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Note

Assay for L-*p*-tyrosine in plasma and brain by column liquid chromatography with electrochemical detection using *m*-tyrosine as the internal standard

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p-Tyrosine is the amino acid precursor of catecholamines as well as of the trace amines tyramine and octopamine [1]. Until recently, the possible role of *p*-tyrosine in brain function has received little attention. The *p*-tyrosine concentration in brain was generally considered to be sufficient to fully saturate tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis. However, studies by Wurtman et al. [2] have indicated that the rate of catecholamine synthesis is somewhat dependent on *p*-tyrosine concentrations under certain conditions of enhanced catecholamine neuronal cell firing; and haloperidol, a dopamine receptor blocker that stimulates dopamine synthesis, has been found to lower striatal *p*-tyrosine levels [3]. *p*-Tyrosine levels strongly influence the synthesis rates of tyramine and octopamine, since the Michaelis constant (K_M) of the enzyme that converts *p*-tyrosine to tyramine, aromatic L-amino acid decarboxylase, is about 8.4 m*M* [4], which far exceeds the concentration of *p*-tyrosine in rat brain (0.088 m*M*) [5]. Evidence for this is provided by the finding that *p*-tyrosine administration in rats elevates the brain and urine concentration of both tyramine and octopamine metabolites [1, 6]. Therefore, in view of the increasing understanding of the key role of *p*-tyrosine in regulating both catecholamine and trace amine metabolism, determining tissue *p*-tyrosine levels is essential in order to fully understand drug-induced changes in amine function.

Tissue *p*-tyrosine levels are most commonly determined by a fluorometric procedure based on the reaction with 1-nitroso-2-naphthol [7]. However, the advent of electrochemical detectors for liquid chromatography (LC) has led several laboratories to assay tyrosine by this technique [3, 8—14]. LC with

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electrochemical detection (LC—ED), which has become the method of choice for analyzing catecholamines, offers the advantages of speed, specificity and sensitivity. However, unlike the catecholamines, *p*-tyrosine requires a relatively high potential applied to the working electrode. Such a high electrode potential reduces the specificity of the detector and can lead to oxidation of components of the mobile phase, increasing the background noise. At a high cell potential, oxidation of various compounds in biological samples may occur, especially when the samples are processed with little or no clean-up. The oxidation products may coat the cell surface, thereby resulting in a gradual decay in detector sensitivity over the course of an analysis [12]. This loss in detector sensitivity is made more serious by the fact that the applied potential is on the steep portion of the voltammogram, since potentials at or near the plateau of the curve are impractical due to the high background noise that would result. In order to minimize this effect, we sought an internal standard for which the detector response changes in parallel to that of *p*-tyrosine. To our knowledge, no internal standard is used in any of the LC—ED methods for *p*-tyrosine. Two potential internal standards, 3-nitro-L-tyrosine and α-methyl-L-*p*-tyrosine, were found unsuitable due to their long retention times and poor electrochemical activities [11]. We have found that *m*-tyrosine is a suitable internal standard, and we now report on its use in a method for assaying *p*-tyrosine in brain and plasma samples.

EXPERIMENTAL

The following chemicals were obtained commercially and used without further purification: L-*p*-tyrosine (Aldrich, Milwaukee, WI, U.S.A.); DL-*m*-tyrosine and DL-*o*-tyrosine (Sigma, St. Louis, MO, U.S.A.); 1-octanesulfonic acid, sodium salt (Eastman Kodak, Rochester, NY, U.S.A.); HPLC-grade methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); chloroacetic acid, potassium phosphate monobasic, EDTA disodium salt, 60% perchloric acid, sodium metabisulfite and sodium hydroxide pellets (Fisher Scientific, Pittsburgh, PA, U.S.A.).

A Model 153 LC system (Bioanalytical Systems, West Lafayette, IN, U.S.A.) included a PM-11 single-piston pump, a flow-through pulse damper, a 5-μm Biophase ODS guard column (30 × 4.6 mm) and analytical column (250 × 4.6 mm), a TL-5A glassy carbon thin-layer electrode cell, an LC-3A amperometric detector (set at 0.9 V vs. an Ag/AgCl reference electrode and at the 10 nA range) and a single-pen strip chart recorder. Samples were injected either manually into a 200-μl sample loop or automatically with a 710B WISP autoinjector (Waters Assoc., Milford, MA, U.S.A.). The mobile phase consisted of 0.1 *M* chloroacetic acid, 0.1 *M* KH_2PO_4, 1 mg/ml sodium octyl sulfonate, 1 m*M* EDTA and 2% methanol and was adjusted to a final pH of 4.00. After degassing the buffer by vacuum filtering through a 0.2-μm nylon filter (47 mm diameter; Rainin Instruments, Woburn, MA, U.S.A.), it was pumped through the liquid chromatograph at a flow-rate of 2.0 ml/min. Plasma samples were prepared by mixing 100 μl of plasma with 100 μl of an internal standard solution (containing 100 μg/ml *m*-tyrosine) and 800 μl of distilled water, and 20 μl were injected directly into the liquid chromatograph. Brain

