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Fluorometric Determination of Some Primary Aromatic Amines with 4-Methoxy-*m*-phenylenediamine[1]

Hirokazu Taniguchi,* Tomohiko Yoshida, Tsuneo Kobayashi, and Saburo Nakano

Meiji College of Pharmacy,[2] 1-35-23 Nozawa, Setagaya-ku, Tokyo

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A sensitive fluorometric method for the determination of primary aromatic amines is described. The procedure consists of diazotization of the amino group followed by coupling with 4-methoxy-*m*-phenylenediamine (MPD), and reaction of the resulting azo compound with ammoniacal cupric sulfate.

By this reaction, primary aromatic amines are derivatized to intensely fluorescent compounds, and a method for the determination of *p*-aminobenzoic acid (PABA) was established. A linear relationship was observed between the PABA concentration and the fluorescence intensity in the range of 0.001—1.0 μg/ml.

The proposed method is applicable to the determination of other primary aromatic amines.

Keywords——fluorometric determination; diazotization; coupling; thin-layer chromatography; aromatic amines; 4-methoxy-*m*-phenylenediamine; *p*-aminobenzoic acid

Fluorometric procedures have been used extensively in primary aromatic amine analysis.[3–7] A fluorescence reaction with *o*-phthalaldehyde has been found by Wachsmuth *et al.*[3] Various phthalaldehyde derivatives[4,5] and N-(1-naphthyl)ethylenediamine[6] have been used for the determination of sulfanilamides. *p*-Dimethylaminobenzaldehyde[7] has been used for the determination of *m*-toluidine and sulfanilamide. Dombrowski and Pratt[8] have reported the most sensitive fluorometric method for primary aromatic amines, by the use of 2,6-diaminopyridine. The method involves diazotization of the aromatic amine, followed by coupling with 2,6-diaminopyridine as the fluorescent reagent, reaction of the resulting azo compound with ammoniacal cupric sulfate and removal of excess reagent. We have investigated a simple and sensitive method using a non-fluorescent *m*-phenylenediamine derivative instead of 2,6-diaminopyridine, and we have found that 4-methoxy-*m*-phenylenediamine (MPD) can be used as a reagent for the analysis of aromatic amines.

Several colorimetric methods[9,10] have been used for the analysis of *p*-aminobenzoic acid (PABA). In addition, 2,6-diaminopyridine[8] has been used for the fluorometric determination of PABA. However, the 2,6-diaminopyridine method requires separation of the intermediate product, the azo compound, by benzene extraction, and removal of the benzene by evaporation with a stream of nitrogen gas, so that this method is tedious. Therefore we have examined a fluorometric method with MPD and established it as a simple and sensitive method.

The proposed method is also applicable to the determination of other primary aromatic amines.

Experimental

Apparatus——The measurements of excitation and emission spectra were made with a Hitachi 650-10S spectrofluorophotometer. The fluorometric procedures for determination were carried out with a Hitachi FPL-2 fluorophotometer.

Reagents and Materials——MPD Solution: 6×10^{-5} M MPD dihydrochloride in acetate buffer (1 M) of pH 5 was prepared freshly before use.

Hydrochloric Acid (0.04 N): 1.8 ml of concentrated HCl (reagent grade) was diluted to 500 ml with redistilled H_2O.

Potassium Nitrite Solution (0.1 M): 0.8511 g of potassium nitrite (purchased from E. Merck) was dissolved in 100 ml of redistilled H_2O.

Ammonium Sulfamate Solution: 3% ammonium sulfamate (reagent grade) was prepared with redistilled H_2O.

Ammonia (10%): 38 ml of 28% ammonia (reagent grade) was diluted to 100 ml with redistilled H_2O.

$CuSO_4 \cdot 5H_2O$ (0.06%): $CuSO_4 \cdot 5H_2O$ (0.06 g) was dissolved in 100 ml of redistilled H_2O.

Quinine Sulfate Solution: Solutions of 10 μg/ml, 3 μg/ml, 0.3 μg/ml and 0.03 μg/ml of quinine sulfate in 0.1 N H_2SO_4 were prepared.

All other chemicals and solvents were of reagent grade.

