Chem. Pharm. Bull.
33(1) 242–248 (1985)

Color Reaction between Keto Acid or Hydroxy Acid and 9-(2′-Carboxyphenyl)-4,5-dibromo-2,3,7-trihydroxy-6-fluorone–Aluminum(III) Complex and Its Application to the Determination of β-Phenylpyruvic Acid[1]

YOSHIKAZU FUJITA, ITSUO MORI,* SHOKO KITANO,
and YOZO KOSHIYAMA

*Osaka College of Pharmacy, 2–10–65, Kawai, Matsubara,
Osaka 580, Japan*

(Received April 26, 1984)

A color reaction involving a keto acid or hydroxy acid, a metal ion such as Al(III), and a xanthene dye was studied. A method for the spectrophotometric determination of keto acids or hydroxy acids (*e.g.*, β-phenylpyruvic acid, PPA) with 9-(2′-carboxyphenyl)-4,5-dibromo-2,3,7-trihydroxy-6-fluorone(bromohydroxyhydroquinonephthalein, Br.Qn.Ph.) and Al(III) was established. A detection test for PPA on a spot plate was also examined. This method is based on the formation of the Br.Qn.Ph.–Al(III)–PPA complex, which has an absorption maximum at around 570 nm. The present method could be used to determine up to 20 μg/10 ml PPA, and the apparent molar absorptivity was calculated to be $8.4\times10^4\,\mathrm{dm^3\,cm^{-1}\,mol^{-1}}$. This method is superior to the other spectrophotometric methods in sensitivity. Recovery of PPA added to human urine was good.

Keywords——keto acid; hydroxy acid; β-phenylpyruvic acid; 9-(2′-carboxyphenyl)-4,5-dibromo-2,3,7-trihydroxy-6-fluorone; aluminum(III); color reaction; spectrophotometry

It is well known that Fe(III) has been used for the detection and determination of various carboxylic acids:[2] for example, in the hydroxamate reaction. However, methods using Fe(III) are not sufficiently sensitive and selective. Recently, a high-performance liquid chromatographic (HPLC) method with Fe(III) for the determination of keto acids and hydroxy acids has been reported.[3]

On the other hand, we noticed that xanthene dye–Al(III) complex solution in the presence of a keto acid or hydroxy acid exhibited a red shift as compared with the xanthene dye–Al(III) complex solution, and we found that the increase in absorbance of xanthene dye–Al(III) solution was proportional to the concentration of keto acid or hydroxy acid. A method using a xanthene dye–Al(III) complex offers the advantages of sensitivity and selectivity as compared with methods using Fe(III).

In this paper, a color reaction involving a keto acid or hydroxy acid, a metal ion such as Al(III), and a xanthene dye was studied, and the fundamental conditions for a spectrophotometric determination of β-phenylpyruvic acid (PPA),[4] which is greatly increased in urine and serum of patients with phenylketonurea, were investigated using 9-(2′-carboxyphenyl)-4,5-dibromo-2,3,7-trihydroxy-6-fluorone (bromohydroxyhydroquinonephthalein, Br.Qn.Ph.) and Al(III). A detection test for PPA on a spot plate was also examined.

Experimental

Reagents and Materials——PPA Standard Solution: A stock solution (1.0×10^{-2} M, M = mol dm^{-3}) of PPA (Sigma Chemical Co.) was prepared by dissolving PPA in water, and the working solution was prepared by suitable dilution of this stock solution as required. These solutions were stored in a cool, dark place.

