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Short Communication

High-performance liquid chromatographic separation of *p*-hydroxyphenylpyruvic acid

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ABSTRACT

This paper reports the development of a reliable method for the separation and determination of *p*-hydroxyphenylpyruvic acid by high-performance liquid chromatography. An aqueous solution of *p*-hydroxyphenylpyruvic acid was injected into a Cosmosil C_{18}-5 column with a mobile phase consisting of acetonitrile–acetic acid–water (18:1:81, v/v/v) with detection at 283 nm. Three peaks were observed on the chromatogram, and were identified as the keto–enol tautomers and the decomposition product *p*-hydroxybenzaldehyde. The enol form and *p*-hydroxybenzaldehyde were determined without derivatization and the keto form as its oxime from their peak heights by comparison with those of known amounts of the enol form, *p*-hydroxybenzaldehyde and the oxime standard of the keto form.

INTRODUCTION

p-Hydroxyphenylpyruvic acid (HPPA) is formed from tyrosine by tyrosine aminotransferase [1], and a large amount of HPPA is excreted in the urine of patients with tyrosinosis [2] or tyrosinaemia [3,4]. It is well known that HPPA in aqueous solution undergoes tautomeric changes [5] and also decomposes to *p*-hydroxybenzaldehyde (HBA) [6,8]. The observation which led us to the present study was that HPPA is quite unstable. Several methods have been reported for the high-performance liquid chromatographic (HPLC) separation of HPPA as the free form [3,4,9,10] or after the derivatization [1,11–15]. However, these methods lack specificity in varying degrees as quantitative techniques, because the degradation of HPPA has not been taken into consideration. Therefore, in this paper we describe a reliable method for the determination of HPPA by HPLC.

EXPERIMENTAL

Materials

HPPA was purchased from Sigma (St. Louis, MO, USA) and HBA and hydroxylamine hydrochloride from Tokyo Kasei (Tokyo, Japan). HPLC-grade acetonitrile was obtained from Wako (Osaka, Japan). All of the other chemicals used were of reagent-grade purity.

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Preparation of the oxime of the keto form

The oxime of the keto form was prepared with hydroxylamine hydrochloride as described by Lancaster *et al.* [16]. A mixture of sodium hydroxide (4 g), HPPA (125 mg) and hydroxylamine hydrochloride (1 g) in 50 ml of water was heated at 60°C for 30 min. After acidification to pH 1–2 with concentrated hydrochloric acid, crude powder was obtained by slow partial evaporation of the ether extract and purified by recrystallization from a mixture of acetone and chloroform: yellow powder, m.p. 149–152°C; analysis, calculated for $C_9H_9O_4N$, C 55.39, H 4.65, N 7.18; found, C 55.15, H 4.61, N 7.23%; λ_{max}, 275; ε, 1560 in the mobile phase.

Preparation of the oxime of HBA

A mixture of HBA (100 mg) and hydroxylamine hydrochloride (1.2 g) in 50 ml of water was treated in a similar way to the preparation of the keto-form oxime; yellow powder, m.p. 115–120°C; analysis, calculated for $C_7H_7O_2N$, C 61.31, H 5.11, N 10.22; found, C 61.02, H 5.15, N 10.08%; λ_{max}, 266; ε, 13 616 in the mobile phase.

High-performance liquid chromatography

A model 5A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 283 nm and a model 7125 syringe-loading sample injector with a 20-μl loop (Rheodyne, Cotati, CA, USA) were used. The quantitative HPLC separations were performed at ambient temperature on a 250 × 4.6 mm I.D. Cosmosil C_{18}-5 (Nacalai Tesque, Kyoto, Japan) with a mobile phase consisting of acetonitrile–acetic acid–water (18:1:81, v/v/v) at a flow-rate of 1.5 ml/min. The components were determined by an absolute calibration method.

RESULTS AND DISCUSSION

We have already reported the vibrational spectroscopic study of phenylpyruvic acids including HPPA [17]; HPPA exists in the enol form in the solid state and in organic media. On the other hand, HPPA in aqueous medium is gradually converted into the ketonic tautomer, and the degradation to HBA also takes place simultaneously. The changes are rapid in alkaline solutions. These phenomena are observed in UV spectra (Fig. 1). At pH values greater than 7.4, the conversion of the enol to keto form and the degradation to HBA requires only a few minutes, while the spectral change is relatively slow in acidic solutions.