Recommended Procedure——A sample solution is prepared containing 0.001—1.0 μg/ml of PABA in 0.04 N HCl. A 5 ml portion of this sample solution is placed in a test tube fitted with a stopper, which is immersed in an ice bath, and 1 ml of 0.1 M potassium nitrite is added to the solution. After 10 min, the test tube is taken out of the ice bath and 1 ml of ammonium sulfamate solution is added. This solution is allowed to stand for 5 min, then 1 ml of MPD solution is added. The whole is mixed thoroughly, and allowed to stand at room temperature for 10 min, then 1 ml of 10% ammonia and 1 ml of 0.06% $CuSO_4 \cdot 5H_2O$ are added to the mixture. The resulting solution is placed in a boiling water bath for 10 min, then cooled to room temperature in an ice bath and diluted to 20 ml with ethanol. The relative fluorescence intensity is measured with a Hitachi FPL-2 fluorophotometer (primary filter, No. 365; secondary filter, No. 47).

The sensitivity of the fluorophotometer is adjusted with 3 μg/ml, 0.3 μg/ml or 0.03 μg/ml quinine sulfate solution.

The excitation and emission spectra (excitation maximum; 358 nm, emission maximum: 462 nm) are shown in Fig. 1.

Results and Discussion

Conditions for the Determination of PABA

The conditions for determination were examined with 0.01 μg/ml of PABA.

Effect of Hydrochloric Acid Concentration——The optimum concentration of hydrochloric acid was examined when the other parameters were fixed. As maximum and constant fluorescence intensity was observed in the range of 0.01—0.1 N hydrochloric acid, 0.04 N hydrochloric acid used.

Potassium Nitrite Concentration——The effect of potassium nitrite concentration used for diazotization was examined. As maximum and constant fluorescence intensity was observed in the range of 0.05—0.2 M potassium nitrite, 0.1 M potassium nitrite was used.

Effect of the Temperature for Diazotization——The effect of the temperature of diazotization of *p*-aminobenzoic acid on the fluorescence intensity was examined. As maximum and constant fluorescence intensity was obtained in the range of 0—5°, the diazotization was performed in an ice bath. A reaction time of 10 min was suitable.

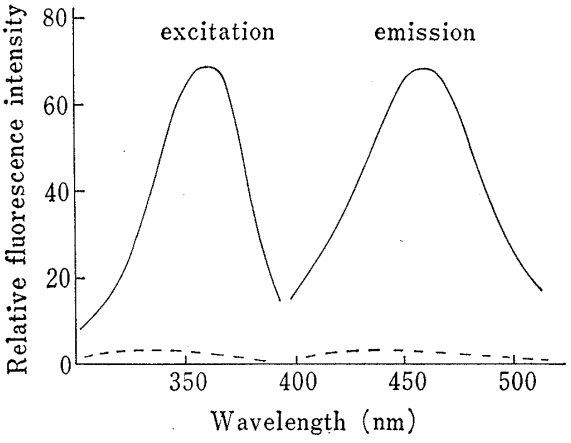

Fig. 1. Excitation and Emission Spectra of the Measurement Solution for PABA

——: 10 ng/ml of PABA,
----: reagent blank.

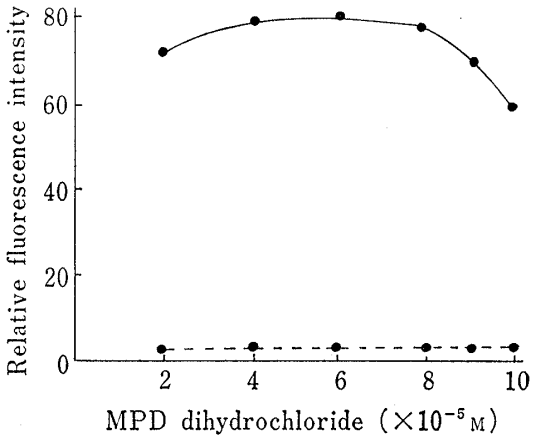

Fig. 2. Effect of MPD Dihydrochloride Concentration in MPD Solution

——: 10 ng/ml of PABA,
----: reagent blank.

