[Chem. Pharm. Bull. 19(11)2241—2246(1971)]

UDC 547.571.04.09 : 547.484.2.03.04

Colorimetric Determination of Pyruvic Acid with *p*-Dimethylaminobenzaldehyde[1]

MITSUYOSHI KAGEURA, YOSUKE OHKURA and TSUTOMU MOMOSE

Faculty of Pharmaceutical Sciences, Kyushu University[2]

(Received January 30, 1971)

A new colorimetric method for the determination of pyruvic acid is presented on the basis of a new color reaction of the acid with *p*-dimethylaminobenzaldehyde in dimethylsulfoxide solution in the presence of strong alkali. The method is selective for pyruvic acid among ketonic acids, but interfered by acetone, acetaldehyde, bilirubin, glucose, D-glucuronolactone and ascorbic acid.

Pyruvic acid is related to many enzyme reactions and plays important roles in biological bodies. The major methods proposed for the colorimetric determination of the acid were based on coloration of its 4-nitrophenylhydrazone[3] or 2,4-dinitrophenylhydrazone[4] in an alkaline solution and on color reaction with salicylaldehyde.[5] These methods, however, are not selective for pyruvic acid, and interfered by some ketonic acids.

Recently, we found that *p*-dimethylaminobenzaldehyde(*p*-DBA) reacted selectively with pyruvic acid to give a yellow color in aqueous dimethylsulfoxide (DMSO) solution in the presence of strong alkali, though the reagent has been used for the detection and determination of some active methylene compounds in the other conditions. This finding was successfully utilized in the colorimetric determination of pyruvic acid.

Experimental[6]

Reagents[7]——*p*-DBA Solution: Dissolve 500 mg of *p*-DBA in 60 ml of DMSO, dilute with H_2O to 100 ml under tap-water cooling, and store under the protection from light. This solution is stable for at least 6 months.

Sodium Hydroxide Solution: Prepare 25% aqueous solution.

Pyruvic Acid Standard Solutions: Dissolve 110.0 mg of pure sodium pyruvate (99.7%)[8] in H_2O and dilute to 500 ml. Dilute this solution, which corresponds to 2 mM (176 μg/ml as pyruvic acid) solution, to give 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mM solutions (105.6, 88.0, 70.4, 52.8, 35.2 and 17.6 μg/ml as pyruvic acid). Add 1 drop of $CHCl_3$ per 100 ml of each solution as a preservative and store in a refrigerator. These solutions are stable for at least 3 months.

Standard Procedure——To 1.0 ml of the test solution placed in a test-tube having a 20 ml mark, add 3.0 ml of *p*-DBA solution and 1.0 ml of NaOH solution, and mix well. Warm the mixture in a water-bath at 37° for 45 min, then cool in running water for 1 min. Add H_2O to the mark and mix by inversion. Measure the absorbance at 420 mμ against the reagent blank within 30 min and read the concentration of pyruvic acid from a calibration curve prepared as described below.

---

1) This forms "Organic Analysis LXXVII". Part LXXVI: Y. Ohkura, Y. Watanabe and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **19**, 1842 (1971).
2) Location: *Katakasu, Fukuoka.*
3) H.D. Dakin and H.W. Dudley, *J. Biol. Chem.*, **15**, 127 (1913).
4) G.D. Lu, *Biochem. J.*, 33, 249 (1939); S.L. Bonting and S. Bonting, *Arch. Biochem. Biophys.*, **58**, 100 (1955).
5) S. Berntsson, *Anal. Chem.*, **27**, 1659 (1955).
6) Absorption spectra and absorbances were measured by a Shimadzu SV-50A Spectrophotometer and a Hitachi 139 Spectrophotometer, respectively, in a cell of 10 mm optical length.
7) All reagents used were JIS Reagent Grade unless otherwise stated.
8) Prepared and determined by the method described by T. Momose, S. Moritani and Y. Ohkura, *Bunseki Kagaku*, in press.

NII-Electronic Library Service

Calibration Curve——Treat three 1.0 ml aliquots of each pyruvic acid standard solution and of water for blanks as described in the standard procedure, and measure the absorbances against the pooled blank. The calibration curve thus drawn up is a straight line which passes through zero. The absorbance for a 1 mM pyruvic acid solution is 0.780.