The liquid chromatogram of HPPA in an aqueous solution of pH 3.0 shows three peaks corresponding to the keto and enol forms and the decomposition product HBA, as in Fig. 2A. The solution was treated with a large excess of hydroxylamine hydrochloride to give the oximes of the keto form and HBA, and subjected to HPLC (Fig. 2B). In Fig. 2A, peak E is observed as a single peak on injection of a freshly prepared methanolic solution of HPPA and does not disappear on addition of hydroxylamine hydrochloride. From these results, we may conclude that peak E corresponds to the enol form.

The retention time of peak A in Fig. 2A agreed with that of the HBA standard. In addition, peak A disappeared on addition of hydroxylamine hydrochloride, resulting in another peak, A-O (Fig. 2B), the retention of which agreed with that of the authentic HBA oxime. Thus peak A was identified as HBA.

The eluate corresponding to peak K in Fig. 2A was pooled and evaporated to a small volume. The solution again gave the same three peaks as those in Fig. 2A. On the other hand, when peak K was pooled in a vial containing a hydroxylamine hydrochloride solution and chromatographed again, only one peak, K-O, which has the same retention as that of the authentic keto-form oxime, was observed. Thus peak K was identified as the keto form.

Yu and Bailey [1] observed two peaks corresponding to the keto form of HPPA and its oxime derivative on their chromatograms. However, it seems that the keto form reported by them was HBA and its oxime was HBA oxime. It was impossible to prepare the keto-form standard because of tautomerism and instability in an aqueous solution. Besides, the absorption of the keto form is low, and the peak on the chromatogram is overlapped by that of an unknown impurity. Therefore, it was necessary to determine the keto form as its oxime derivative by comparing with the oxime standard. The reaction yield with hydroxylamine by using an HBA solution (10 μg/ml) was examined and a complete conversion of HBA to the oxime was achieved in a few minutes at pH 3 when an equal volume of

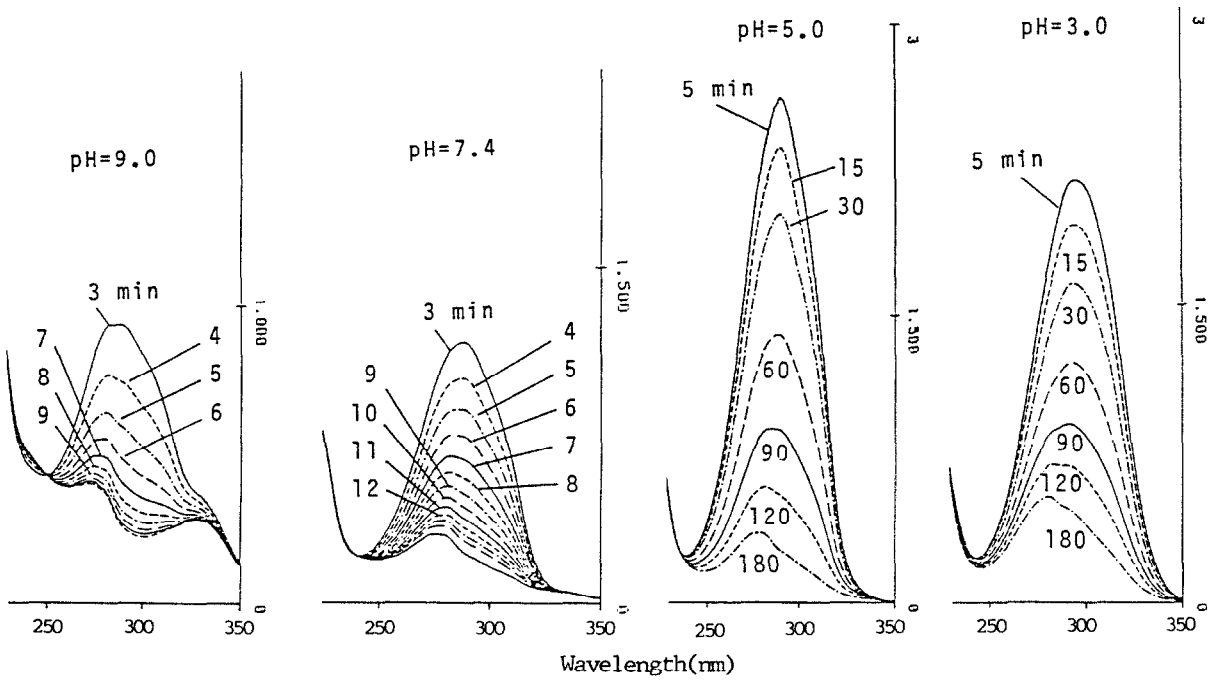

Fig. 1. Changes in UV spectra of HPPA in aqueous solutions (25 μg/ml) due to tautomerism and decomposition. The solutions were prepared using various buffers (pH 9.0, 7.4, 0.1 *M* phosphate; 5.0, 3.0, 0.1 *M* acetate). The values represent the time (min) after the preparation of solutions.