samples were homogenized in 2 ml of 0.1 *M* perchloric acid containing 0.05% EDTA using a Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) at a setting of 6 for 15 s. After centrifugation at 15 000 *g*, 450 μl of the supernatant fraction were transferred to a clean tube, mixed with 50 μl of the internal standard solution, and 20 μl of the mixture were injected into the liquid chromatograph. The remainder of the supernatant solution was used for assaying catecholamines. Standards were prepared by adding various amounts of a stock *p*-tyrosine solution (100 μl/ml) to distilled water to make the final concentration between 1 and 5 μg/ml.

RESULTS AND DISCUSSION

The following compounds were examined as potential internal standards for assaying *p*-tyrosine by LC—ED: *o*-tyrosine, *m*-tyrosine, α-methyl-*p*-tyrosine, *p*-hydroxyphenylpropionic acid, *p*-hydroxyphenylacetic acid and *p*-hydroxymandelic acid. Of these compounds, only *m*-tyrosine and *o*-tyrosine had reasonable retention times (t_R) and were completely resolved from *p*-tyrosine under the conditions used. The voltammograms for these compounds indicated that

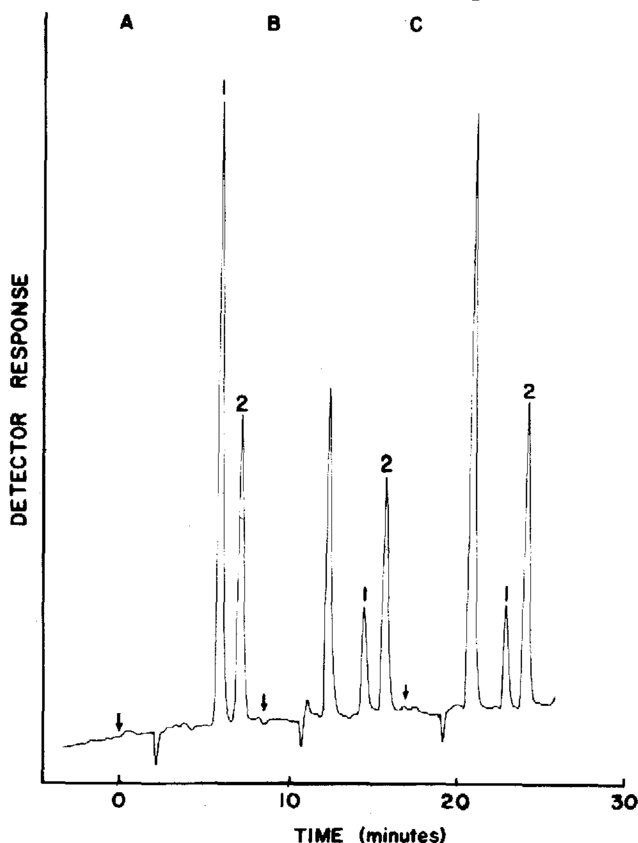

Fig. 1. Chromatograms for the analysis of *p*-tyrosine (peak 1) using the internal standard, *m*-tyrosine (peak 2). (A) Standard containing 5 μg/ml *p*-tyrosine (10 μl injected); (B) plasma sample of a control rat; (C) plasma sample of a chlorpromazine-treated rat. Chlorpromazine (20 mg/kg) was injected intraperitoneally 4.5 h before the animal was killed and trunk blood collected into a heparin-containing beaker.

the oxidation potentials were in the order *m*-tyrosine > *p*-tyrosine > *o*-tyrosine. Although accurate oxidation potentials could not be determined since plateaus in these curves were not reached over the range of the applied potentials used, they were estimated to be approximately 0.93 V for *m*-tyrosine, 0.89 V for *p*-tyrosine, and 0.86 V for *o*-tyrosine. The retention times for the three tyrosine isomers were 5.44 min for *p*-tyrosine, 6.51 min for *m*-tyrosine and 10.47 min for *o*-tyrosine. Typical chromatograms for a standard and rat plasma samples are shown in Fig. 1. There were no peaks in these samples that interfered with either the *p*-tyrosine or *m*-tyrosine peaks. However, late eluting peaks (t_R = 48 min for plasma samples and t_R = 23 and 27 min for brain samples) were usually observed, but we were able to prevent these peaks from interfering with the *p*-tyrosine and *m*-tyrosine peaks of subsequent samples by carefully choosing the injection times. This was greatly simplified by using the autoinjector, since it maintained a precise time interval between injections.

