
2) Location: *Sagisu, Fukushima-ku, Osaka, 553, Japan.*
3) W.T. Caldwell and E.C. Kornfeld, *J. Am. Chem. Soc.*, **64**, 1695 (1952).
4) M.A. Phillips, *J. Chem. Soc.*, **1941**, 9.

NII-Electronic Library Service

Ethanol: Reagent-grade EtOH (1000 ml) was distilled after dissolving 10 g of Na metal in it.
Acetone (or Triethylamine): Reagent-grade acetone (or triethylamine) was distilled by the usual method.
Ether, benzene, hydrochloric acid, acetic acid, and dimethyl sulfoxide (DMSO) were of reagent grade.

Standard Procedure——One milliliter of sample solution containing epinephrine (2—20 μg) was pipetted into a test tube equipped with a reflux condenser, then 1.0 ml of 2-CNP solution and 5.0 μl of triethylamine were added, and the solution was mixed thoroughly. The mixture was heated at 150° for 30 min, further heated at 100° for 5 min without a reflux condenser and cooled under running water.

After adding 5.0 ml of ether to the reaction mixture, it was shaken vigorously for 5 min followed by centrifugation at 3000 rpm for 5 min. Next, 4.0 ml of the ether layer of the tube was transferred into a test tube and evaporated to dryness at 70° for 5 min. The residue was dissolved in 200 μl of acetone as the test solution.

A 50 μl aliquot of the test solution was spotted on a silica gel plate and developed with a mixture of benzene–acetic acid–ether (2.1 : 1 : 0.9, v/v) for 40 min in a chromatographic chamber saturated with solvent vapor.

After drying the plate for 15 min in air at room temperature, a spot ($Rf \fallingdotseq 0.41$) which appeared dark under ultraviolet illumination (254 nm) was scraped off, and the material was extracted with 5.0 ml of ethanol by shaking for 5 min, followed by centrifugation at 3000 rpm for 5 min. At the same time, a reagent blank and a phosphorescence standard solution were prepared by treating 1.0 ml of 0.02 N hydrochloric acid and 1.0 ml of a working standard solution of epinephrine, respectively, as described above.

Within 2 hr, the phosphorescence intensity of the extract was measured at 526 nm with excitation at 380 nm, taking the intensities of the reagent blank and the phosphorescence standard solution as zero and one arbitrary unit, respectively.

The concentration of epinephrine in the sample solution was then obtained from the calibration curve by calculation.

Preparation of Phosphorescent Compound——5-Nitro-2-{methyl[2-(3,4-dihydroxyphenyl)-2-hydroxyethyl]-amino}-pyridine (I)[5]: To a solution of 0.72 g of epinephrine suspended in 3.0 ml of DMSO, 0.32 g of 2-CNP and 0.5 ml of triethylamine were added. The solution was heated in an oil bath at 100° for 1 hr. The reaction mixture was poured into 80 ml of cold water, then the precipitate was collected by filtration. The precipitate was dissolved in 30 ml of ethyl acetate and then the solution was concentrated to about 1.0 ml. The resulting solution was chromatographed on a silica gel column (1.5×23 cm) and the column was eluted with ethyl acetate. The eluate was collected in a 50 ml flask and then concentrated to 0.5 ml. On adding a small amount of ether, yellow needles were obtained, mp 147—148°, yield, 0.37 g (31%). *Anal.* Calcd for $C_{14}H_{15}N_3O_5$: C, 54.96; H, 5.05; N, 13.47. Found: C, 55.08; H, 4.95; N, 13.66. IR ν_{max}^{Nujol} cm^{-1}: 3350 (OH, broad), 1530, 1333 (NO_2). NMR (δ in DMSO-d_6) ppm: 3.10 (3H, singlet, >N–CH_3), 3.71 (2H, doublet, $J_1=J_2=6.0$ Hz, –CH_2CH–), 4.73 (1H, triplet, $J_1=J_2=6.0$ Hz, –CH_2CH–), 6.63—6.79 (4H,multiplet, benzene ring protons and pyridine ring proton at the 3 position), 8.16 (1H, quartet, $J_1=9.0$, $J_2=3.0$ Hz, pyridine ring proton at the 4 position), 8.95 (1H, $J=3.0$ Hz, pyridine ring proton at the 6 position).

Results and Discussion

Phosphorescent Compound

The phosphorescence of the final extract showed an excitation maximum at 380 nm and emission maxima at 500 and 526 nm (Fig. 1). The phosphorescent compound was prepared in crystalline form and was identified as I bassed on the elemental analysis data and infrared and nuclear magnetic resonance spectra. Figure 1 shows the phosphorescence excitation and emission spectra of I dissolved in the same solvent system as the final extract. These spectra coincided with those of the final extract, indicating that I was the sole phosphorescent compound formed in the procedure.

