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NEW SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR *p*-TYROSINE AMINOTRANSFERASE ASSAY

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SUMMARY

A rapid, sensitive and specific procedure has been developed for the determination of *p*-tyrosine aminotransaminase activity. The assay is based on high-performance liquid chromatography (HPLC) separation and electrochemical detection of the pyruvate product, which has been derivatized with hydroxylamine to form a stable oxime. Using this method the product at the low pmol level can be measured. A comparison of the kinetic parameters of the rat liver tyrosine aminotransferase and rat brain non-specific aspartate aminotransferase towards *p*-tyrosine has been made.

INTRODUCTION

Tyrosine aminotransferase (TAT) (L-tyrosine: 2-oxoglutarate aminotransferase, E.C. 2.6.1.5) is probably the major route of tyrosine metabolism in mammals[1]. The enzyme is inducible by several amino acids and hormones such as glucocorticoids, dexamethasone, glucagon, insulin and dibutyryl-3,5[1]-cyclic AMP (see ref. 2 for review). Its activity can be elevated under stress conditions. *p*-Tyrosine is the precursor amino acid for the catecholamines, *p*-tyramine and probably *p*-octopamine and *p*-synephrine; it is of considerable interest to find out whether *p*-tyrosine transamination can shunt this amino acid away from amine biosynthesis[3].

Several methods for TAT assay have been developed. Diamondstone[4] reported a rapid and convenient procedure based on the conversion, under alkaline conditions, of the product *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde, which could then be measured spectrophotometrically. This procedure seems to be the most popular and widely used. A number of other spectrophotometric[5,6] and radioisotopic methods[7,8] have also been developed.

In this paper, we describe a new high-performance liquid chromatographic

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(HPLC) procedure with electrochemical detection to determine TAT activity. It permits a considerable increase in sensitivity using a simplified procedure.

EXPERIMENTAL

Materials

Wistar male rats (200 g) were used. *p*-Tyrosine, *p*-hydroxypyruvate, '5-pyridoxal phosphate, α-ketoglutarate, ethylenediamine tetra aceticv acid (EDTA) and diethyldithiocarbamate were purchased from Sigma (St. Louis, MO, U.S.A.); HPLC-grade acetonitrile was obtained from Caledon (Ontario, Canada) and octyl sodium sulfate from Kodak (Rochester, NY, U.S.A.).

Preparation of tyrosine aminotransferase

The enzyme was partially purified from rat liver as described previously[9]. Pooled tissue (39 g) was homogenized in 5 volumes of 0.04 *M* phosphate buffer (pH 7.5) using a Polytron homogenizer. After centrifugation, the supernatant was fractionated by ammonium sulfate precipitation (30–70% saturation). The protein precipitates were dissolved in phosphate buffer, dialyzed overnight and then separated by DEAE cellulose chromatography in a column (40 × 2.6 cm I.D.) equilibrated with 0.01 *M* phosphate buffer (pH 6.8). Elution was carried out using a linear gradient of sodium chloride (0–0.4 *M*). The fractions containing tyrosine aminotransferase were pooled and precipitated by ammonium sulfate. Rat brain mitochondrial membrane fragments which contain non-specific aspartate aminotransferase, were prepared as previously described[10].

Assay of TAT activity

In a typical assay, the reaction mixture (250 μl) contained the enzyme preparation mixed with 0.04 *M* phosphate buffer (pH 7.5), 1 m*M* *p*-tyrosine, 1 m*M* ketoglutarate, 20 μ*M* pyridoxal phosphate and 200 μ*M* diethyldithiocarbamate. The enzyme incubations were started by addition of the enzyme preparation. After 10 min at 37°C the reaction was terminated by addition of 250 μl 0.25 *M* sodium hydroxide containing 0.15% hydroxylamine. The mixtures were then further heated at 60°C for 15 min after the addition of 500 μl 0.5 *N* perchloric acid; this mixture was then centrifuged at 13 000 *g* for 10 min. The products in the supernatant were separated by HPLC and analyzed by electrochemical detection.