We observed, as previously reported by Lasley et al. [12], that the detector sensitivity declined over time. This decline varied from day to day, and generally was greater while assaying plasma samples. Fig. 2 illustrates the decrease in the peak height of *p*-tyrosine of a standard injected repeatedly during an assay of plasma samples. More importantly, this figure shows that despite the decline in detector sensitivity, the ratio of *p*-tyrosine to *m*-tyrosine peak heights remained relatively constant. The slope of the plot of *p*-tyrosine/*m*-tyrosine peak-height ratios was virtually zero in most assays; but even when a definite negative slope was evident, it was never greater than 2% of the slope of the plot of *p*-tyrosine peak heights.

These results clearly indicate that *m*-tyrosine is a suitable internal standard for the assay of *p*-tyrosine and should greatly improve the precision of the assay. An internal standard is particularly important when the samples are

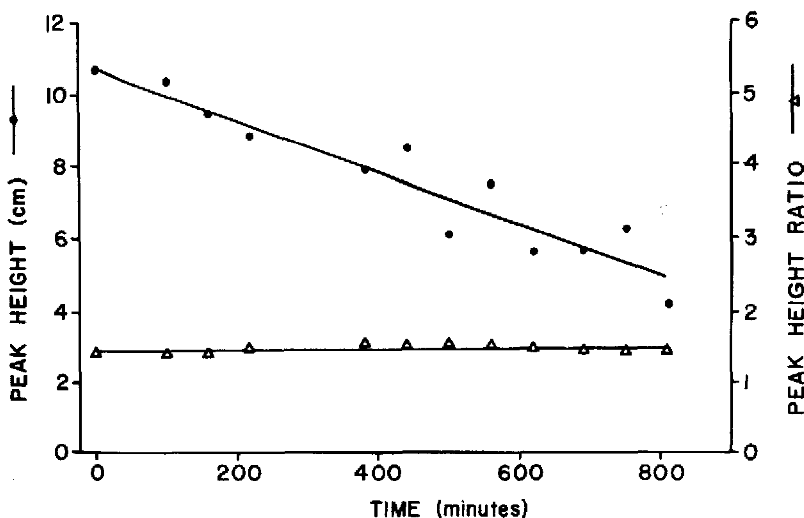

Fig. 2. Plots of the peak height of *p*-tyrosine (•) and of the *p*-tyrosine/*m*-tyrosine peak-height ratios (△) versus time for a standard containing 5 μg/ml *p*-tyrosine and 10 μg/ml *m*-tyrosine injected repeatedly over a single assay run.

injected manually, since we use the method of partially filling the sample loop in order that various volumes can be injected without changing the sample loop. Without an internal standard, the precision of the assay would be limited by the error of the injection volume.

The internal standard virtually eliminates the dependence of the results on detector sensitivity, since the detector sensitivity toward *m*-tyrosine and *p*-tyrosine decline in parallel. As a result, when *m*-tyrosine is used as an internal standard, the standards need to be injected less frequently. Without using an internal standard, we have found it necessary to inject a standard after every fifth sample.

Plasma *p*-tyrosine levels for the experiment shown in Fig. 1 were determined to be (mean ± S.E.) 19.94 ± 2.26 μg/ml for a group of saline-treated rats ($n = 6$) and 8.33 ± 0.43 μg/ml for a group of chlorpromazine-treated rats ($n = 6$). The decrease of plasma *p*-tyrosine levels in drug-treated animals to 42% of control was statistically significant ($p < 0.001$, two-tailed Student's *t*-test). In the same groups of animals, brain tyrosine concentrations were reduced to 59% of control (to 7.36 ± 0.48 μg/g vs. 12.56 ± 0.88 μg/g in control animals; $p < 0.001$ by a two-tailed Student's *t*-test). The decrease in *p*-tyrosine in plasma is consistent with, though somewhat larger than that previously found using a fluorometric assay by Tagliamonte et al. [15], who reported that plasma tyrosine levels were lowered to 63% of control 1.5 h after 10 mg/kg chlorpromazine. However, in contrast to our results, those investigators found no decline in brain *p*-tyrosine levels. This discrepancy could be due to either the greater specificity of the LC—ED assay or to differences in the time point or drug dose. The finding that chlorpromazine lowers brain *p*-tyrosine concentrations as has been previously reported for haloperidol [3], a butyrophenone which though structurally dissimilar to chlorpromazine is also a potent dopamine receptor blocker, suggests that this effect on brain *p*-tyrosine is a general feature of dopamine receptor antagonists.

In summary, by using *m*-tyrosine as an internal standard we have developed an improved assay procedure for determining both brain and plasma concentrations of *p*-tyrosine by LC—ED.

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