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Note

High-performance liquid chromatography assays of phenylpyruvate and phenylpyruvate oxidase

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Phenylketonuria (PKU) is an inborn error of metabolism affecting phenylalanine metabolism. The normal catabolic path of phenylalanine to tyrosine is blocked owing to the deficiency of the enzyme phenylalanine hydroxylase. The lack of phenylalanine hydroxylase activity in phenylketonurics causes dramatically elevated levels of blood phenylalanine. Much of the accumulated phenylalanine is shunted to make phenylpyruvate, also causing a significant rise in blood levels of this metabolite. Phenylpyruvate has profound inhibitory effects on many enzyme systems and, therefore, may create severe metabolic problems for phenylketonurics. A recent review is provided by Scriver and Clow [1]. Mammalian liver contains the enzyme, phenylpyruvate oxidase (EC 1.14.2.2), which converts phenylpyruvate to the non-toxic *o*-hydroxyphenylacetate. It is clear, however, from plasma analyses, that the activity of mammalian phenylpyruvate oxidase is not sufficient to cope with the conversion of the grossly elevated levels of phenylpyruvate found in PKU patients. As shown by previous studies [2], assistance in such conversion could be provided by an immobilized phenylpyruvate oxidase hollow fiber reactor.

Studies on phenylpyruvate oxidase have been hampered by difficult, tedious and often inaccurate assay methods. Manometric [3] and colorimetric [4] methods were among the first to be used but these were replaced by spectrophotometric [5] and radiochemical procedures [6] in the 1970s. The radiochemical assay, based on a procedure originally developed by Kobayashi [7] for histidine decarboxylase, is the most commonly used today. While the method is relatively sensitive, it is hampered by the fact that it is both time-consuming and expensive. Radiolabelled phenylpyruvate is not available commercially and is typically prepared by reacting ^{14}C-labelled phenylalanine

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with amino acid oxidase (EC 1.4.3.2) [2]. Conversion is incomplete and the amount of product formed has to be checked by an enol—borate assay [8]. Owing to the relative instability of the substrate and the fact that small amounts of radioactive impurities could interfere with the assay, the radiolabelled substrate is prepared immediately before use. The preparation involves a 1-h incubation followed by a chromatographic step.

This paper describes a new high-performance liquid chromatographic (HPLC) procedure that is sensitive, rapid, uses readily available substrate and avoids the requirement of radioactive materials. The method can be used for the assay of blood phenylpyruvate and *o*-hydroxyphenylacetate levels as well as for determining the enzymatic activity of phenylpyruvate oxidase preparations.

EXPERIMENTAL

Phenylpyruvate, *o*-hydroxyphenylacetate, dithiothreitol, catalase, 2,6-dichlorophenol-indophenol and sulfosalicylic acid were purchased from Sigma (St. Louis, MO, U.S.A.); ultrapure, enzyme-grade ammonium sulfate was from Schwartz-Mann (Spring Valley, NJ, U.S.A.); HPLC-grade methanol was from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); HPLC-grade phosphoric acid was purchased from Spectrum Chemical Manufacturing (Redondo Beach, CA, U.S.A.) and sucrose was from Mallinckrodt (Paris, KY, U.S.A.). Deionized and then glass-distilled water was used for chromatography and for the preparation of all reagents.

HPLC was performed with a Waters Assoc. (Milford, MA, U.S.A.) system consisting of a U6K injector, Model 6000A pump, Model 45 pump, data module M730 and system controller M720 together with an LDC (Riviera Beach, FL, U.S.A.) Spectro Monitor III variable-wavelength detector. The column used was a Waters 10-μm μBondapak C_{18} (30 cm × 3.9 mm) reversed-phase unit.

Phenylpyruvate and *o*-hydroxyphenylacetate were separated by HPLC using a linear gradient beginning with 1% phosphoric acid in water and going to 100% methanol in 1% phosphoric acid in 20 min at a flow-rate of 1.5 ml/min.

A crude preparation of phenylpyruvate oxidase was made by dicing fresh rabbit liver (150 g) and homogenizing it in ice-cold 0.25 *M* sucrose (400 ml) for 2 min in a blender. The suspension was centrifuged at 35,000 *g* for 30 min in a Sorvall RC2B refrigerated centrifuge. The resulting supernatant fraction was filtered through cheese cloth and glass wool and the filtrate brought to 35% ammonium sulfate saturation by addition of the solid salt. The pH was maintained at 7 by the addition of 10% ammonium hydroxide. When the ammonium sulfate was completely dissolved the mixture was kept on ice for 30 min. It was then centrifuged at 35,000 *g* for 1 h in a Sorvall RC2B centrifuge. The supernatant fraction from this step was used as a source of phenylpyruvate oxidase.