The detection limit (taken as the concentration giving a phosphorescence signal amounting to twice the background noise) of I in ethanol was 0.15 ng/ml.

Effect of 2-CNP Concentration

The concentration of 2-CNP affected the phosphorescence development. Figure 2 shows that the concentration of 2-CNP should be maintained at more than 200 times that of epinephrine by weight to obtain a constant phosphorescence intensity.

5) 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 using tetramethylsilane as an internal reference (chemical shifts are shown as δ values).

NII-Electronic Library Service

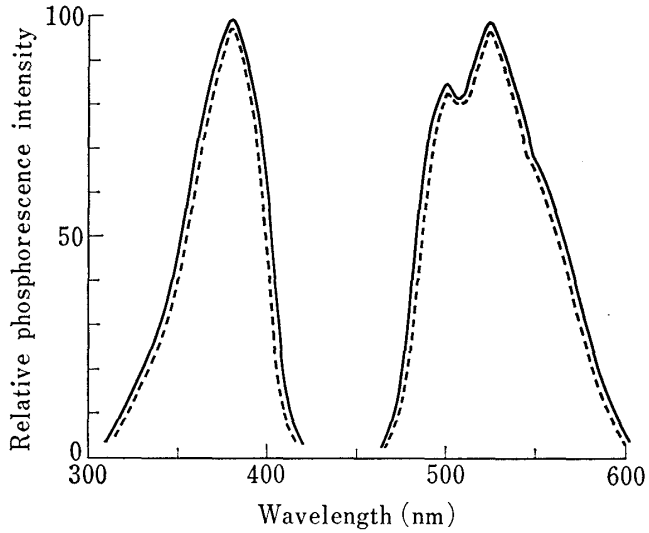

Fig. 1. Phosphorescence Excitation and Emission Spectra of the Final Extract and of I (uncorrected)

------: final extract (one milliliter of 5 μg/ml epinephrine solution was treated according to the standard procedure). Lifetime: 0.32 sec.
——: I dissolved in the same solvent system as the final extract at a concentration of 0.5 μg/ml. Lifetime: 0.34 sec.

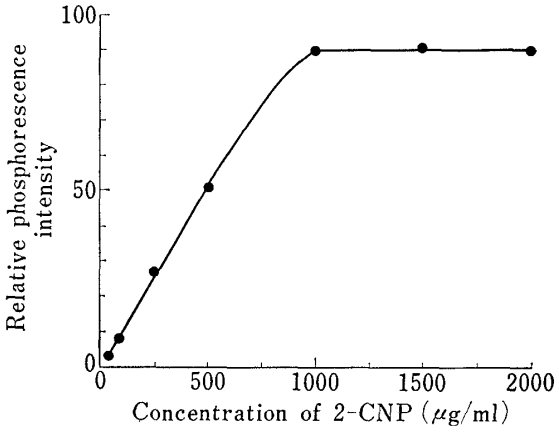

Fig. 2. Effect of 2-CNP Concentration on the Phosphorescence Development

One milliliter portions of 5 μg/ml epinephrine solution were treated according to the standard procedure with various concentrations of 2-CNP.

Correlation between the Reaction Time and the Temperature

The effects of reaction time and temperature on the phosphorescence development are shown in Fig. 3. The time required for the optimum phosphorescence development was 40 min at 120° and 30 min at 150°. No significant difference was observed in the final phosphorescence intensity of the reaction mixture.

Therefore, a reaction time of 30 min and a temperature of 150° were selected for the standard procedure, in order to minimize the experimental time requirement.

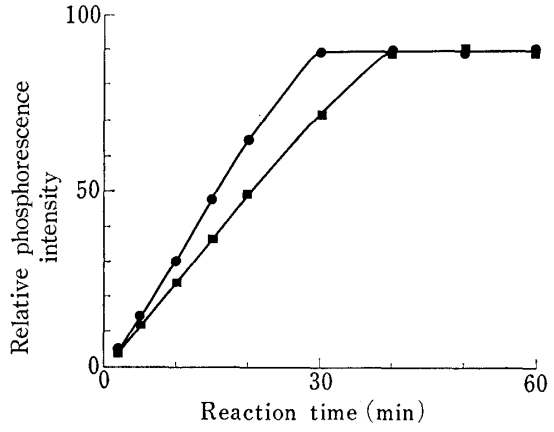

Fig. 3. Effects of Reaction Temperature and Reaction Time on the Phosphorescence Development

One milliliter portions of 5 μg/ml epinephrine solution were treated according to the standard procedure for various reaction times at 120 or 150°.
—■—: 120°. —●—: 150°.