Result and Discussion

Choice of the Solvent for *p*-DBA

The solvent of *p*-DBA influenced the color development of active methylene compounds. DMSO, ethanol, dimethylformamide (DMF) and dimethylacetamide (DMA) were tested to find the selective and sensitive conditions for pyruvic acid by spot test technics. For some ketonic acid and carbonyl compounds, those solvents were compared by the absorbances of developed colors. The results, shown in Table I and II, demonstrated that *p*-DBA reacted with pyruvic acid more selectively and sensitively in DMSO at lower temperature. The re-agent in ethanol showed also a sensitive response with pyruvic acid, but sometimes gave a high blank.

Table I. Colorations and Limits of Detection (μg/drop)of Active Methylene Compounds with *p*-DBA[a]

Compound	Coloration[b]	Reaction temperature 20° Solvent for *p*-DBA				Reaction temperature 70° Solvent for *p*-DBA			
		DMSO	EtOH	DMF	DMA	DMSO	EtOH	DMF	DMA
Sodium pyruvate[c]	Y	0.05	0.05	0.1	0.1	0.1	5	0.5	0.5
2-Oxoglutaric acid[c]	Y	10	10	10	10	1	5	1	0.5
Oxalacetic acid[c]	OY	1	1	2	2	0.5	0.5	1	1
Sodium acetoacetate[c]	Y	20	20	—	—	1	5	—	—
Ethyl acetoacetate[d]	Y	10	1	500	50	0.5	50	10	5
Ethyl cyanoacetate[d]	GY	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05
Acetaldehyde[c]	Y	0.2	0.5	0.5	1	—	—	—	—
Propionaldehyde[c]	Y	0.1	0.1	—	—	—	—	—	—
n-Butylaldehyde[c]	GY	1	4	40	0.5	0.05	0.05	8	—
Acetone[c]	Y	0.1	0.1	0.5	0.5	0.5	0.5	0.5	0.5
Methyl ethyl ketone[c]	Y	5	2	10	5	0.5	0.05	1	5
Methyl *n*-propyl ketone[d]	Y	5	5	5	2	0.5	1	10	5
Diethyl ketone[d]	OY	40	40	—	—	1	0.1	—	—
Acetylacetone[d]	Y	10	10	—	—	1	0.1	—	—
Cyclohexanone[d]	Y	2	2	2	2	0.05	0.05	0.05	0.05
2-Methylcyclohexanone[d]	Y	15	50	50	50	0.05	0.05	0.1	0.05
3-Methylcyclohexanone[d]	Y	5	10	5	—	0.05	0.05	0.05	—
4-Methylcyclohexanone[d]	Y	2	2	2	—	0.05	0.1	5	—
Cyclopentanone[d]	OY	0.2	0.2	—	—	1	0.2	—	—
Acetophenone[c]	OY	0.3	0.5	—	—	5	1	—	—
2-Hydroxyacetophenone[d]	Y	5	5	10	5	0.05	0.05	0.1	0.05
Cyanoacetophenone[d]	OY	50	50	50	50	0.05	0.05	5	0.05
Resorcinol[c]	Y	50	50	—	—	1	5	—	—
Orcinol[c]	Y	50	50	—	—	5	10	—	—
Phloroglucinol[d]	Y	50	50	50	50	5	0.5	50	50
Androsterone[d]	YG	10	10	—	—	0.5	5	—	—
Dehydroepiandrosterone[d]	YG	10	10	—	—	0.5	1	—	—
Creatinine[c]	Y	10	10	—	—	5	10	—	—
Pyrrole[d]	Y	10	10	—	—	1	0.1	—	—
Indole[d]	Y	1	5	—	—	0.05	0.1	—	—

a) One drop of sample solution was treated with 1 drop of 0.4% *p*-DBA solution (dissolved in 50% (v/v) DMSO, EtOH, 50% (v/v) DMF or 50% (v/v) DMA) and 3 drops of 8% NaOH solution for 20 min.
b) O: orange; G:green; Y: yellow.
c) dissolved in water
d) dissolved in the same solvent as that for *p*-DBA

NII-Electronic Library Service

Table II. Absorbances Given by Ketonic Acids and Some Carbonyl Compounds[a)]

