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Phosphorescence from 3,4-Dihydroxyphenyl Derivatives in Basic Solution and Its Use for the Assay of Epinephrine and Norepinephrine in Urine

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Phosphorescence derived from 3,4-dihydroxyphenyl derivatives such as catecholamines in aqueous basic solution and its use for the assay of epinephrine and norepinephrine in urine by high-performance liquid chromatography are described. Intense phosphorescence is obtained from epinephrine and norepinephrine when the amines are allowed to stand in 0.02 M sodium hydroxide at room temperature for 20 min. The limits of detection for epinephrine and norepinephrine are 2×10^{-8} and 4×10^{-8} M (1 and 2 pmol in 50 μl of sample required for the phosphorimetric procedure), respectively. As little as 5 and 10 pmol of epinephrine and norepinephrine, respectively, in 2.0 ml of urine can be assayed.

Keywords——phosphorimetry; 3,4-dihydroxyphenyl derivative; catecholamines; epinephrine; norepinephrine; urine sample; high-performance liquid chromatographic separation

It has been reported that 3,4-dihydroxyphenyl derivatives such as catecholamines are converted into phosphorescent compounds on standing in basic solution.[1,2]

We have investigated the conditions of the phosphorescence development from 3,4-dihydroxyphenyl derivatives in aqueous basic solution, by using norepinephrine as a model compound, with the aim of developing a quantitative microanalysis procedure for catecholamines. We found that intense phosphorescence could be derived from epinephrine and norepinephrine. This finding was applied to the assay of these catecholamines in urine, after separation by alumina adsorption and ion exchange high-performance liquid chromatography (HPLC).

Experimental

Chemicals——All chemicals and solvents were of reagent grade, unless otherwise noted. Deionized and distilled water was used. L-Epinephrine, L-norepinephrine, dopamine hydrochloride, L-dopa, DL-3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid and DL-3,4-dihydroxyphenylethyleneglycol (all from Sigma) were used without further purification. 3,4-Dihydroxybenzaldehyde (Wako) was recrystallized from *n*-hexane. Acid-washed alumina (Woelm) was prepared in the usual manner[3] and stored in a desiccator.

Apparatus——A Hitachi MPF-3 spectrofluorimeter equipped with a xenon lamp and a Hitachi QPD 33 recorder were used throughout. The slit widths in the exciter and analyzer were both set at 10 nm for the fluorescence and phosphorescence measurements. The fluorescence spectra and intensities were measured in conventional quartz cells of 1×1 cm optical path length. The phosphorescence spectra and intensities were measured at liquid nitrogen temperature (77°K) with the spectrometer equipped with a Hitachi phosphoroscope attachment in open quartz sample tubes (1.0 mm i.d.; 5.0 mm o.d.; 200 mm long; sample volume, *ca.* 20 μl). The tube was rotated at *ca.* 1500 rpm by using a sample tube rotating assembly to minimize the signal fluctuation.[2,4] The spectra are uncorrected. The phosphorescence lifetimes were measured on a Hitachi V-550 synchroscope. pH was measured with a Hitachi-Horiba M-7 pH meter at 25°C.

A Shimadzu LC-3A liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (50-μl loop).

HPLC Column——A stainless-steel tube (250×2.6 mm i.d.) was packed with Hitachi Gel 3011-C (cation exchanger; particle size, 5 μm) by the slurry technique. The column can be used for more than 500 injections with only a small decrease in the theoretical plate number.

Urine Collection——Twenty-four-h urine samples were collected in dark glass bottles containing 10 ml of concentrated hydrochloric acid from healthy volunteers (22—51 years of age). Urine samples were stored

at −20°C and used within 2 d.

Standard Phosphorimetric Procedure——To an aliquot of sample solution (50 μl), a mixture (50 μl) of sodium hydroxide and methanol (8: 2, v/v) was added; the methanol in the mixture served to form a homogeneously frozen matrix at 77°K and the sodium hydroxide concentration in the final solution was 0.02 M. The resulting solution was allowed to stand in air at room temperature (25°C) for 20 min to develop the phosphorescence. The phosphorescence intensity of the mixture was measured at the emission maximum wavelength with irradiation at the excitation maximum (see Table 1).

Assay Procedure for Epinephrine and Norepinephrine in Urine——A urine sample (20 ml) was adjusted to pH 1—2 with 0.2 M hydrochloric acid and heated for 20 min in a boiling-water bath. To the resulting solution, 0.2 ml of 0.2 M disodium ethylenediaminetetraacetate and 0.2 g of acid-washed alumina was added. With gentle stirring, the mixture was adjusted to pH 8.4 with 0.2 M sodium hydroxide. The alumina was then transferred to a glass filter (5×0.5 cm i.d., G4) and washed with two 1-ml portions of water. A 0.4 ml portion of 0.2 M perchloric acid was then added to the filter and the catecholamines were eluted while the alumina was being stirred with a glass rod. A 50-μl volume of the filtrate was injected into the chromatograph. The eluate from the chromatograph was taken dropwise into a fraction collector; the volume of 1 drop was *ca.* 50 μl. The mobile phase comprised 0.1 M citric acid and 0.05 M sodium citrate, and the flow rate was 1.2 ml/min (78 kg/cm²). The column temperature was 50°C.

