[Chem. Pharm. Bull. 27(12)3167—3170(1979)]

UDC 547.576.08 : 543.426.061

Fluorometric Determination of Vanillin

TSUNETOSHI KAITO, KAZUHIKO SAGARA, and KONOMI IKUNAGA

Research Laboratories, Taisho Pharmaceutical Co., Ltd.[1]

(Received May 31, 1979)

Vanillin reacts with *o*-phenylenediamine·2HCl in absolute ethanol to form a strongly fluorescent product which has excitation and emission maxima at 344 nm and 400 nm, respectively. Based on this reaction, a method for the fluorometric determination of vanillin was established. A linear relationship was observed between the vanillin concentration in the range from 0.005 to 2 μg/ml and the fluorescence intensity. The fluorescent product was assigned as 2-(4-hydroxy-3-methoxyphenyl)benzimidazole by comparison with the authentic material obtained by synthesis.

Keywords——vanillin; hydroxybenzaldehydes; *o*-phenylenediamine dihydrochloride; fluorometry; 2-(4-hydroxy-3-methoxyphenyl)benzimidazole

1,2-Diaminonaphthalene[2] is an excellent reagent for the fluorometric determination of aliphatic and aromatic aldehydes such as benzaldehyde and cinnamaldehyde, but it cannot be applied to vanillin because it exhibits very weak fluorescence. The *o*-aminothiophenol method[3] has therefore been used for the determination of vanillin. However, this method is tedious and time-consuming. We sought to devise a simple method for the fluorometric determination of vanillin and found that vanillin develops very strong fluorescence when dissolved in absolute ethanol with *o*-phenylenediamine dihydrochloride (*o*-PDA·2HCl), followed by heating on a water bath. This method is simpler and more sensitive than the *o*-aminothiophenol method.[3] The structure of the fluorescent product is proposed.

Experimental

Materials——Vanillin, benzaldehyde and other chemicals were of reagent grade. Other aldehydes were obtained from Tokyo Kasei Kogyo Co., Ltd.

Reagents——*o*-Phenylenediamine dihydrochloride (*o*-PDA·2HCl) ethanol solution: 15 mg of *o*-PDA·2HCl was freshly dissolved in 100 ml of absolute ethanol to give a 0.015% solution when required. KOH ethanol solution: 0.6 g of KOH was dissolved in 2.5 ml of water and diluted to 100 ml with absolute ethanol. After standing for 24 hr, the clear supernatant of the solution was used as 0.1 N KOH ethanol solution. A 5% HCl methanol solution was obtained from Tokyo Kasei Kogyo Co., Ltd.

Apparatus——A Hitachi MPF-2A recording spectrofluorometer was used for measurements of fluorescence spectra and their intensities. A Shimadzu UV-200 double-beam spectrophotometer was used for measurements of absorption spectra.

Assay Procedure——Two ml of the test solution containing 75 ng of vanillin in one ml of absolute ethanol was placed in a 10 ml volumetric flask. One ml of 0.015% *o*-PDA·2HCl ethanol solution was added and the mixture was heated for 60 min at 75° in the tightly stoppered flask. After cooling to room temperature, the whole was diluted to 10 ml with 0.1 N KOH ethanol solution. The fluorescence intensity of the solution was measured at 400 nm with excitation at 344 nm.

Results and Discussion

Conditions for Determination

Effect of the Concentration of the *o*-PDA·2HCl Ethanol Solution——No fluorescence was observed when *o*-PDA base was added to the test solution. However, fluorescence developed

---

[1] Location: *1-403, Yoshino-cho, Omiya, Saitama, 330, Japan.*

[2] Y. Ohkura and K. Zaitsu, *Talanta*, **21**, 547 (1974); K. Zaitsu and Y. Ohkura, *Chem. Pharm. Bull.* (Tokyo), **23**, 1057 (1975).