Compound (100 μg/ml)	Solvent for p-DBA			
	DMSO	EtOH	DMF	DMA
Pyruvic acid	0.309	0.288	0.165	0.166
2-Oxoglutaric acid	0.002	0.002	0.001	0.001
Oxalacetic acid	0.015	0.015	0.013	0.011
Acetoacetic acid	0.001	0.001	0.001	0.001
Acetone	0.029	0.027	0.019	0.015
Acetaldehyde	0.084	0.057	0.056	0.034
Acetophenone	0.048	0.059	0.041	0.034
Cyclohexanone	0.003	0.003	0.002	0.002
4-Methylcyclohexanone	0.003	0.003	0.002	0.002
Blank[b)]	0.030	0.035	0.025	0.025

a) The reaction conditions were the same as those described in Table I, but at the reaction temperature of 20°. The absorbance was measured at 420 mμ against the reagent blank.
b) measured against water.

Reaction Conditions

The absorption spectra of reaction mixture has the maximum at 415 mμ (Fig. 1). A relatively large absorbance of the reagent blank at the maximum may slightly vary sometimes and affects the value of pyruvic acid to be determined, and hence the wave length, 420 mμ, was used for the procedure to ensure a low and constant blank.

The color intensity increases with increasing concentration of p-DBA (Fig. 2), but the reagent frequently deposits in the concentration above 0.6% in the reaction mixture. Therefore, 0.5% was selected as the maximum. At this concentration of reagent, a constant color intensity is obtained for the reaction time of 45—120 min.

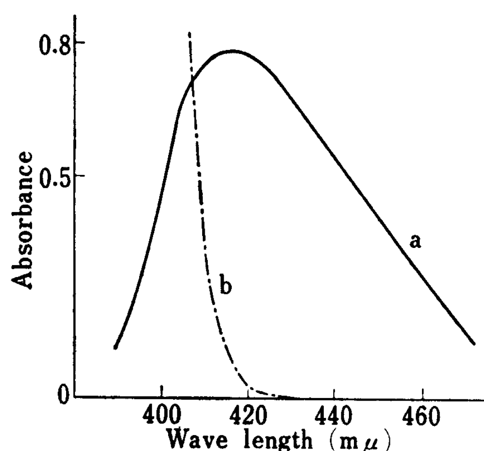

Fig. 1. Absorption Spectra of the Reaction Mixture and the Reagent Blank

a, 1.0 ml of 1mM pyruvic acid solution was treated as in the standard procedure; b, the corresponding reagent blank measured against water.

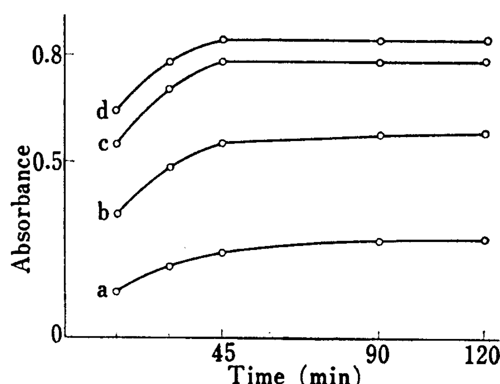

Fig. 2. Effect of the Concentration of p-DBA and the Reaction Time on the Color Development

1.0 ml of 1 mM pyruvic acid solutions were treated as in the standard procedure with the concentrations of p-DBA a, 0.1; b, 0.3; c, 0.5; d, 0.7%.

The concentration of DMSO in the reagent solution was limited to 60% (by volume) to avoid the heat generated when a more concentrated solvent was diluted with the sample solution in the procedure. The heat caused an erroneous absorbance because the color development was largely affected by the reaction temperature as described later.

NII-Electronic Library Service

The concentration of sodium hydroxide affected the color development, and the prescribed concentration, 25%, gave the maximum intensity over the reaction times of 45 to 120 min (Fig. 3). With a more concentrated sodium hydroxide solution, *p*-DBA precipitated from the reaction mixture.

