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Note

Determination of tyrosine hydroxylase activity by high-performance liquid chromatography with on-line radiochemical detection

EIJA SCHULTZ and ERKKI NISSINEN*

Orion Pharmaceutical Co , Research Centre, P O Box 65, SF 02101 Espoo 10 (Finland)

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Tyrosine hydroxylase (TH), also called tyrosine 3-monooxygenase (EC 1 14 16.2), catalyses the first step in catecholamine biosynthesis, i.e the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (DOPA) in the presence of pterin cofactor This enzyme is present in the brain, in peripheral noradrenergic nerves and in the adrenal medulla [1] TH activity is rate-limiting [2] and hence of particular importance in the regulation of catecholamine biosynthesis

Many methods have been developed to assay TH activity. Since the enzyme activity is very low, the radiochemical assays using [3,5-^{3}H]tyrosine [3, 4] or ^{14}C-labelled tyrosine [1, 5] as the substrate have been frequently used. Fluorometric methods [6, 7] also offer enough sensitivity but require, as in most radiochemical assays, the isolation of the product before it can be quantitated. TH activity can also be assayed by coupled-enzymatic [8] or non-enzymatic [9] decarboxylation of DOPA formed from L-[1-^{14}C]tyrosine In these methods, the evolved $^{14}CO_2$ is trapped and counted

High-performance liquid chromatography (HPLC) with electrochemical detection is a fast and sensitive method to assay TH activity in different tissues [10—12]. Recently, HPLC with on-line radiochemical detection has been shown to be a better method than fraction collection and subsequent liquid scintillation counting when radioactivity in HPLC eluates is measured [13]. It is also sensitive enough to assay low enzyme activities [14].

Here we describe a rapid and sensitive assay for TH activity by HPLC with on-line radiochemical detection This assay permits accurate determinations of the enzyme activity in bovine adrenals.

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EXPERIMENTAL

Chemicals

L-Tyrosine, catalase from bovine liver, D,L-6-methyl-5,6,7,8-tetrahydropterine (6-MTHP) and dithiothreitol (DTT) were purchased from Sigma (St Louis, MO, U S A) L-[U-^{14}C] Tyrosine (specific activity 500 mCi/mmol) and aqueous counting scintillant (ACS) were from Amersham International (Little Chalfont, U.K) Octane sulphonate was obtained from Eastman-Kodak (Rochester, NY, U S A), 3-iodotyrosine from Fluka (Buchs, Switzerland) and methanol (HPLC grade) from Orion Pharmaceutical (Espoo, Finland) All other reagents were of analytical grade and purchased from commercial sources

Tissue preparation

Fresh bovine adrenals were collected on dry ice and stored at −70°C until used For TH assay, the adrenal cortex was removed and the medulla was cut into pieces Tissue pieces were homogenized 1 4 (w/v) in 0 25 *M* sucrose using a Polytron homogenizer The crude homogenate was further homogenized with Potter-Elvehjem homogenizer and finally sonicated twice for 30 s at 40 W The fine homogenate was centrifuged at 40 000 *g* for 30 min The supernatant was dialysed in an Amicon ultrafiltration apparatus Model 8050 using PM 10 membrane-a (Amicon, Danvers, MA, U S A) and diluted with 50 m*M* sodium phosphate (pH 7 5) containing 1 m*M* DTT to a protein concentration of ca 20 mg/ml All the above steps were carried out at 4°C The enzyme solution was stored in small aliquots at −20°C without loss of activity at least for one month. Bio-Rad protein assay kit (Bio-Rad Labs , Richmond, CA, U S A) was used for protein determinations

Assay

Assay of TH activity was carried out in a total volume of 225 μl, which contained the following components 100 μl of 1 *M* sodium acetate (pH 6.0), 10 μl (3500 U) of catalase, 25 μl of 10 m*M* 6-MTHP dissolved in 0.5 *M* ascorbic acid, and 40 μl of enzyme preparation. The reaction was started by the addition of 50 μl of 0 4 m*M* [^{14}C] tyrosine containing ca 120 000 dpm (specific activity 2 7 mCi/mmol) The blank was incubated without 6-MTHP The reaction was stopped after 10 min incubation at 37°C in open vials by the addition of 25 μl of 4 *M* perchloric acid The protein precipitate was removed by centrifugation and a 20-μl aliquot of the supernatant was injected into the liquid chromatograph for on-line radiochemical detection