[3] T. Uno and K. Taniguchi, *Bunseki Kagaku*, **21**, 76 (1972).

upon addition of a small amount of HCl or H_2SO_4 to the non-fluorescent solution. The change in the intensity of fluorescence was examined on adding hydrogen chloride methanol solution in the range of 1 to 10 mol per mol of *o*-PDA base. As shown in Fig. 1, the maximum intensity was obtained with 2 mol of hydrogen chloride. *o*-PDA·2HCl ethanol solution, therefore, was selected for the present procedure, and a concentration of 0.015% *o*-PDA·2HCl was chosen based on the fact that the intensity reached a constant value at concentrations higher than 0.013%.

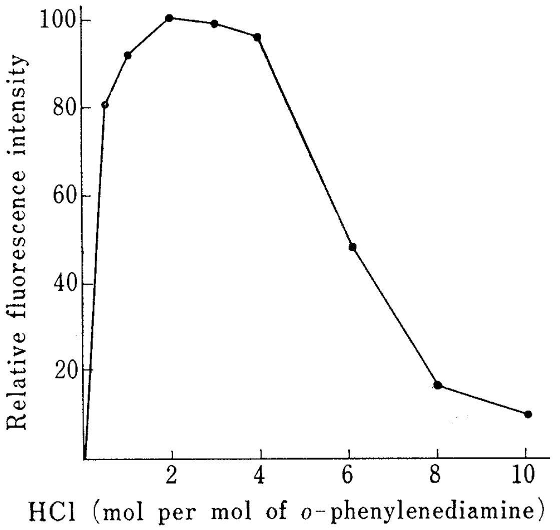

Fig. 1. Relation between the Amount of HCl Relative to *o*-Phenylenediamine and the Fluorescence Intensity

Vanillin: 5×10^{-7} M.
o-Phenylenediamine: 8.3×10^{-4} M.
Reaction time and temp.: 60 min, 75°.

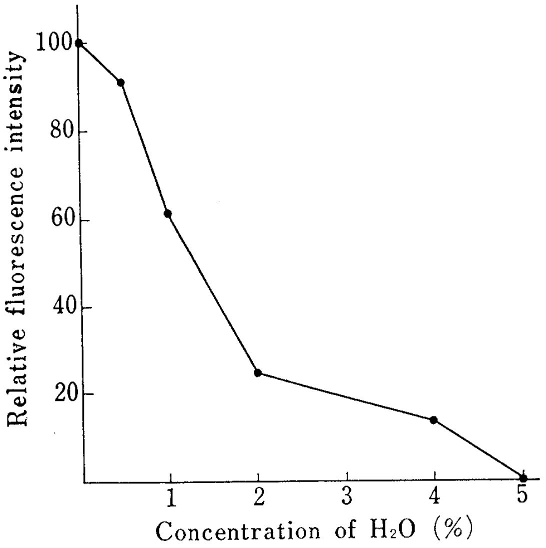

Fig. 2. Effect of Concentration of Water on the Fluorescence Intensity

Vanillin: 5×10^{-7} M.
Reaction time and temp.: 60 min, 75°.

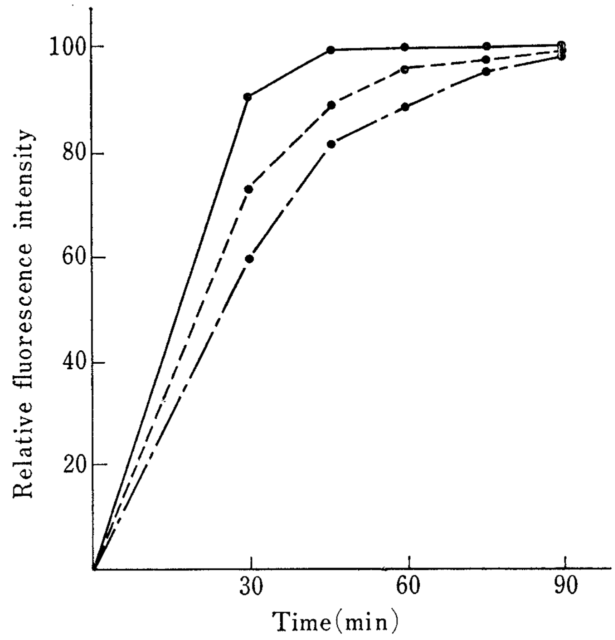

Fig. 3. Effects of Reaction Temperature and Time on the Fluorescence Intensity

——: 75°, ------; 70°, —-—: 60°.
Vanillin: 5×10^{-7} M.
o-Phenylenediamine·2HCl: 0.015%.