Effect of Reaction Time and Temperature on the Coupling of MPD——The reaction time for coupling was varied from 3 to 90 min and the temperature was varied from 60° to 100° in the proposed method. Maximum and constant fluorescence intensity was obtained after 5 min and at a temperature above 90°, so a reaction time of 10 min on a boling water bath was adopted.

Effect of Ammonium Sulfamate Concentration——For the decomposition of excess nitrite, the most suitable ammonium sulfamate concentration was in the range of 2—7%. Consequently, 3% ammonium sulfamate was used.

Effect of MPD Concentration——As maximum and constant fluorescence intensity was observed in the range of 4×10^{-5}—8×10^{-5} M MPD dihydrochloride (Fig. 2), 6×10^{-5} M MPD dihydrochloride was used.

In addition, the utility of polyamino compounds such as *m*-phenylenediamine, 4-chloro-*m*-phenylenediamine, 4-methyl-*m*-phenylenediamine, 3,5-diaminobenzoic acid, 1,2,4-triaminobenzene and 2,6-diaminopyridine as reagents for PABA was examined. It was found that these compounds were inferior to MPD.

Effect of pH on the Coupling——The coupling of MPD with diazotized PABA was pH-dependent. When the MPD dihydrochloride was dissolved in the buffer solution of pH 3.75—5.75, maximum and constant fluorescence intensity was obtained. In the case of buffer solution of pH below 3 or above 6, the fluorescence intensity decreased to about 30% compared with pH 3.75—5.75. Consequently, acetate buffer solution (1 M) of pH 5 was used.

Effect of Cupric Sulfate and Ammonia Concentrations——As maximum and constant fluorescence intensity was observed in the range of 0.05—0.08% $CuSO_4\cdot5H_2O$, 0.06% $CuSO_4\cdot5H_2O$ was used.

When a concentration of ammonia higher than 5% was adopted, the fluorescence intensity was maximum and constant. Consequently, 10% ammonia was used in the proposed method.

Effect of Solvent——Table I showed the effect of the solvent used to dilute the reaction mixture on the fluorescence intensity. When ethanol was used as the diluent, maxiumm fluorescence intensity was obtained. Consequently, ethanol was chosen as the diluent.

Table I. Solvent Effects on the Fluorescence Intensity of the Reaction Product

Solvent	λ_{ex}[a] (nm)	λ_{em}[b] (nm)	R.F.I.[c]
Water	358	480	43.8
Methanol	356	467	69.0
Ethanol	358	462	75.0
Acetone	360	462	67.5
Dioxane	361	462	65.0
Pyridine	364	465	67.0
Dimethylformamide	362	466	66.0

a) Excitation maximum.
b) Emission maximum.
c) Relative fluorescence intensities, uncorrected; fluorescence readings at the same instrument sensitivity.

Calibration Curve and Stability of the Fluorescence——The calibration curve for PABA was linear from 0.001 to 1.0 μg/ml.

The background in the proposed method was very weak and the detection limit of the proposed method, for a signal-to-noise ratio of 2, was found to be 0.001 μg/ml of PABA. The standard deviations for sample solutions having PABA concentrations of 0.001, 0.01, 0.1 and 1.0 μg/ml were calculated to be 0.50, 0.44, 0.42 and 0.36, respectively (ten measurements each).

The fluorescence that developed was stable for at least 12 hr at room temperature.

Table II. Fluorescence Characteristics of Amine Solutions reacted with 4-Methoxy-*m*-phenylenediamine