Dye Solutions: All dyes were prepared in 2.0×10^{-3} M or 1.0×10^{-3} M methanol solution: Br.Qn.Ph., *o*-hydroxyhydroquinonephthalein (Qn.Ph.), 2′-hydroxyphenylfluorone (Salicyl. fl.), 2′-chlorophenylfluorone (Cl.Ph.fl.) and 2′-nitrophenylfluorone (NO_2.Ph.fl.) were prepared according to the literature.[5] Phenylfluorone (Ph.fl.), bromopyrogallol red (BPR), pyrogallol red (PR), gallein (Gall.), chromazurol S (CAS), xylenol orange (XO), pyrocatechol violet (PV), 1-(2-pyridylazo)-2-naphthol (PAN), aluminon and alizarin S were prepared by dissolving pure grade materials purchased from Dojindo Lab., Sigma Chemical Co. or Tokyo Kasei Kogyo Co., Ltd.

Al(III) Solution: A solution of Al(III) (1.0×10^{-3} M) was prepared by dissolving a suitable quantity of aluminum potassium sulfate in water.

Surfactant Solutions: All cationic surfactants were prepared as 5.0×10^{-2} M 20%-methanol solutions. Cationic surfactants used were tetradecyldimethylbenzylammonium chloride (Zep.), benzethonium chloride (Benz.), cetyltrimethylammonium chloride (CTAC) and cetylpyridinium chloride (CPC). All nonionic and anionic surfactants were prepared in 1.0 or 2.0% aqueous solutions. Nonionic and anionic surfactants used were polyvinyl pyrrolidone (PVP, K-30), polyvinyl alcohol (PVA, $n=500$), polyoxyethylenesorbitan monolaurate (LT-221) and sodium dodecyl sulfate (SDS).

Buffer Solution: A 0.2 M hydrochloric acid–0.2 M sodium acetate buffer solution was used.

All other chemicals were of analytical-reagent grade, unless otherwise noted. Double-distilled water was used.

Apparatus——The absorption spectra and values of absorbance were measured using a Shimadzu UV-240 recording spectrophotometer with 1.0-cm matched silica cells. The values of pH were measured with a Hitachi-Horiba F-7 AD glass electrode pH meter.

Standard Procedure for the Spectrophotometric Determination of PPA——The following components were mixed in a 10-ml calibrated flask; 0.75 ml of 1.0×10^{-3} M Al(III) solution, 0.5 ml of 5.0×10^{-2} M Zep. solution, 0.5 ml of 2.0% PVP solution, 3.0 ml of the buffer solution (pH 3.2), 1.0 ml of 2.0×10^{-3} M Br.Qn.Ph. solution, and a sample solution containing up to 20 μg of PPA. The mixture was diluted to 10 ml with water, kept at 40 °C for 20 min, and then cooled to room temperature. The absorbance of the reaction mixture was measured at 570 nm against the reagent blank.

Procedure for the Detection of PPA on a Spot Plate——The following components were mixed in the cavity of a spot plate; 0.02 ml of 5.0×10^{-4} M Al(III) solution, 0.02 ml of 5.0×10^{-2} M Zep. solution, 0.1 ml of the buffer solution (pH 3.2) and 0.02 ml of 2.0×10^{-3} M Br.Qn.Ph. solution. Then 0.02 ml of the sample solution was added, and the mixture was well mixed with a glass rod. A positive response was indicated by the color change of the mixture from pale red to reddish-violet.

Results and Discussion

Color Reaction and Absorption Spectra

The absorption spectra of Br.Qn.Ph., Br.Qn.Ph.–Al(III), Br.Qn.Ph.–Al(III)–PPA and Br.Qn.Ph.–Al(III)–salicylic acid (SA) solutions are shown in Fig. 1.

On the addition of PPA or SA to the Br.Qn.Ph.–Al(III) solution, the absorption maximum of Br.Qn.Ph.–Al(III)–PPA or Br.Qn.Ph.–Al(III)–SA solution at around 570 nm was distinctly observed; its absorbance was proportional to the concentration of PPA or SA.