The eluate was treated according to the standard phosphorimetric procedure except for the use of 0.05 M sodium hydroxide to neutralize citric acid in the eluate and make the final solution alkaline. Peak heights in the resulting chromatogram were used for the quantification of epinephrine and norepinephrine. To prepare a calibration curve, 2.0 ml aliquots of standard solutions containing 0.05—3 nmol each of epinephrine and norepinephrine were subjected to the assay procedure in place of a urine sample.

Results and Discussion

Phosphorescence from Norepinephrine

The native phosphorescence of norepinephrine in basic solution (Fig. 1, a) decreases in intensity with time when the solution is allowed to stand in air at room temperature, and disappears after 5—10 min.[2] Then, a new phosphorescence appears with excitation maxima near 300 nm and 360 nm and an emission maximum at around 460 nm (Fig. 1, b). The new phosphorescence increases in intensity with time in 0.02—1.2 M sodium hydroxide to reach a maximum after 10—15 min at room temperature, and the resulting phosphorescence is stable for more than 30 min (Fig. 2, b—d). At sodium hydroxide concentrations of less than 0.002 M, the phosphorescence develops more slowly and is unstable (Fig. 2, a). The most intense and constant phosphorescence is obtained in the presence of 0.016—0.024 M sodium hydroxide. A sodium hydroxide concentration of 0.02 M and a standing time for 20 min were thus selected for the standard phosphorimetric procedure.

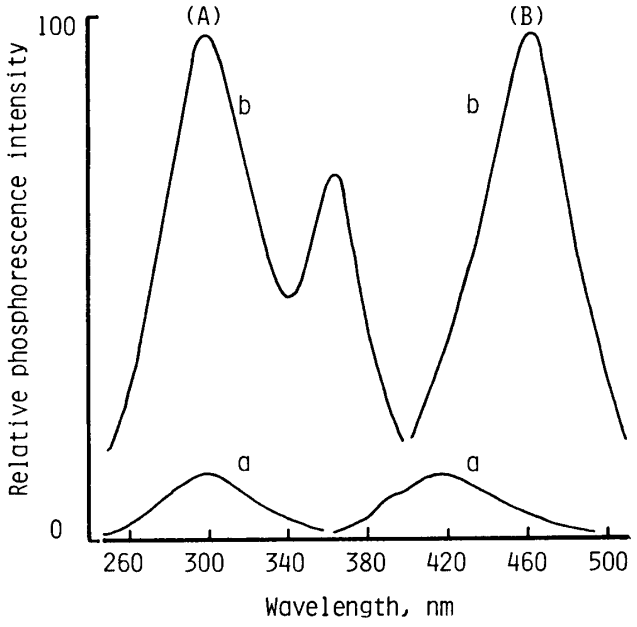

Fig. 1. Phosphorescence Spectra of Epinephrine in Basic Solution

Portions (50 μl) of aqueous solution of 1×10^{-5} M norepinephrine were treated according to the standard phosphorimetric procedure for various periods (at 25°C). Standing time: a, 0.5; b, 20 min. (A) excitation; (B) emission spectra.

The intensity of the phosphorescence produced under the conditions of the phosphorimetric procedure was proportional to the concentration of norepinephrine up to 1×10^{-4} M. The precision of the standard procedure was examined on 1×10^{-7} and 1×10^{-5} M norepinephrine solutions ($n=20$ in each

case). The coefficients of variation were 6.7 and 8.8%, respectively. This suggests that the phosphorescence measurement is not so precise. Without the use of the sample tube rotating assembly in the phosphorescence measurement, however, only irregular intensities were observed.

On the other hand, the native fluorescence of norepinephrine disappears almost immediately in basic solution and then a new fluorescence appears with an excitation maximum near 400 nm and an emission maximum around 500 nm; this has been ascribed to formation of the trihydroxyindole derivative from norepinephrine.[1] The fluorescence in 0.02 M sodium hydroxide increases in intensity with time to reach a maximum after *ca.* 1 min, then decreases rapidly with time and is quenched completely after *ca.* 15 min at room temperature (Fig. 2, e). Winefordner *et al.* stated that the phosphorescence was based on the formation of the trihydroxyindole derivative from norepinephrine.[1] However, the above observations indicate that the new phosphorescence is due to an unknown product which is probably formed *via* the trihydroxyindole derivative produced from norepinephrine.