Correlation between the reaction temperature and the reaction time was shown in Fig. 4, and indicated that a higher temperature produced a less intense color at a longer reaction time and the color developed even at room temperature. The selected temperature, 37°, is easily obtainable in a laboratory and gives an appropriate and almost constant color intensity for the reaction time longer than 45 min. From the results described above, the reaction time of 45 min was selected as the optimum for the procedure. And the reaction mixture should be diluted with water to stop the color development as described in the procedure.

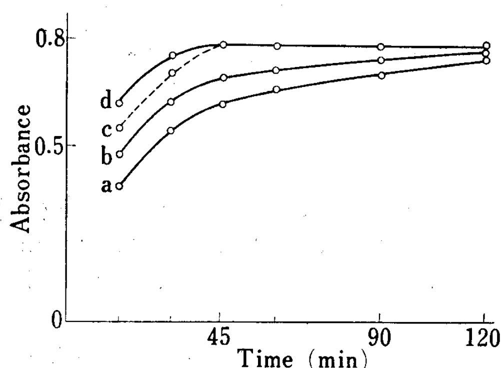

Fig. 3. Effect of the Concentration of Sodium Hydroxide and the Reaction Time on the Color Development

1.0 ml of 1 mM pyruvic acid solutions were treated as in the standard procedure with the concentrations of sodium hydroxide a, 15; b, 20; c, 25; d, 28%.

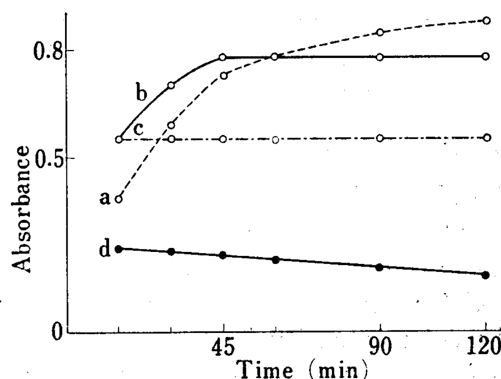

Fig. 4. Effect of the Reaction Temperature and Reaction Time on the Color Development

1.0 ml of 1 mM pyruvic acid solutions were treated as in the standard procedure at the reaction temperatures a, 25°; b, 37°; c, 60°; d, 100°.

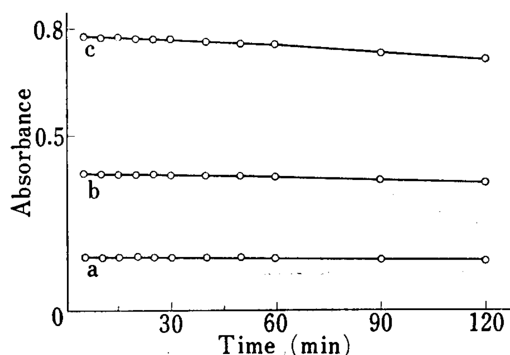

Fig. 5. Stability of the Color Developed

1.0 ml of pyruvic acid solutions (a, 0.2; b, 0.5; c, 1 mM) were treated by the standard procedure and left standing at 20°.

Stability of the Color Developed

The color developed under the conditions of standard procedure was stable for 30 min for a higher concentration of pyruvic acid (1 mM) and 60 min for a lower one (0.2 mM) when left standing at room temperature (Fig. 5). Therefore, the absorbance should be measured in general within 30 min after the reaction mixture was diluted with water.

Precision of the Method

This was studied with respect to repeatability and reproducibility. The repeatability was obtained by carrying out 30 determinations on a 0.5 mM pyruvic acid solution at the same time. The standard deviation was 0.0023 mM (coefficient of variation, 0.46%). The reproducibility was examined by repeating the procedure 5 times on a 0.6 mM pyruvic acid solution, freshly prepared reagent solutions and pyruvic acid solution being used and performing 3 determinations each time. The standard deviation obtained was 0.0039 mM (coefficient of variation, 0.66%).

NII-Electronic Library Service

Interfering Compounds

Interfering compounds were tested with respect to their colorations and the effects on the coloration of pyruvic acid. 2-Oxoglutaric and acetoacetic acids gave neither absorbance, nor interference with the absorbance given by pyruvic acid. Oxalacetic acid showed a weak absorbance which corresponded to only 4% of that given by the same concentrated pyruvic acid, and the additive absorbance was observed in the coexistence of pyruvic acid (Table III, a). These results indicates that the present method is fairly selective for pyruvic acid among ketonic acids.