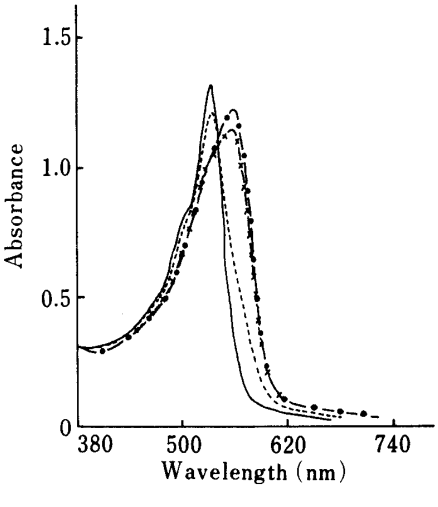

Fig. 1. Absorption Spectra of Br.Qn.Ph., Br.-Qn.Ph.–Al(III), Br.Qn.Ph.–Al(III)–PPA and Br.Qn.Ph.–Al(III)–SA Solutions at pH 3.2

PPA and SA, 1.0×10^{-5} M; Al(III), 5.0×10^{-5} M; Br.Qn.Ph., 1.0×10^{-4} M; Zep., 2.5×10^{-3} M; PVP, 0.5 ml of 2.0% PVP solution/10 ml; reference, water. ——, Br.Qn.Ph., -----, Br.Qn.Ph.–Al(III); —●—, Br.Qn.Ph.–Al(III)–PPA; —×—, Br.Qn.Ph.–Al(III)–SA.

The effect of dyes in this reaction system was studied by measuring the difference of absorbance between a dye–Al(III)–PPA or a dye–Al(III)–SA solution and the dye–Al(III) solution. The use of xanthene dyes increased the absorbance of dye–Al(III) solution, and CAS remarkably decreased the absorbance of CAS–Al(III) solution. As for other dyes such as XO, PV, PAN and alizarin S, only small differences of absorption spectra between dye–Al(III)–PPA or dye–Al(III)–SA and dye–Al(III) solutions were observed. Br.Qn.Ph. was superior to the other xanthene dyes tested in terms of sensitivity. The results are given in Table I.

The effect of metal ions on the color-reaction system was investigated. Al(III), Ga(III) and In(III) were effective among various metal ions tested: Al(III), Ga(III), In(III), Fe(III), Cu(II), Zn(II), Th(IV), Sc(III), La(III), *etc.* The reaction sensitivity among Al(III), Ga(III) and In(III), in spite of the differing optimal pH values, decreased in the sequence: Al(III) > Ga(III) > In(III).

Next, the color reactions between the Br.Qn.Ph.–Al(III) solution and various carboxylic acids were compared by measuring the absorbance at 570 nm against the Br.Qn.Ph.–Al(III) solution. Among various carboxylic acids tested, reaction sensitivities with keto acids or hydroxy acids, especially α-keto acids, were far larger than those with the other carboxylic acids, as shown by the following examples—tartaric acid > malic acid ≫ succinic acid; PPA > phenyllactic acid; pyruvic acid > lactic acid. The results are shown in Table II.

On the basis of these results, PPA was chosen for the purpose of fundamental investigations on the determination of keto acids or hydroxy acids using Br.Qn.Ph.–Al(III) complex.

Effect of pH

The maximum and constant absorbance of the Br.Qn.Ph.–Al(III)–PPA solution at 570 nm against a Br.Qn.Ph.–Al(III) solution was obtained in the pH range of 3.0—3.6 when the final solution was adjusted with 3.0 ml of 0.2 M hydrochloric acid–0.2 M sodium acetate buffer solution.