A phenylpyruvate oxidase assay reagent was prepared weekly. It consisted of 0.82 g of phenylpyruvic acid, 2.25 mg of 2,6-dichlorophenol-indophenol, 60 mg of dithiothreitol and 500 μl of catalase in 50 ml of 0.1 *M* imidazole—HCl buffer, pH 7.0. For the assay of phenylpyruvate oxidase activity 0.5-ml samples of the liver extract were preincubated for 5 min in a shaking waterbath at

37°C. The reaction was initiated by adding the extract to 1.0 ml of the assay reagent in the waterbath. The samples were removed at various intervals and the reaction was stopped by the addition of 2.5 ml of ice-cold 6% sulfosalicylic acid and the samples were held on ice for 30 min with periodic shaking. The samples were then centrifuged at 12,000 *g* for 10 min and the supernatant fractions were held at −80°C until immediately prior to HPLC analysis.

RESULTS AND DISCUSSION

Separation of phenylpyruvate and *o*-hydroxyphenylacetate on a reversed-phase C_{18} column is dependent upon these compounds being in their neutral form. This was accomplished by using a solvent phase brought to approximately pH 2 by the addition of phosphoric acid. Using the methanol gradient described, *o*-hydroxyphenylacetate is eluted with a retention time of 8.4 min at a methanol concentration of 40% (Fig. 1) while phenylpyruvate is eluted with a retention time of 13.7 min with 68% methanol (Fig. 2). Excellent linearity was observed between peak heights and metabolite concentrations up to 1000 nmol. Phenylpyruvate oxidase assays were carried out as described in the experimental section and the rate of phenylpyruvate conversion was monitored by HPLC. The sulfosalicylic acid is eluted at the void volume of the column. The peak heights for phenylpyruvate were converted to nanomoles of phenylpyruvate using a standard curve. A typical assay is shown in Fig. 3. The rate of phenylpyruvate conversion is linear up to approximately 10 min but the reaction then slows down, probably because of product inhibition [5]. The

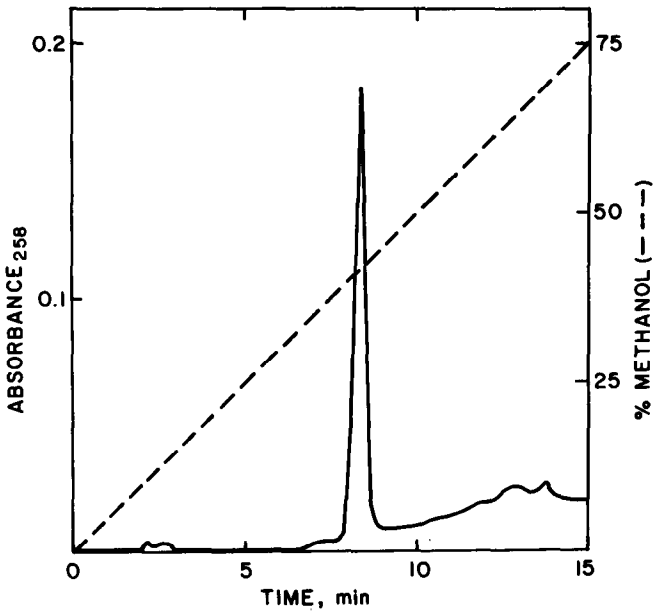

Fig. 1. Elution profile of *o*-hydroxyphenylacetate on reversed-phase HPLC. *o*-Hydroxyphenylacetate was eluted by 40% methanol with a retention time of 8.4 min from a 5-μm C_{18} column (30 cm × 3.9 mm). The column was pre-equilibrated with 1% phosphoric acid in water and a linear gradient from 0% to 100% methanol in 1% phosphoric acid was run in 20 min at a flow-rate of 1.5 ml/min. The column was monitored at 258 nm.