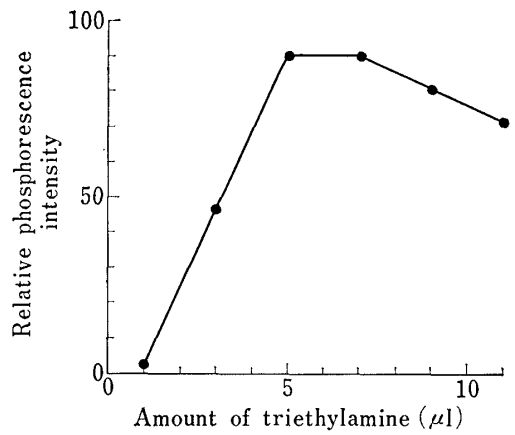

Fig. 4. Effect of Amount of Triethylamine on the Phosphorescence Development

One milliliter portions of 5 μg/ml epinephrine solution were treated according to the standard procedure with various amounts of triethylamine.

NII-Electronic Library Service

Effect of Amount of Triethylamine

Hydrogen chloride produced in the reaction of 2-CNP with epinephrine interfered with the condensation reaction forming I, and hydrochloric acid used in the sample solution also interfered with this reaction. Therefore, these interfering factors had to be removed from the reaction system. For this purpose, triethylamine was used, and the effects of various amounts were examined.

As shown in Fig. 4, the maximum phosphorescence intensity was obtained in the range from 5 to 7 μl of triethylamine, and the spectra scarcely changed over the range tested. Thus, 5 μl of triethylamine was used in the standard procedure.

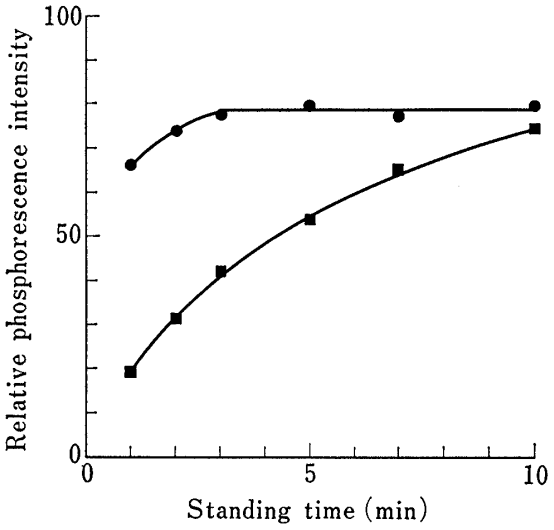

Fig. 5. Effects of Standing Time and Temperature on the Removals of Acetone and Triethylamine

One milliliter portions of 5 μg/ml epinephrine solution were treated according to the standard procedure for various standing times at 70 or 100°.
—■—: 70°. —●—: 100°.

Table I. Effect of Solvent on the Extraction of I from the Reaction Mixture[a)]

Solvent	Relative phosphorescence intensity
Ether	100
Chloroform	59
Ethyl acetate	12

a) One milliliter portions of 5 μg/ml epinephrine solution were treated according to the standard procedure with various organic solvents.

Effect of Standing Time on the Removals of Acetone and Triethylamine

The effect of standing time on the removals of acetone and triethylamine from the reaction mixture was examined at 70 and 100°.

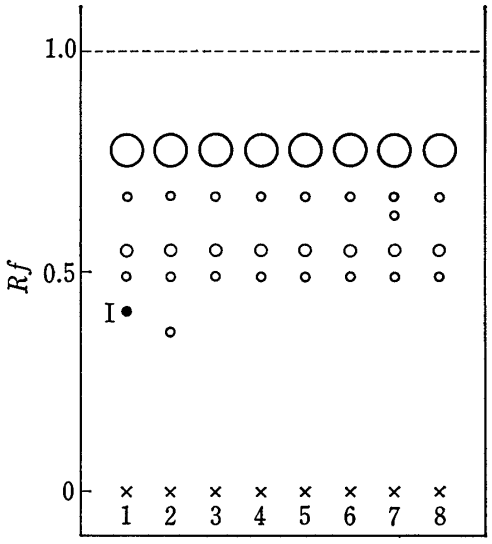

Fig. 6. Thin-Layer Chromatogram of Reaction Mixture of 2-CNP with Amines

One milliliter portions of 10 μg/ml amine solutions were treated according to the standard procedure.
1: epinephrine, 2: norepinephrine, 3: tyrosine, 4: metanephrine, 5: normetanephrine, 6: phenylalanine, 7: 3,4-dihydroxyphenylalanine, 8: 3-hydroxytyramine.