Table I. Effect of Dyes

Dye	Absorbance difference[a] at λ_{max}			
	PPA		SA	
Br.Qn.Ph.	0.840	570	0.648	570
Qn.Ph.	0.080	545	0.040	545
Ph.fl.	—[b]	—	—[b]	—
Salicyl.fl.	0.220	545	0.328	545
Cl.Ph.fl.	0.338	535	0.370	530
NO_2.Ph.fl.	0.175	560	0.110	560
PR	0.030	555	0.048	555
BPR	0.332	625	0.342	625
Gall.	0.018	560	0.046	575
CAS	−0.805	635	−0.782	635
XO	0	—	0	—
PV	0	—	0	—
PAN	0	—	0	—
Alizarin S	0	—	0	—
Aluminon	—[b]	—	—[b]	—

PPA and SA, 1.0×10^{-5} M; Al(III), 5.0×10^{-5} M; dye, 1.0×10^{-4} M; Zep., 2.5×10^{-3} M; PVP, 0.5 ml of 2.0% PVP solution/10 ml; pH, 3.2; reference, water.

a) Difference of absorbance between dye–Al(III)–PPA (or SA) and dye–Al(III) solutions.

b) Turbidity.

TABLE II. Reaction between Various Carboxylic Acids and Br.Qn.Ph.–Al(III) Complex

Carboxylic acid	Added (M)	Absorbance at 570 nm
PPA	1.0×10^{-5}	0.840
SA	1.0×10^{-5}	0.648
Phenyllactic acid	1.0×10^{-5}	0.192
Homogentisic acid	1.0×10^{-5}	0.092
Oxalacetic acid	1.0×10^{-5}	0.195
Ketomalonic acid	1.0×10^{-5}	0.780
α-Ketoglutaric acid	1.0×10^{-5}	0.200
Pyruvic acid	1.0×10^{-5}	0.080
Lactic acid	1.0×10^{-4}	0.120
Oxalic acid	1.0×10^{-5}	0.375
Tartaric acid	1.0×10^{-4}	0.650
Malic acid	1.0×10^{-4}	0.455
Succinic acid	1.0×10^{-4}	0
Maleic acid	1.0×10^{-4}	0
Citric acid	1.0×10^{-5}	0.270
Levulinic acid	1.0×10^{-4}	0
Glutamic acid	1.0×10^{-4}	0
m-Hydroxybenzoic acid	1.0×10^{-4}	0

PPA, 8.2 μg/10 ml; Al(III), 5.0×10^{-5} M; Br.Qn.Ph., 1.0×10^{-4} M; Zep., 2.5×10^{-3} M; PVP, 0.5 ml of 2.0% PVP solution/10 ml; pH, 3.2; reference, the Br.Qn.Ph.–Al(III) solution.

Effect of Surfactants

Among various surfactants examined, Zep., a cationic surfactant, was most effective with respect to sensitivity; the maximum and constant absorbance was obtained over the range of 0.15 to 0.8 ml of 5.0×10^{-2} M Zep. solution in the final volume of 10 ml. However, the use of Zep. alone was unfavorable as regards the reproducibility of color development. This disadvantage could be virtually eliminated by using PVP solution together with Zep. solution. The results are given in Table III.

Effect of Al(III) and Br.Qn.Ph. Concentrations

The effects of the amounts of Al(III) and Br.Qn.Ph. were examined by varying the molar ratio of Br.Qn.Ph. to Al(III), the amount of PPA being kept constant. The results indicated that the molar ratio of Br.Qn.Ph. to Al(III) should be greater than 2. On the other hand, the less the amount of Al(III), the better was the color contrast between sample and reagent.

Accordingly, all further work was carried out with 7.5×10^{-5} M Al(III) and 2.0×10^{-4} M Br.Qn.Ph. solutions (final concentrations).

Sequence of Addition of the Reagents and Stability of the Developed Color

The same absorbance was obtained even when the order of the addition of the reagents was varied. The color formation in this reaction system did not occur instantaneously at room temperature (15—25 °C), more than 120 min being required for completion of the color reaction. Experiments on the effect of time and temperature proved that the maximum and constant absorbance was obtained on heating at 40 °C for 15—25 min. The absorbance remained unchanged for at least 90 min after the solution had been cooled to room temperature.