Effect of the Concentration of Water during the Reaction Period——When the test solution was heated with *o*-PDA·2HCl, the fluorescence intensity decreased with increase in the concentration of water. This effect was examined by varying the concentration of water from 0 to 5%. As shown in Fig. 2, the fluorescence intensity fell by *ca.* 40% in the presence of 1% water in absolute ethanol and by *ca.* 75% in the case of 2% water. On the basis of these results, absolute ethanol was used as a solvent to prepare the test solution and the *o*-PDA·2HCl solution.

Effect of Reaction Temperature and Time——The reaction temperature was varied from 60 to 75° and the reaction time from 30 to 90 min in the standard procedure. The maximum and nearly constant fluorescence intensity was obtained at 75° for 60 min, as shown in Fig. 3.

Effect of KOH Concentration——The fluorescence intensity was two or more times higher in an alkaline medium than in neutral and acidic media. A maximum and constant value of intensity was obtained when the reaction solution was diluted with 0.05 to 0.2 N KOH ethanol solution. Therefore, 0.1 N KOH ethanol solution was selected.

Calibration Curve and Stability of the Fluorescence——The calibration curve for vanillin was linear from 0.005 to 2 μg/ml. The fluorescence intensity was virtually unchanged for at least 2 hr.

Identification of the Fluorescent Product

It was assumed by analogy with the result for *o*-aminothiophenol[3] that the fluorescent product might be a benzimidazole derivative. The corresponding compound, 2-(4-hydroxy-3-methoxyphenyl)benzimidazole, was synthesized according to the method of Boehringer *et al.*[4] The thin layer chromatography (TLC) behavior, as well as the excitation and emission spectra and ultraviolet absorption spectra of this compound (Figs. 4 and 5) coincide well with those of the reaction solution. The reaction process is thus thought to be as shown in Chart 1.

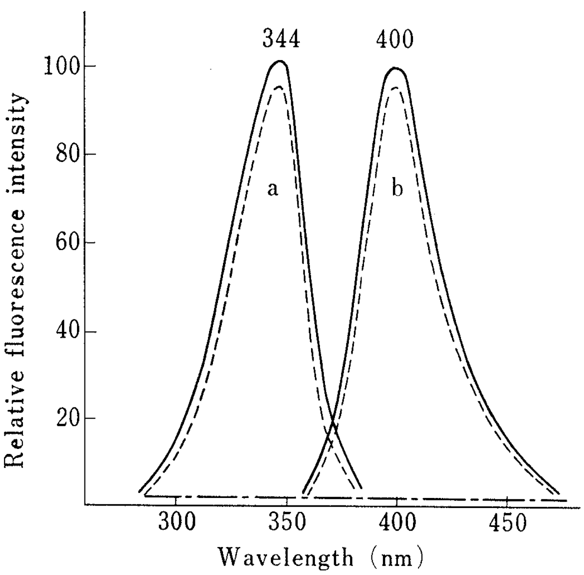

Fig. 4. Excitation and Emission Spectra

a: excitation spectra. b: emission spectra.
——: synthetic product in 0.1 N KOH ethanol solution (1.3×10^{-5} M).
------: reaction solution (vanillin; 1.3×10^{-5} M).
—·—: reagent blank solution.