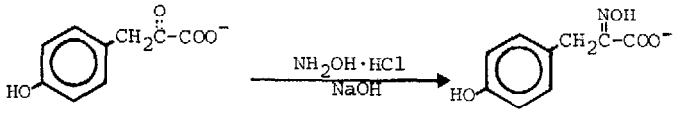
HPLC analysis

Chromatographic separations were performed as previously described[11] at ambient temperature on a 250 × 4.6 mm I.D. Ultrasphere I.P. analytical column packed with C_{18}, spherical 5-μm particles (Beckman, Toronto, Canada). A 30 × 4.6 mm I.D. Brownlee MPLC RP-18 SPHERI-5 guard column (Technical Marketing Associaties, Calgary, Canada) was installed between the Waters WISP 710B automated sample injector and analytical column. A 20-μl aliquot of each prepared sample was injected onto the column. The mobile phase, containing 75 m*M* monobasic sodium phosphate, 1 m*M* sodium octyl sulfate, 500 μ*M* EDTA, and 12.5% acetonitrile, was adjusted to pH 2.75 with orthophosphoric acid and pumped through the column at 1.0 ml/min using a Waters Model 6000A solvent delivery system (Millipore, Missis-

sauga, Canada). Prior to the addition of acetonitrile, the buffer was filtered through a 0.22-μm Millipore filter. Degassing of the mobile phase was accomplished by vacuum. The detector consisted of a BAS Model TL-5A thin-layer amperometric electrode (Mandel, Rockwood, Canada) at 0.9 V *versus* an Ag/AgCl reference electrode. Signals from the detector were integrated by peak area using a Spectra-Physics Computing integrator, SP-4290 (Technical Marketing Associates).

RESULTS AND DISCUSSION

As can be seen in Fig. 1, a clean chromatographic separation of *p*-hydroxyphenylpyruvate was obtained from the *p*-tyrosine transamination reaction catalyzed by partially purified rat liver TAT. Unfortunately, *p*-hydroxyphenylpyruvate is quite unstable[12] and so it is necessary to form the relatively stable oxime derivative as shown in the reaction scheme below as described by Lancaster *et al.*[13] in routine assessments of TAT activity.



This oxime derivative is readily separated from *p*-hydroxyphenylpyruvate as can be seen in Fig. 1B. Under the experimental conditions described above, a complete conversion of *p*-hydroxyphenylpyruvate to the oxime is achieved.

The measurement of the products of the TAT catalyzed reactions is based on HPLC separation followed by electrochemical detection; the sensitivity is extremely high[14]. Hydrodynamic voltammograms have indicated that an oxidation potential of more than 1 V is needed to bring about a total oxidation of this oxime derivative (results not shown). A somewhat lower oxidation potential (0.9 V) for the oxime derivative of *p*-hydroxyphenylpyruvate is applied in this assay. This is to prevent

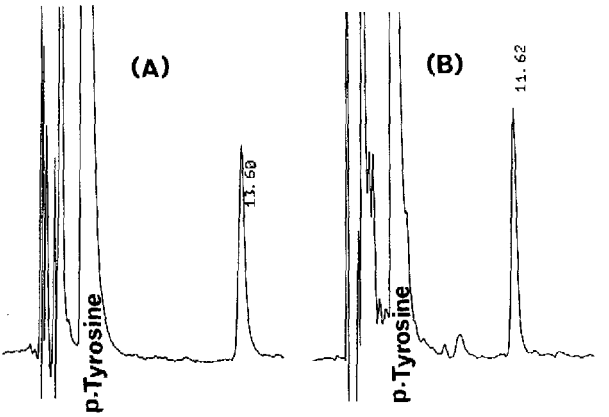


Fig. 1. High-performance liquid chromatogram of the *p*-tyrosine transaminated product *p*-hydroxyphenylpyruvate (A) and its oxime derivative (B) as catalyzed by rat liver TAT. The assay conditions are described in Experimental. The retention time of *p*-tyrosine, *p*-hydroxyphenylpyruvate and its oxime derivative are 5.1, 13.6 and 11.6 min respectively.