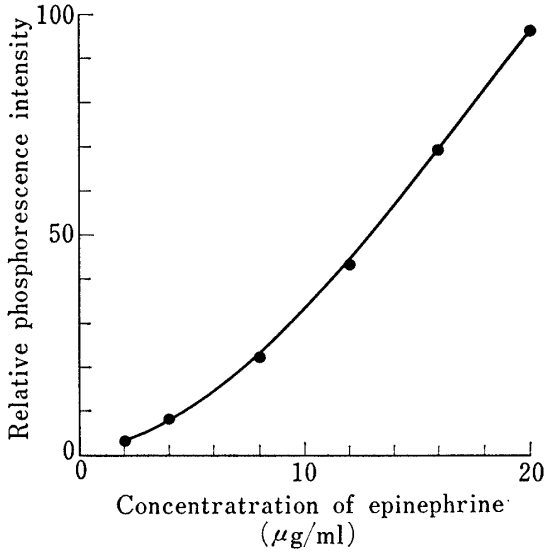

Fig. 7. Calibration Curve for Epinephrine

NII-Electronic Library Service

Figure 5 shows that a constant phosphorescence intensity was obtained for standing times from 3 to 10 min at 100°, but not at 70°. A time of 5 min and a temperature of 100° were thus selected as the optima for the standard procedure.

Effect of the Solvent on the Extraction of I

The effect of the solvent on the extraction of I from the reaction mixture was examined using ether, chloroform, and ethyl acetate. As shown in Table I, the maximum phosphorescence intensity was obtained with ether. The effect of the amount of ether on the extraction of I was also examined using 5, 10, 15, and 20 ml, and constant phosphorescence intensity was obtained for all amounts tested.

Thus, ether was selected as the extraction solvent, and 5 ml was used for the standard procedure.

Separation of I

Epinephrine and related compounds, such as norepinephrine, tyrosine, metanephrine, normetanephrine, phenylalanine, 3,4-dihydroxyphenylalanine, and 3-hydroxytyramine, are present in biological fluids, and these related compounds reacted with 2-CNP to form phosphorescent compounds interfering with the determination of epinephrine.

Therefore, for the determination of epinephrine, I should be separated from the reaction products of the related compounds.

Conditions suitable for the separation of I were examined using silica gel plates and four kinds of benzene–acetic acid–ether mixture. As shown in Fig. 6, effective separation of I from the reaction products of the related compounds was obtained by using a mixture of benzene–acetic acid–ether (2.1: 1: 0.9, v/v) as a developing solvent.

Calibration Curve

Figure 7 shows the calibration curve for epinephrine in the concentration range from 2 to 20 μg/ml. The relationship between the phosphorescence intensity and the concentration was not linear. The nonlinearity was probably caused by the adsorption of a certain amount of I on the silica gel plate in the concentration range tested.

This curve was fitted to a second-order equation by a suitable statistical procedure.

Phosphorescence Stability of the Final Extract

The phosphorescence stability of the final extract was examined by subjecting 1.0 ml of 5 μg/ml epinephrine solution to the standard procedure. The phosphorescence intensity of the final extract under room light and at room temperature was stable for 2 hr with a coefficient of variation of about 5%.

Table II. Regression Analysis for the Determination of Epinephrine in the Presence of Normetanephrine[a)]

Sample No.	Components in 1 ml of mixed sample solution (μg)		Found (μg)
	Epinephrine (X)	Normetanephrine	Epinephrine (Y)
1	2.00	100.42	2.51
2	4.00	100.42	4.44
3	7.99	100.42	7.51
4	11.99	100.42	12.60
5	15.98	100.42	16.45
6	19.98	100.42	19.79

a) Regression equation: $Y=0.9832X+0.403$, $s=0.48$, $r=0.998$.

NII-Electronic Library Service

Regression Analysis

Regression analysis for the determination of epinephrine in the concentration range from 2.00 to 19.98 μg/ml was carried out using six mixed sample solutions in which normetanephrine was present in ratios of 5: 1 to 50: 1 relative to epinephrine.

As shown in Table II, the calculated relation between the theoretical (X) and the experimental (Y) values indicated that the present method correctly determined epinephrine with a correlation coefficient of 0.998.

Advantage of This Method

For the determination of epinephrine, the present method is inferior to the fluorometric methods[6] in sensitivity, but it offers the advantage that epinephrine can be determined in the presence of related compounds, such as norepinephrine, tyrosine, metanephrine, normetanephrine, phenylalanine, 3,4-dihydroxyphenylalanine, and 3-hydroxytyramine.

6) S. Udenfriend, "Fluorescence Assay in Biology and Medicine," Vol. II, ed. by B. Horecker, N.O. Kaplan, J. Marmur, and H.A. Scheraga, Academic Press, Inc., New York, 1969, p. 217.

NII-Electronic Library Service