Calibration Curve for PPA and Repeatability

The calibration curve for PPA was constructed according to the standard procedure described in Experimental. It was found that Beer's law held in the concentration range up to

TABLE III. Effect of Surfactants

Surfactant	Absorbance at λ_{max}	
Zep.	0.422	570
Zep. + PVP	0.422	570
Zep. + LT-221	0.320	570
Benz.	0.408	565
CPC	0.325	565
CTAC	0.315	565
PVP	0.336	555
LT-221	0.306	550
PVA	0.062	550
SDS	0.012	550

PPA, 8.2 μg/10 ml; Al(III), 7.5×10^{-5} M; Br.Qn.Ph., 2.0×10^{-4} M; surfactant, 2.5×10^{-3} M or 0.5 ml of 1.0% surfactant solution/10 ml; pH, 3.2; reference, the Br.Qn.Ph.–Al(III) solution.

20 μg of PPA in the final volume of 10 ml. The apparent molar absorptivity at 570 nm was estimated to be 8.4×10^4 dm^3 mol^{-1} cm^{-1}. The sensitivity, according to Sandell's scale, was 0.0019 μg cm^{-2} for PPA. The coefficient of variation (*C.V.*) for 6 replicate determinations was 0.98% for 8.2 μg of PPA, whereas the *C.V.* in the absence of PVP (Zep. alone) was 6.41%.

Effects of Foreign Substances

Interference by various substances was studied. Among metal ions examined, Cu(II) and Fe(III) interfered, though other metal ions such as Co(II), Zn(II) and Mg(II) did not interfere in 20-to 100-fold excess over PPA. Among the anions examined, fluoride ion in small amounts and phosphate ion in large amounts interfered.

As mentioned above, oxalic acid, L-ascorbic acid, pyruvic acid, *etc.* gave rise to considerable positive errors, even at low concentrations, due to colored complex formation in this reaction system. The influences of large amounts of phosphate ion and uric acid could be conveniently eliminated by precipitation as the magnesium salt and the barium salt, respectively, in weakly basic media. In addition, the effects of interfering ions and substances, even when present in fairly large excess compared with PPA, could be minimized by using a standard addition method.

On the other hand, various organic compounds such as D-glucose, albumin, amino acids and creatinine, and drugs such as ampicillin and chlorpheniramine showed only minor effects on the determination of PPA. The results are summarized in Table IV.

Recovery of PPA Added to Human Urine

Recovery of PPA added to human urine was examined. Satisfactory results were obtained in the following way. Exactly 10 ml of human urine was taken and adjusted to about pH 8 with 0.2 M ammonia–0.2 M ammonium chloride solution. Next, 0.5—1.0 ml of 10^{-1} M magnesium nitrate and 10^{-1} M barium chloride solutions were added as required, and the solution was diluted to 50 ml with water and filtered. A 0.2 ml aliquot of the filtrate was taken and PPA content was determined according to the standard procedure. Recovery (the average of 5 determinations for 8.2 μg of PPA) was 98.3%.

Detection Test for PPA on a Spot Plate

For clinical use, a simple and sensitive method for the detection of PPA is desirable. Thus, a detection test for PPA on a spot plate was also examined as described in the previous report.[6] Satisfactory results were obtained when detection tests were carried out according to the procedure described in Experimental. The detection limit for PPA on a spot plate was 0.5 μg in 0.02 ml of the sample solution.

Table IV. Effects of Foreign Substances on the Determination of PPA

Substance	Added		PPA found (μg/10 ml)
	(μg/10 ml)	Molar ratio[a]	
—	—	—	8.2
Cu(II) (nitrate)	1.6	1/2	11.1
Fe(III) (sulfate)	8.4	3	10.5
Co(II) (nitrate)	5.9×10	20	8.2
Mg(II) (chloride)	2.4×10^2	200	8.2
Sodium chloride	2.9×10^4	1000	8.2
Sodium fluoride	4.2	2	9.6
Potassium iodide	1.7×10^4	200	12.4
$H_2PO_4{}^{2-}$ (potassium)	9.7×10	20	16.0
Oxalic acid	2.3×10	1/2	10.1
Pyruvic acid	8.8	2	11.7
Lactic acid	2.3×10	5	8.3
L-Ascorbic acid	8.8	1	8.3
Creatinine	2.8×10^3	50	10.2
Phenylalanine	8.2×10^3	100	9.5
Folic acid	4.4×10	2	8.2
Urea	6.0×10^3	200	8.2
D-Glucose	9.0×10^3	100	8.2
Albumin	5.0×10^3	—	10.0
Ampicillin	3.5×10^3	20	8.2
Chlorpheniramine	2.7×10^3	20	8.2