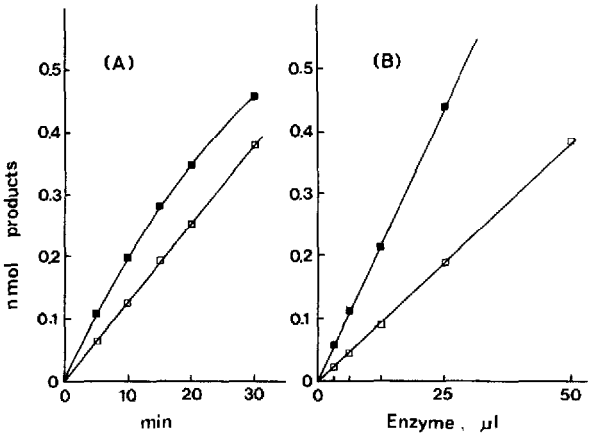


Fig. 2. (A) The initial rates of rat liver TAT (■) and rat brain non-specific aspartate aminotransferase (□) catalyzed transamination of *p*-tyrosine. Partially purified liver TAT (1.1 µg protein) and washed rat brain mitochondria (750 µg protein) were applied. The reactions were terminated at different time intervals by the addition of perchloric acid. (B) Transamination of *p*-tyrosine by partially purified rat liver TAT (■) (protein 15 mg/ml) with increasing amounts of enzyme. The incubation period for the liver enzyme was 10 min and for the brain enzyme 30 min.

rapid fouling of the working electrode and thus increases the stability of the detection. Using this method, *p*-hydroxyphenylpyruvate and its oxime derivative in the low pmol range can be detected and this permits TAT activity in the crude enzyme preparation from rat liver to be measured using as little as 10 µg protein. This is the most sensitive method for TAT assay so far described.

The enzymatic reaction is linear with time for at least 10 min using the rat liver enzyme and for at least 30 min when rat brain mitochondrial membrane fragments are used (Fig. 2A). The reaction is also linear with increasing enzyme concentrations (Fig. 2B).

When the results of this HPLC method were compared with those obtained using Diamondstone's spectrophotometric procedure, they agreed quite well. The

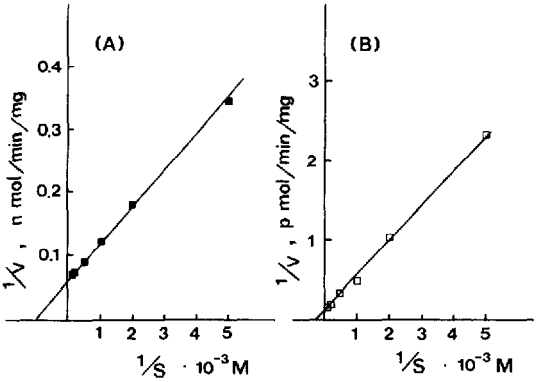


Fig. 3. Lineweaver–Burk plots for the transamination of *p*-tyrosine by rat liver TAT (A) and rat brain mitochondrial bound non-specific aspartate aminotransferase (B). The assay conditions are described in Experimental, except that substrate concentrations are varied [V = velocity, nmol · (mg protein)$^{-1}$ · min^{-1}; S = substrate concentration of *p*-tyrosine].

HPLC method is, however, at least twenty times more sensitive. A typical HPLC analysis of the oxime derivative of *p*-hydroxyphenylpyruvate requires 15 min and if the HPLC system is equipped with an automated injector more than 90 samples can be analyzed in a 24-h period without supervision. The assay is linear with time and protein concentration, demonstrating that it is also suitable for kinetic studies. For example, a Lineweaver–Burk plot of the rat liver soluble TAT and of the rat brain mitochondrial bound non-specific aminotransferase utilising *p*-tyrosine as substrate is shown in Fig. 3. The brain enzyme has a rather low apparent V_{max} value (10.0 ± 1.0 pmol/min/mg protein) and a relatively high K_m value (3.35 ± 0.68 m*M*) whereas the liver soluble TAT exhibits a much higher V_{max} (17.4 ± 0.3 nmol/min/mg protein) and a lower K_m (1.04 ± 0.07 m*M*). This finding supports the suggestion that *p*-tyrosine transamination is perhaps insignificant in normal brain metabolism of this particular amino acid[15].

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