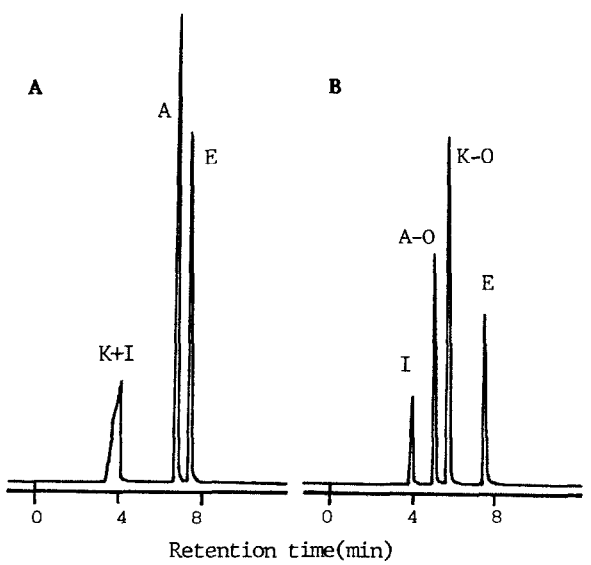

Fig. 2. Chromatograms of HPPA in an aqueous solution of pH 3 (A) and after addition of hydroxylamine hydrochloride (B). Chromatographic conditions: see text. Peaks: A = HBA; A-O: HBA oxime; E = enol form; I = impurity; K = keto form; K-O = keto-form oxime.

hydroxylamine hydrochloride solution (10 mg/ml) was added. At pH 9 the reaction proceeded rather slowly. The keto form is considered to react with hydroxylamine in the same manner as with HBA.

We propose the following equations:

$$\left.\begin{array}{l}[E_0] \rightleftarrows [E] \\ [K_0] \rightleftarrows [K^*] \\ [A_0] \rightleftarrows [A^*]\end{array}\right] \xrightarrow{+NH_2OH} \left[\begin{array}{l}[E] \\ [K_0\text{-}O]+[K^*\text{-}O]=[K\text{-}O] \\ [A_0\text{-}O]+[A^*\text{-}O]=[A\text{-}O]\end{array}\right]$$

where $[E_0]$, $[K_0]$ and $[A_0]$ are the initial concentrations of the enol form, the keto form and HBA in the sample solution, respectively. $[K^*]$ and $[A^*]$ are concentrations produced from E_0 during the period from initial injection to next one after the reaction with hydroxylamine.

$$[K_0] = [K_0\text{-}O] = [K\text{-}O] - [K^*\text{-}O]$$
$$[E_0] = [E] + [K^*\text{-}O] + [A^*\text{-}O] = [E] + [K^*\text{-}O] + [A^*\text{-}O]$$

$$[A^*\text{-}O] = [A\text{-}O] - [A_0\text{-}O] = [A\text{-}O] - [A_0]$$
$$[K^*\text{-}O] = [E_0] - [E] - [A^*\text{-}O] = [E_0] - [E] - ([A\text{-}O] - [A_0])$$