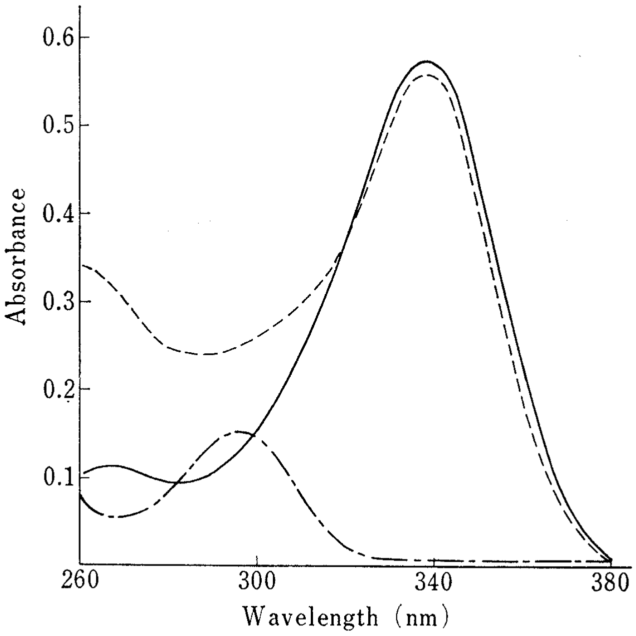

Fig. 5. Absorption Spectra

——: synthetic product in 0.1 N KOH ethanol solution (1.3×10^{-5} M).
------: reaction solution (vanillin; 1.3×10^{-5} M).
—·—: reagent blank solution.

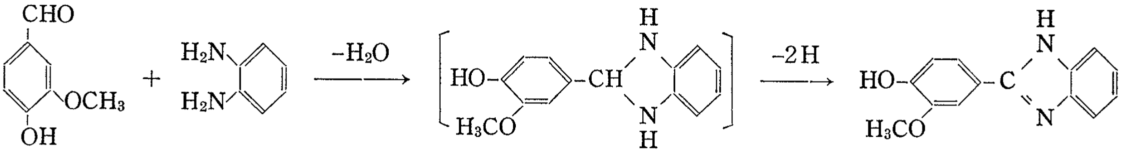

Chart 1

Relative Fluorescence Intensities of Similar Compounds

The fluorescence intensities of eight hydroxybenzaldehydes and benzaldehyde were determined at a final concentration of 1×10^{-7} M at the appropriate maximum excitation

---

[4] A. Boehringer, E. Boehringer, I. Liebrecht, and J. Liebrech, Brit. Patent 713133 (1954) [*C.A.*, **50**, 6520 (1956)].

Table I. Fluorescence Characteristics of Similar Compounds

Compound	Excitation λ_{max} (nm)	Emission λ_{max} (nm)	R.F.I.[a,b]	R.F.I.[a,c]
Vanillin	344	400	100.0	100.0
Isovanillin	336	444	4.9	0
o-Vanillin	360	422	21.0	11.3
p-Hydroxybenzaldehyde	332	382	123.2	71.0
m-Hydroxybenzaldehyde	300	360	1.8	0.4
o-Hydroxybenzaldehyde	358	408	19.5	19.1
3,4-Dihydroxybenzaldehyde	—	—	0	0
2,4-Dihydroxybenzaldehyde	358	400	84.8	72.5
2,5-Dihydroxybenzaldehyde	—	—	0	0
Benzaldehyde	308	400	12.6	0

a) R.F.I.: relative fluorescence intensity.
b) Measured at the appropriate maximum excitation and emission wavelengths.
c) Measured at the maximum excitation and emission wavelengths of vanillin.
Final concentration: 1×10^{-7} M.

and emission wavelengths and at those of vanillin. As shown in Table I, *o*-vanillin and in particular isovanillin showed lower intensities than vanillin, and 3,4-dihydroxybenzaldehyde did not show any fluorescence even at a final concentration of 1×10^{-6} M. Among the compounds examined, *p*-hydroxybenzaldehyde gave the highest value.

Acknowledgement The authors are grateful to Professor A. Tsuji, Showa University, for his valuable advice during this work. The authors also thank Dr. I. Tanaka, Director of Taisho Research Laboratories, for his permission to publish this paper.