Fluorometric Determination of Phenylpyruvic Acid with 1,4-Dimethyl-3-carbamoylpyridinium Chloride

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A fluorometric method for the determination of phenylpyruvic acid (PPA) has been established. Benzaldehyde formed from PPA by alkaline decomposition is quantified by means of its fluorogenic reaction with 1,4-dimethyl-3-carbamoylpyridinium chloride. PPA can be determined in the range of 0.1—3 nmol/50 μl with the relative standard deviation of 4.0—4.1%. About 70% recovery was obtained when a urine sample (0.1 ml) added with 5 nmol of PPA was extracted and then determined by the proposed method. The method should be useful for the biochemical diagnosis of phenylketonuria.

Keywords—phenylpyruvic acid; benzaldehyde formation; 1,4-dimethyl-3-carbamoylpyridinium chloride; fluorometry; phenylketonuria

Phenylketonuria is a serious inherited disease involving a metabolic dysfunction. The determination of urinary phenylpyruvic acid (PPA) levels is considered to be applicable for the biochemical diagnosis of phenylketonuria, and several methods have been reported. However, at an early stage in the disease, the applicability of methods such as a qualitative test with test paper1) and conventional spectrophotometric methods using 2,4-dinitrophenylhydrazine,2) ferric chloride,3) diazotized p-chloroaniline4) and xanthene dye-aluminum(III) complex5) is often limited by their low sensitivity. These methods also lack selectivity for PPA. Although gas chromatography6) and high-performance liquid chromatography with fluorescence detection7) are sensitive, the chromatographic steps are time-consuming.

PPA is known to give benzaldehyde when treated with alkali.8) Further, we have recently proposed a sensitive and selective fluorometric method for determining aromatic aldehydes based on a fluorogenic reaction with 1,4-dimethyl-3-carbamoylpyridinium chloride (DCP-C1) in the presence of alkali.9) Fortunately, benzaldehydes having a hydroxy group, which would be expected to be formed from hydroxyphenylpyruvic acids, did not generate fluorescence in this method. It is shown here that PPA can be determined by reacting DCP-C1 with benzaldehyde produced by alkaline decomposition of PPA. A fluorometric method for determining PPA is proposed.

Experimental

Chemicals and Apparatus—DCP-C1 was synthesized by the published method.9) A stock standard solution of PPA was prepared from PPA sodium salt (Sigma Chemical Co.) at 1 mM concentration; diluted solutions were also prepared by using water. Water used was purified on a Milli RO-Milli Q system (Millipore Ltd.). All other chemicals used were of analytical-reagent grade.

A Hitachi F-3000 fluorescence spectrometer equipped with a xenon lamp was used with a 10 × 10 mm quartz cell at room temperature; spectral bandwidths of 5 nm were used in both excitation and emission. All fluorescence excitation and emission spectra are uncorrected.

Recommended Procedure—Aqueous sample solution (50 μl) was taken in a 1.5 ml glass-stoppered test tube, and 50 μl of 5M aqueous sodium hydroxide solution containing 50 mM sodium pyrosulfite was added. The tube was heated
in a boiling water bath for 30 min, and then cooled in a water bath. Then 1 ml of 15 mM aqueous DCP-C1 solution was added. The mixture was incubated in a water bath at 37 °C for 50 min and then cooled to room temperature in a water bath. The fluorescence intensity was measured with excitation at 436 nm and emission at 506 nm.

**Extraction of PPA from Urine**——A urine sample (0.1 ml) was placed in a 10-ml glass-stoppered test tube, and 1 ml of water, 0.5 g of sodium chloride and 0.2 ml of 1 M hydrochloric acid were added. The mixture was extracted with 2-ml portions of diethyl ether (twice) by shaking for 5 min. To 3 ml of the combined ether extract, 0.5 ml of 2.5 M aqueous sodium hydroxide solution containing 25 mM sodium pyrosulfite was added. The mixture was shaken for 5 min. The organic layer was discarded, then a 0.1-ml aliquot of the aqueous layer was submitted to the fluorometric determination.

**Results and Discussion**

**Fluorescence Excitation and Emission Spectra**

The fluorescence excitation and emission spectra of the reaction mixture of PPA were identical with those obtained in the reaction of benzaldehyde with DCP-C1. The wavelengths of maximal fluorescence excitation and emission of PPA were 436 nm and 506 nm, respectively.

**Reaction Conditions**

Analytical application of the decomposition reaction of PPA to benzaldehyde has not been reported. Thus the reaction conditions for benzaldehyde formation from PPA were first studied. Holcomb et al. reported that conversion of p-hydroxyphenylpyruvic acid to p-hydroxybenzaldehyde was accomplished in 2—3 min at room temperature in 1 M sodium hydroxide. However, a higher temperature was necessary for the reaction of PPA in aqueous sodium hydroxide to form benzaldehyde. As shown in Fig. 1, constant fluorescence intensity was obtained on heating in a boiling water bath for 30—45 min, whereas longer times were necessary below 70 °C. Thus, reaction in a boiling water bath for 30 min was used.