PPA, 8.2 μg/10 ml; Al(III), 7.5×10^{-5} M; Br.Qn.Ph., 2.0×10^{-4} M; Zep., 2.5×10^{-3} M; PVP, 0.5 ml of 2.0% PVP solution/10 ml; pH, 3.2; reference, the Br.Qn.Ph.–Al(III) solution.

a) Substance/PPA.

In conclusion, a reaction involving a keto acid or hydroxy acid, a metal ion, and a xanthene dye was studied, and fundamental conditions for the spectrophotometric determination of keto acids or hydroxy acids (as PPA) using the Br.Qn.Ph.–Al(III) complex were established. This method was inferior to the HPLC method[3,7] in sensitivity, but it was about 5—10 times more sensitive than the other spectrophotometric method.[8] Recovery of PPA added to human urine was satisfactory and the detection test on a spot plate was also good. Thus, the present method may be applicable to determine PPA in biological and clinical samples, and should be useful for the determination of keto acids or hydroxy acids, as well as the assay of PPA.

References and Notes

1) This paper is Part XLII of a series entitled "Application of Xanthene Derivatives for Analytical Chemistry," Part XLI, *Chem. Pharm. Bull.*, **32**, 4161 (1984).

2) *a*) F. Feigl, "Spot Tests in Organic Analysis," Elsevier, New York, 1956, p. 236; *b*) M. Pesez and J. Bartos, "Colorimetric and Fluorometric Analysis of Organic Compounds and Drugs," Marcel Dekker, New York, 1974, p. 291.

3) S. Tanabe, T. Togawa, S. Naba, K. Kawaguchi, and T. Imanari, Abstracts of Papers, The 104th Annual Meeting of the Pharmaceutical Society of Japan, 1984, p. 560.

4) *a*) K. Tada, *Rinsho I*, **3**, 150 (1977); *b*) T. Ohura, *Taisha*, **19**, 352 (1982); *c*) H. Bickel, F. P. Hudson, and L. I. Woolf, "Phenylketonurea," G. Thieme, Stuttgart, 1971.

5) *a*) N. Osorovitz, *Ber.*, **36**, 1076 (1903); *b*) C. Lieberman, *ibid.*, **34**, 2299, 2617 (1901); *c*) C. Lieberman and S. Lindenbaum, *ibid.*, **37**, 1171, 2728 (1901); *d*) H. Sano, *Bull. Chem. Soc. Jpn.*, **31**, 974 (1958); *e*) M.-L. Lu, T.-C. Liu, I.-I. Yen, and W.-K. Chin, *Hua Hsüeh Hsüeh Pao*, **25**, 353 (1959).

6) Y. Fujita, I. Mori, and S. Kitano, *Bunseki Kagaku*, **32**, E379 (1983).

7) T. Hirata, M. Kai, K. Kohashi, and Y. Ohkura, *J. Chromatogr.*, **226**, 25 (1981).

8) *a*) A. Saifer and A. F. Harris, *Clin. Chim.*, **5**, 203 (1959); *b*) E. Zelnicek and O. Podahradska, *Clin. Chem. Acta*, **25**, 179 (1969); *c*) S. P. Coburn, J. D. Mahuren, and R. W. Fuller, *Clin. Chem.*, **17**, 378 (1971); *d*) G. Isshiki, *Medical Technology*, **6**, 1083 (1978).