Acetone, acetaldehyde and bilirubin gave a positive response and interfered with the method, as shown in Table III, a. Glucose, D-glucuronolactone and ascorbic acid scarcely gave the absorbance, but reduced the absorbance given by pyruvic acid, as shown in Table III, b.

Androsterone and dehydroepiandrosterone showed neither coloration, nor interference at a concentration of 250 μg/ml. Thiamine hydrochloride did not affect even at a concentration of 500 μg/ml.

Other substances tested which might be present in biological samples showed neither coloration, nor interference with the color development of pyruvic acid even at a concentration of 1000 μg/ml. These included formaldehyde, 3-hydroxybutyric acid, lactic acid, inositol, urea, creatine, creatinine, allantoin, alloxan, L-alanine, L-aspartic acid, L-tyrosine and L-tryptophane.

TABLE IIIa. Effects of Interfering Compounds

Compound tested (μg/ml)	Absorbance given by		
	Compound tested	Pyruvic acid (μg/ml)	Pyruvic acid+ compound tested
2-Oxoglutaric acid (100)	0.003	0.443 (50)	0.445
Oxalacetic acid[a] (100)	0.035	0.440 (50)	0.474
Acetoacetic acid (100)	0.002	0.445 (50)	0.445
Acetone (20)	0.090	0.221 (25)	0.308
Acetaldehyde (20)	0.151	0.220 (25)	0.371
Bilirubin (100)	0.177	0.222 (25)	0.400

a) Freshly prepared solution was used within 1 hr, otherwise a higher absorbance was observed due to the formation of pyruvic acid.

TABLE IIIb.

Compound	Absorbance given by the compound tested Concentration (μg/ml)								
	0	20	60	100	200	400	600	800	1000
Glucose	—	0.001	0.001	0.002	0.004	0.006	0.007	0.008	0.009
Glucose+pyruvic acid (50μg/ml)	0.440	0.435	0.425	0.415	0.388	0.352	0.332	0.317	0.307
D-Glucuronolactone	—	0.000	0.000	0.000	—	—	—	—	0.001
D-Glucuronolactone+pyruvic acid (50 μg/ml)	0.440	0.429	0.400	0.381	—	—	—	—	0.227
Ascorbic acid	—	0.000	0.000	0.000	0.000	—	0.000	—	0.001
Ascorbic acid+Pyruvic acid (50 μg/ml)	0.440	0.435	0.420	0.400	0.391	—	0.386	—	0.375

Comparison with Other Method

The present method gave 8 times higher absorbance in pyruvic acid than that given by the salicylaldehyde method,[5] and was compared with a 2,4-dinitrophenylhydrazine method

NII-Electronic Library Service

modified for the serum transaminase assay[9] regarding some ketonic acids. In the modified method, 2 mM 2,4-dinitrophenylhydrazine solution and an alkaline solution containing 0.24 N sodium hydroxide and 0.1 M potassium sodium tartarate were used to obtain a linear calibration curve of pyruvic acid and suppress the color development of 2-oxoglutaric acid. The results are shown in Table IV and indicate that the present method is sensitive as well as the 2,4-dinitrophenylhydrazine method, and is more selective for pyruvic acid. Therefore, the present method may be suitable for the assay of enzymes which are concerned with pyruvic acid.

Studies on the color reaction mechanism will be published in the near future.

TABLE IV. Comparison of the Absorbances shown by the Present Method and the 2,4-Dinitrophenylhydrazine Method

Compound (100 μg/ml)	Present method	2,4-Dinitrophenylhydrazine method
Pyruvic acid	0.886	0.929
2-Oxoglutaric acid	0.004	0.089
Oxalacetic acid	0.036	0.308
Acetoacetic acid	0.000	0.010

Acknowledgement This work was partly supported by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, to which we are indebted. Thanks are also due to Misses Y. Moribe and K. Yamada for their technical assistance.

9) Ts. Momose, Y. Ohkura, Ta. Momose, M. Otsuka, T. Kanzaki, K. Morita and S. Ando, *Rinshobyori*, 17, (supplement) 192 (1969).

NII-Electronic Library Service