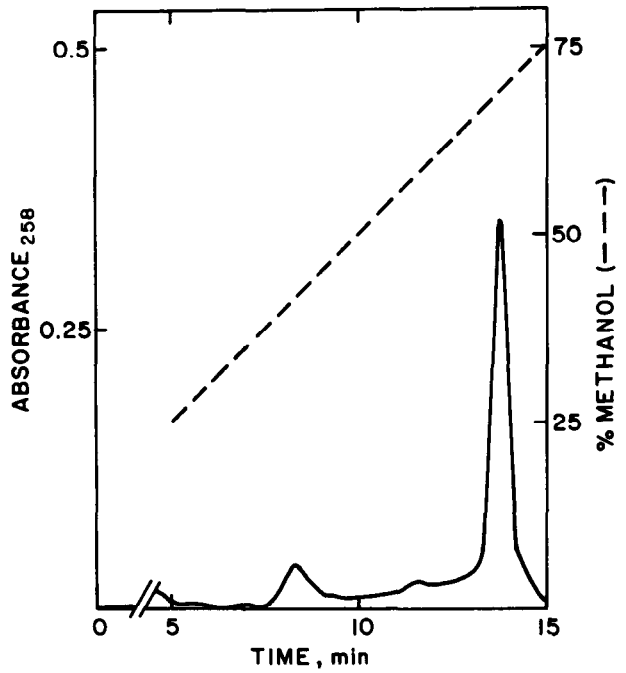

Fig. 2. Elution profile of phenylpyruvate on reversed-phase HPLC. Conditions were as described in Fig. 1. Phenylpyruvate was eluted by 68% methanol with a retention time of 13.7 min from a 5-μm C_{18} column (30 cm × 3.9 mm).

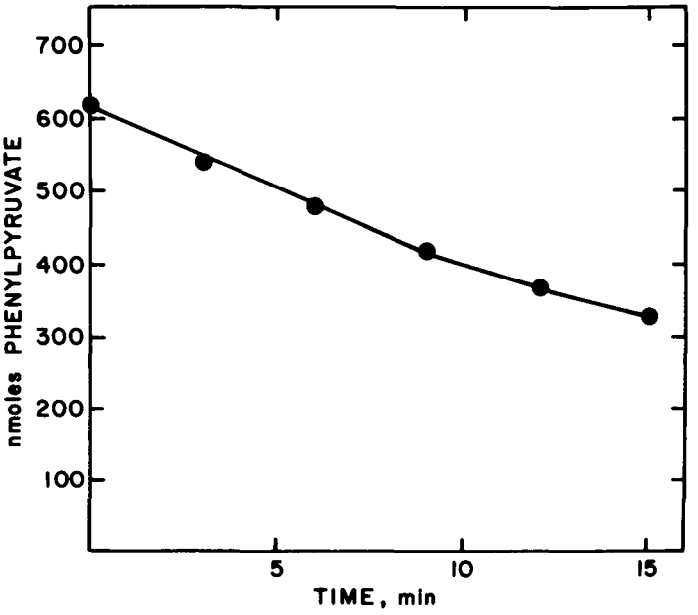

Fig. 3. HPLC assay of a crude phenylpyruvate oxidase preparation. Samples (0.5 ml) of the crude phenylpyruvate oxidase were preincubated for 5 min at 37°C. The reaction was initiated by addition of 1.0 ml of the substrate containing assay reagent, at 37°C, as described in the experimental section. At the indicated time intervals samples were taken from the water bath and the reaction stopped by addition of 2.5 ml of cold 6% sulfosalicylic acid. After 30 min on ice, the samples were centrifuged and the phenylpyruvate concentrations determined by HPLC.

activity of phenylpyruvate oxidase can also be measured in terms of appearance of the reaction product, *o*-hydroxyphenylacetate. In practical terms, it has proven more convenient to measure the decrease in phenylpyruvate concentration with time, to obtain initial rates, rather than attempting to measure very low concentrations of *o*-hydroxyphenylacetate.

The peak heights for phenylpyruvate, obtained from control assay samples lacking enzyme, provide a means for monitoring spontaneous breakdown of phenylpyruvate during the assay period. Little degradation of phenylpyruvate was observed up to 30 min at 37°C. The HPLC assay procedure also provides an internal check on the actual phenylpyruvate content of the assay reagent, thus avoiding one of the difficulties associated with the radiometric assay procedure. The HPLC assay reagent proved stable up to one week when stored at 4°C, but beyond that period increasing breakdown of phenylpyruvate was observed. It is therefore recommended that the reagent be prepared fresh weekly.

The phenylpyruvate oxidase assay by HPLC offers many advantages over the radiochemical procedure. The substrate, phenylpyruvate, is commercially available at much lower cost than the radiolabelled compound required to synthesize [^{14}C] phenylpyruvate, and the fact that the substrate does not have to be synthesized reduces subtantially the time for setting up the assay. The high sensitivity of HPLC detectors makes both the enzymes and metabolite assays viable for low concentration levels.

During the course of the work detailed here, Lefeng et al. [9] reported a similar HPLC procedure for the separation and quantitation of serum phenylalanine. The availability of rapid, accurate assays for the simultaneous monitoring of these important metabolites of phenylketonuria should facilitate future studies of this genetic disease.

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