$[K_0]$ is defined by the following equation

$$[K_0] = [K\text{-}O] - ([E_0] - [E]) + ([A\text{-}O] - [A_0])$$

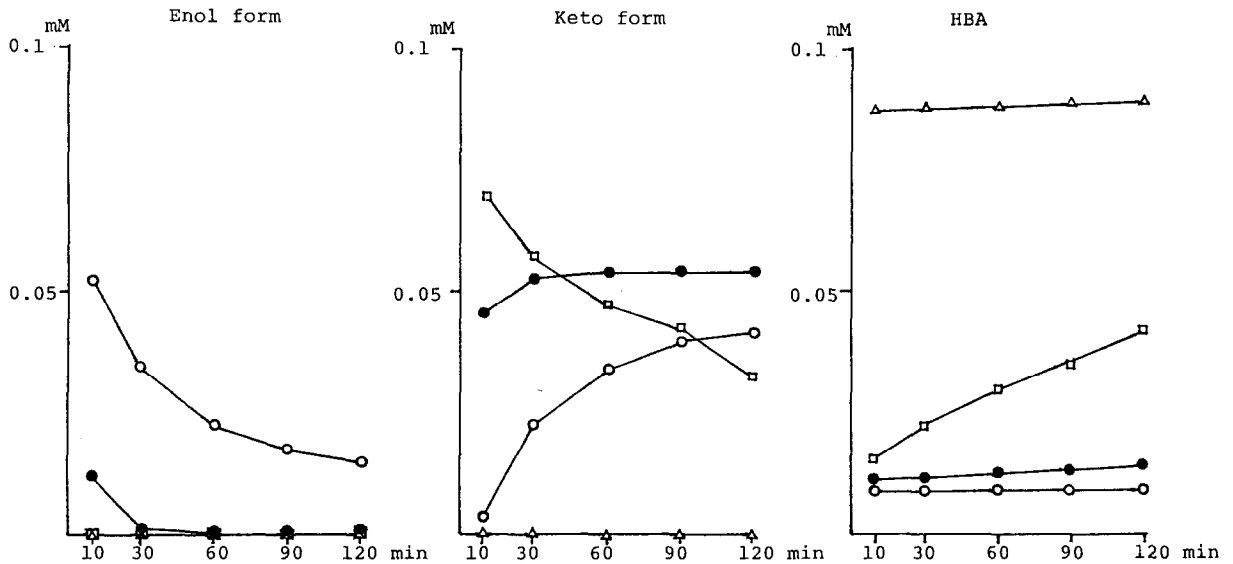

Fig. 3. Effect of pH on the stability of HPPA. ○ = pH 3.0; ● = pH 7.4; □ = pH 10.0; △ = pH 12.0. The sample solutions (0.1 m*M*) of HPPA in 0.05 *M* buffers of several pH values were prepared, and the amounts of the enol and keto forms and HBA were determined.

Thus, $[E_0]$ and $[A_0]$ were measured from their peak heights in Fig. 2A by comparison with those of known amounts of the enol form and HBA. $[K_0]$ was calculated from this equation. Strictly, the enol form is considered to be converted to the keto form or to be decomposed to HBA during running of HPLC. Consequently the values for the enol form tend to be slightly lower.

The effect of pH on the stability of HPPA in aqueous solution was investigated by the present method. An HPPA–methanol solution (450 μg/ml, 2.5 m*M*) was diluted to 0.1 m*M* with 0.05 *M* buffers of several pH values (3.0, acetate; 4.4, 10.0 and 12.0, phosphate). The time dependence of the enol and keto forms and HBA was measured for these solutions. The results are summarized in Fig. 3, from which it can be seen that the conversion from the enol to the keto form proceeds slowly in an acidic solution (pH 3.0) and rapidly in a neutral medium (pH 7.4). HPPA decomposes to HBA gradually at pH 10.0 and instantly at 12.0. A decrease in the amount of the keto form is simultaneously observed at pH 10.0, although no keto form is detected at pH 12.0.

REFERENCES

1 P. H. Yu and B. A. Bailey, *J. Chromatogr.*, 362 (1986) 55.
2 G. Medes, *Biochem. J.*, 26 (1932) 917.
3 H. Todoriki, T. Hayashi and H. Naruse, *J. Chromatogr.*, 310 (1984) 273.
4 G. Rumsby, J. Belloque, R. S. Ersser and J. W. T. Seakines, *Clin. Chim. Acta*, 163 (1987) 171.
5 H. A. Painter and S. S. Zilva, *Biochem. J.*, 41 (1947) 520.
6 K. Schwarz, *Arch. Biochem. Biophys.*, 92 (1961) 168.
7 R. Haavaldsen and T. Norseth, *Anal. Biochem.*, 15 (1966) 536.
8 T. I. Diamondstone, *Anal. Biochem.*, 16 (1966) 395.
9 S. R. Binder and G. Sivorinovsky, *J. Chromatogr.*, 336 (1984) 173.
10 M. L. R. Ramnaraine and M. Tuchman, *J. Chromatogr. Sci.*, 24 (1985) 549.
11 T. Hayashi, H. Tsuchiya, H. Todoriki and H. Naruse, *Anal. Biochem.*, 122 (1982) 173.
12 D. J. Kieber and K. Mopper, *J. Chromatogr.*, 281 (1983) 135.
13 S. Hara, M. Yamaguchi, M. Nakamura and Y. Ohkura, *Chem. Pharm. Bull.*, 33 (1985) 3493.
14 S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 344 (1985) 33.
15 M. Nakamura, S. Hara, M. Yamaguchi, Y. Takemori and Y. Ohkura, *Chem. Pharm. Bull.*, 35 (1987) 687.
16 G. Lancaster, P. Lamm, C. R. Scriver, S. S. Tjoa and O. A. Mamer, *Clin. Chim. Acta*, 48 (1973) 279.
17 K. Hanai, A. Kuwae, S. Kawai and Y. Ono, *J. Phys. Chem.*, 93 (1989) 6013.