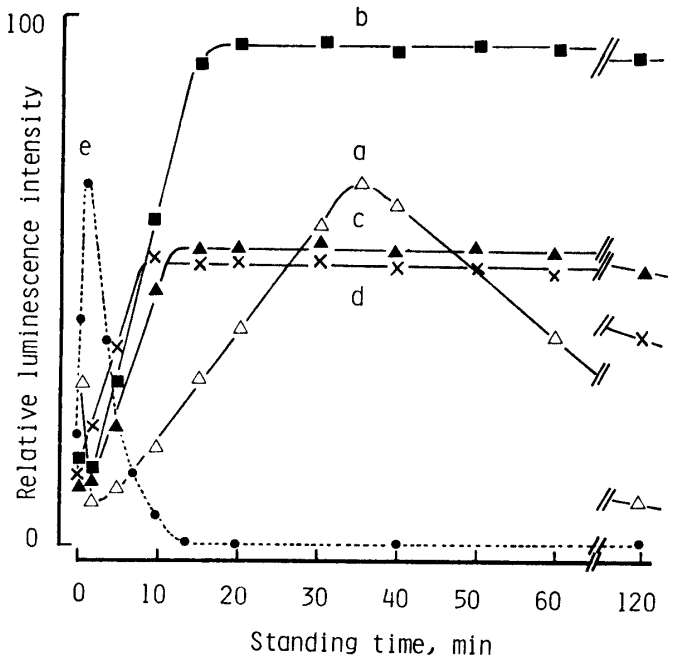

Fig. 2. Effect of Sodium Hydroxide Concentration and Standing Time on the Phosphorescence and Fluorescence from Norepinephrine[a]

Portions (50 μl) of aqueous solutions of 1×10^{-5} M norepinephrine were treated according to the standard phosphorimetric procedure with various concentrations of sodium hydroxide (in the final solutions) for various periods (25°C). a—d, phosphorescence; e, fluorescence. Sodium hydroxide concentration: a,0.002; b and e, 0.02; c, 0.02; c, 0.2; d, 1.2 M.

Phosphorescence from Other 3,4-Dihydroxyphenyl Derivatives

Most 3,4-dihydroxyphenyl derivatives tested phosphoresce under the conditions of the standard phosphorimetric procedure. The excitation and emission maxima, lifetimes and

Table I. Excitation and Emission Maxima of the Phosphorescence from 3,4-Dihydroxyphenyl Derivatives, and the Lifetimes and Limits of Detection

Compound	Excitation maximum[a,b] (nm)	Emission maximum[b] (nm)	Detection limit[c] (M)	(pmol)[d]	Lifetime[b] (s)
Epinephrine	305, 355	450	2×10^{-8}	1	0.3
Norepinephrine	300, 460	460	4×10^{-8}	2	0.3
Dopamine	320, 355	455	8×10^{-7}	40	0.3
Dopa	295, 350	450	9×10^{-7}	45	0.3
3,4-Dihydroxyphenylacetic acid	300, 350	430	1×10^{-6}	50	0.3
3,4-Dihydroxyphenylethyleneglycol	310, 350	460	4×10^{-7}	20	0.3

a) The lower wavelengths were used for excitation in all cases.
b) Concentration of 3,4-dihydroxyphenyl derivatives: 1×10^{-5} M.
c) Detection limit is defined as the amount giving an intensity of twice the blank value.
d) Actual amount in the sample solution (50 μl) required for the phosphorimetric procedure.

limits of detection for these compounds are shown in Table I. The excitation and emission maxima are not characteristic of individual compounds. The limits of detection for epinephrine and norepinephrine are very low, but those for dopamine and dopa are much higher (*ca.* 50 times) than that for epinephrine. The lifetimes were all 0.3 s.

3,4-Dihydroxymandelic acid and 3,4-dihydroxybenzaldehyde did not phosphoresce appreciably, even at a concentration of 1×10^{-5} M.

Assay for Epinephrine and Norepinephrine in 24-h Urine after Separation by HPLC

Acid hydrolysis of conjugated catecholamines in urine sample, adsorption of the amines on alumina and their elution for clean-up of the sample were accomplished according to the reported methods[3,5] with some modifications. HPLC conditions were the same as described in the previous paper.[3]

Figure 3 shows the chromatogram obtained with an authentic sample solution of catecholamines. The peaks for dopa and dopamine were relatively very small, and therefore could not be used for the assay of these amines. Recoveries of epinephrine and norepinephrine (0.55 nmol/2 ml each) were 74.2±5.7 and 77.8±6.8% (mean±standard deviation, $n=10$). The calibration curves were linear up to 3 nmol/2 ml for both epinephrine and norepinephrine.