The effect of sodium hydroxide concentration was then examined (Fig. 2). The highest fluorescence intensity was obtained when 2 M sodium hydroxide was used. However, 5 M solution was chosen, because it gave a better reproducibility and a lower blank value.

Reducing agents tended to decrease the blank fluorescence intensity. Sodium pyrosulfite was selected instead of sodium sulfite, which was used in the previous study, because the former was more effective than the latter. Changes in the concentration of sodium pyrosulfite from 10—50 mM did not affect the fluorescence intensity of PPA; therefore, a concentration of 50 mM was chosen. Sodium hydroxide and sodium pyrosulfite could be used as a mixture.

**Fig. 1. Effect of Reaction Temperature and Time on the Formation of Benzaldehyde from PPA**

PPA (2 nmol/50 μl) was treated by the recommended procedure except that various temperatures and times were used. Temperature: (△) 37 °C; (▲) 50 °C; (○) 70 °C; (●) 95 °C (boiling water bath).

**Fig. 2. Effect of Sodium Hydroxide Concentration on the Fluorogenic Reaction of PPA**

PPA (2 nmol/50 μl) was treated by the recommended procedure except that various concentrations of aqueous sodium hydroxide were used.
Next, reaction conditions for the fluorogenic reaction of benzaldehyde liberated from PPA were examined. The fluorescence intensity was constant at DCP-C1 concentrations of 15—30 mm, and hence a 15 mm solution was used. The relationship between fluorescence intensity and the reaction time and temperature after addition of DCP-C1 was studied. The results obtained were similar to those described in the previous paper. The best result was obtained when the reaction was done at 37 °C, and almost constant fluorescence intensity was obtained after 40—60 min. Thus conditions of 37 °C for 50 min were selected.

The fluorescence was stable for 40 min at room temperature.

**Calibration Curve for Standard Solutions of PPA**

A linear calibration curve was obtained over the concentration range of 0.1—3 nmol of PPA in 50 µl of sample. The relative standard deviations (n = 9) were 4.1% at 0.3 nmol and 4.0% at 2 nmol.

Pyruvic acid, α-ketoglutaric acid, oxalacetic acid and p-hydroxyphenylpyruvic acid showed no fluorescence in the present method even at a concentration of 50 nmol/50 µl.

**Recovery of PPA from Urine**

The extraction procedure used was essentially the same as the method of Hirata and Ohkura except that PPA was conveniently back-extracted from the ether extract into aqueous sodium hydroxide containing sodium pyrosulfite. The recovery test was performed by using normal urine because phenylketonuric urine could not be obtained. The recovery in this method was found to be 70.1 ± 3.3% (mean ± standard deviation) at the PPA concentration level of 5 nmol/0.1 ml urine (n = 4). Fluorescence intensity resulting from blank urine was only about 1.5 times the reagent blank. When the first heating in a boiling water bath for 30 min was omitted in the fluorogenic reaction of urine extract containing PPA, the fluorescence intensity obtained was almost the same as that of the blank urine itself. Thus, in practice the blank value of the urine to be analyzed can be approximated by this fluorescence intensity. The lower limit of determination of PPA in urine was 1 nmol/0.1 ml (164 µg/100 ml). PPA can not be detected in normal urine by this method because of its concentration is very low (below about 0.2 mg in 24-h urine of adults and below about 3 µg in 24-h urine of newborn infants). However, the method should be easily applicable to PPA in urine from patients with phenylketonuria because urinary PPA levels in the disease are 24.0—132 mg/100 ml of urine. The method can be used to distinguish clearly between normal and abnormal samples.

Phenylketonuria is also characterized by elevated levels of blood phenylalanine. It is well known that the urinary excretion of PPA is often delayed for several months as compared to the elevation of blood phenylalanine. Thus, current procedures used in mass screening for phenylketonuria usually consist of bioassay for phenylalanine in blood spotted on filter paper. However, excessive amounts of phenylalanine are also found in blood from patients with hyperphenylalaninemia, in which urinary excretion of PPA does not occur. Distinction between the diseases is necessary, because a phenylalanine-poor diet, which is used in phenylketonuria treatment, is not effective for hyperphenylalaninemia. The present method should be applicable for differential diagnosis.

In conclusion, the method described here seems to be useful for the biochemical diagnosis of phenylketonuria, because its sensitivity permits the determination of PPA in urine from patients even at considerably low levels.

**References**