Amine[a]	λ_{ex}[b] (nm)	λ_{em}[c] (nm)	R.F.I.[d]
Aniline	352	449	77
o-Aminobenzoic acid	337	447	12
m-Aminobenzoic acid	352	451	51
p-Aminobenzoic acid	358	462	70
o-Toluidine	328	440	29
m-Toluidine	351	448	66
p-Toluidine	351	446	77
o-Anisidine	333	444	29.5
m-Anisidine	353	452	40.8
p-Anisidine	354	444	56
o-Aminodiphenyl	329	447	22
p-Aminodiphenyl	361	454	61.8
o-Chloroaniline	331	450	3
m-Chloroaniline	356	456	51.5
p-Chloroaniline	356	455	65
p-Bromoaniline	357	455	36
p-Iodoaniline	357	455	8.3
m-Aminophenol	350	450	3
2,4-Diaminophenol	353	440	10
2,4-Diaminoanisole	353	442	7.6
m-Phenylenediamine	354	450	2
4-Chloro-*m*-phenylenediamine	353	441	4.1
4-Methyl-*m*-phenylenediamine	355	453	1.2
3,5-Diaminobenzoic acid	358	457	11.5
p-Aminosalicylic acid	353	452	1
Sulfanilic acid	358	463	52
p-Aminobenzenesulfonamide	363	479	42
Sulfaguanidine	363	475	47
Sulfamethoxazole	361	466	38
Sulfadiazine	361	465	36.5
Sulfisoxazole	357	463	24
Sulfadimethoxine	352	465	24
Sulfathiazole	365	470	1.5

a) Amine taken: 1.0 μg/ml.
b) Excitation maximum.
c) Emission maximum.
d) Relative fluorescence intensities, uncorrected; fluorescence readings at same instrument sensitivity.

Fluorescence from Some Primary Aromatic Amines

Most primary aromatic amines were expected to fluoresce in a similar manner. The results of examination of some such amines are shown in Table II.

On the other hand, secondary and tertiary aromatic amines and aliphatic amines gave no fluorescence.

Thin Layer Chromatography (TLC) of Reaction Products of Some Primary Aromatic Amines

The fluorescence reaction products from some primary aromatic amines were examined by means of TLC (Table III).

The fluorescence reaction solution was obtained according to the recommended procedure for PABA and the solution were applied to a silica gel (Wako Gel 10-B) TLC plate.

Determination of Other Primary Aromatic Amines

Other primary aromatic amines were determined by the recommended procedure. It was found that ethyl *p*-aminobenzoate, procaine hydrochloride, sulfaguanidine, sulfamethox-

Table III. TLC of Primary Aromatic Amines

Amine	*Rf* values Solvent A [a]	B [b]	C [c]
Aniline	0.57	0.83	0.70
o-Aminobenzoic acid	0	—	0.46
m-Aminobenzoic acid	0.18	—	—
p-Aminobenzoic acid	0.20	0.76	0.60
o-Toluidine	0.51	0.88	0.57
m-Toluidine	0.53	—	—
p-Toluidine	0.56	0.82	0.64
o-Anisidine	0.35	—	0.44
m-Anisidine	0.52	—	—
p-Anisidine	0.53	0.81	0.68
o-Aminodiphenyl	0.58	0.42	—
p-Aminodiphenyl	0.57	0.78	0.70
o-Chloroaniline	0.53	0.41	—
m-Chloroaniline	0.53	—	—
p-Chloroaniline	0.59	0.80	0.65
p-Bromoaniline	0.51	0.59	—
p-Iodoaniline	0.40	0.56	—
Sulfanilic acid	0	0.50	0
p-Aminobenzenesulfonamide	0.12	—	—
Sulfaguanidine	0	0.67	0.14
Sulfamethoxazole	0.27	—	—
Sulfadiazine	0.14	—	—
Sulfisoxazole	0	0.51	0
Sulfadimethoxine	0.21	—	—
Sulfathiazole	0.05	0.20	—
p-Aminosalicylic acid	0.30	0.35	—
m-Aminophenol	0.26	0.32	—

a) Benzene: dioxane: acetic acid=79: 19: 1.
b) Ethyl acetate: methanol: acetic acid=13: 4: 1.
c) *n*-Butyl ether: ethyl acetate: acetic acid=10: 10: 1.

azole, sulfadiazine, sulfisoxazole, and sulfadimethoxine could be determined in the range of 0.01—1.0 μg/ml. The detection limit of these aromatic amines was 0.01 μg/ml in each case.

In conclusion, it was found that 4-methoxy-*m*-phenylenediamine dihydrochloride could be used as a sensitive analytical reagent for primary aromatic amines.

References and Notes

1) This work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August, 1979.
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