In a typical chromatogram obtained with 24-h urine from a normal person (Fig. 4), the peak of dopamine was small and that due to dopa was not observable. The chromatogram is similar to that obtained by the HPLC-fluorimetric method.[3] Without clean-up of the sample, the peaks for epinephrine and norepinephrine were overlapped by a broad and large peak due to unknown substance(s). Recoveries of epinephrine and norepinephrine (0.55 nmol/2 ml each) added to normal urine were 53.5±7.3 and 76.4±7.7% (mean±standard deviation, $n=10$).

The limits of detection in the assay procedure were 5 and 10 pmol (1 and 2 ng) for epine-

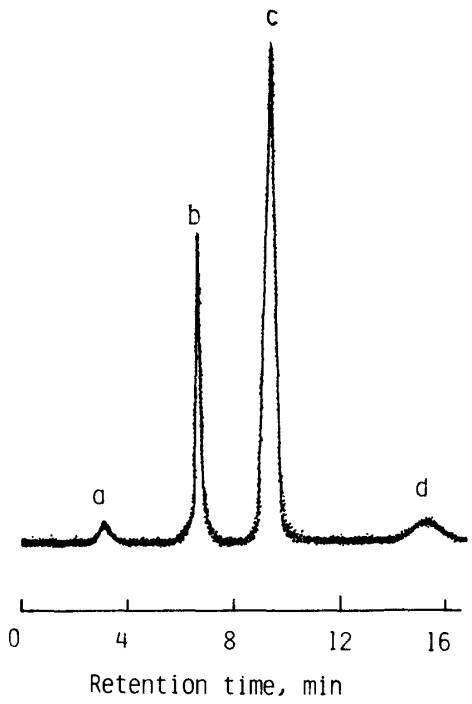

Fig. 3. Chromatogram of Catecholamine Solution

A 2-ml portion of catecholamine solution (2.75×10^{-7} M each; 0.55 nmol/2 ml each) was treated according to the assay procedure. Peak: a, dopa; b, norepinephrine; c, epinephrine; d, dopamine.

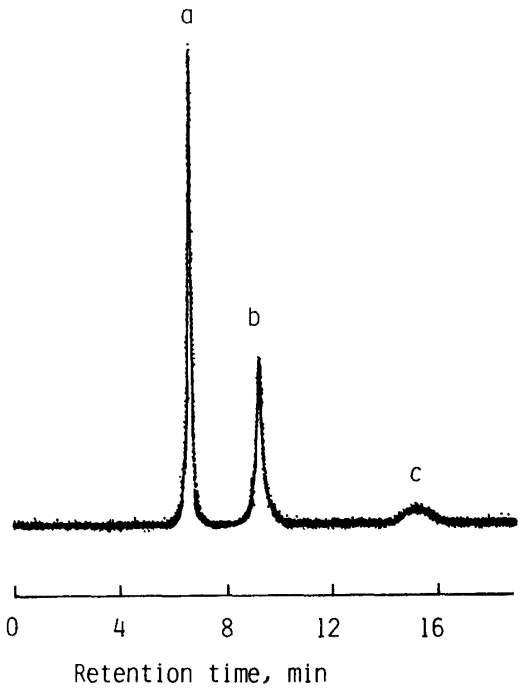

Fig. 4. Chromatogram obtained with Normal 24-h Urine

Peak: a, norepinephrine; b, epinephrine; c, dopamine.

phrine and norepinephrine in 2 ml of urine, respectively. The limit was defined as the amount giving a signal-to-noise ratio of 2 in the chromatogram. The within-day precision was examined by using urine samples with mean values of epinephrine and norepinephrine of 0.11 and 0.62 nmol (20.4 and 105.2 ng)/2 ml (n=15 each), and of 0.26 and 2.97 (45.1 and 505.3 ng)/2 ml (n=15 each), respectively. The coefficients of variation were 7.4 and 7.8% for epinephrine and 6.8 and 8.5% for norepinephrine, respectively.

Although the phosphorescence from epinephrine and norepinephrine was very strong, the sensitivity and the precision of the present assay procedure were not very good. This is due to the high noise level in the phosphorescence measurement (see Figs. 3 and 4). There is scope for improvement in the measurement of phosphorescence in aqueous frozen matrices.

The amounts of epinephrine and norepinephrine in 24-h urine samples of 20 healthy persons assayed by the present method were 54.4±15.0 nmol (9.8±2.7 μg) and 388.2±91.2 nmol (52.2±15.5 μg) (mean±standard deviation), respectively. The values are in agreement with data obtained by other workers.[3]

The structures of the phosphorescent compounds remain unknown.

References and Notes

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