Chromatography

The HPLC system consisted of an Altex Model 110 A pump (Beckman Instruments, Fullerton, CA, U S A), a Rheodyne Model 7125 injector with 20-μl sample loop (Rheodyne, Cotati, CA, U S A.), and a 5-μm Radial-Pak C_{18} radially compressed column with precolumn in a Z-module (Waters Assoc , Milford, MA, U.S A) The mobile phase consisted of 0 1 *M* sodium phosphate and 1 5 m*M* octane sulphonate (pH 3 0) in 15% methanol The flow-rate was 1 5 ml/min. The detection of non-radioactive compounds was carried out with a Waters Model 441 absorbance detector at 254 nm

The on-line liquid scintillation counting was performed with a Flo-One HS flow-through radioactivity detector (Radiomatic Instruments and Chemicals, Tampa, FL, U.S A) The radioactivity detector was interfaced directly with the HPLC system and the counting was performed using an effluent-to-scintillant ratio of 1 3

The counting efficiency (CE) was estimated by pumping a known amount of distintegrations per minute (dpm) in scintillation fluid through the radioactivity detector, which was also connected to the working HPLC system with a mixing ratio of 3 1. Then, CE = 3/4 × cpm × cell volume (2 5 ml) × 100/dpm.

The quantitation of the ^{14}C-label was achieved simultaneously as net dpm per peak, with results being printed out on the Flo-One printer. The radioactivity signal from the detector was also plotted as a histogram using a strip-chart recorder.

RESULTS AND DISCUSSION

The chromatographic pattern of TH assay using [^{14}C]tyrosine as the substrate is shown in Fig 1. The blank (Fig. 1A) shows that under our

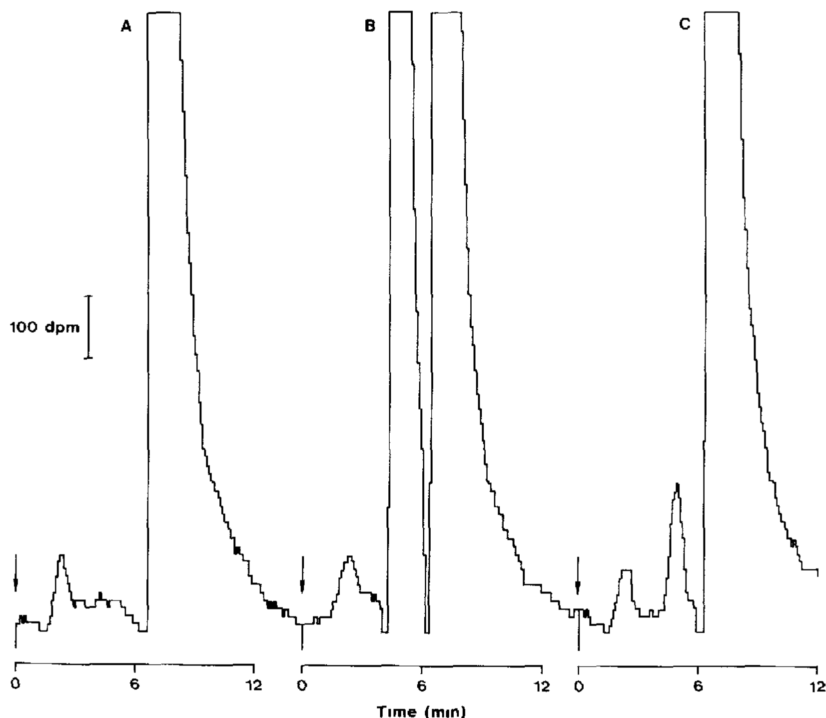

Fig 1 Chromatographic profiles of ^{14}C-labelled reaction products from tyrosine hydroxylase assay using [^{14}C] tyrosine as the substrate (A) Blank, i e reaction mixture without 6-MTHP, (B) standard assay, (C) assay with 50 μM 3-iodotyrosine Chromatographic conditions 5-μm Radial-Pak C_{18} cartridge (100 mm × 5 mm I D) in a Z-module, mobile phase, 15% methanol in 100 mM sodium phosphate buffer (pH 3 0) containing 1 5 mM octane sulphonate, flow-rate, 1 5 ml/min, injection volume, 20 μl, radiochemical detection with sensitivity of 1000 dpm full scale, ^{14}C-counting efficiency, 61%

experimental conditions the enzyme was totally inactive without the addition of the pterin cofactor. The retention time for the product DOPA was 5.1 min, which resulted in baseline separation from the substrate L-tyrosine (Fig. 1B).

When the incubation was performed in the presence of 50 μM 3-iodotyrosine, a specific inhibitor of TH, a 90% inhibition in the enzyme activity was recorded (Fig. 1C). This was further evidence that the peak eluting at 5 1 min was formed from L-tyrosine by the action of TH. Since small molecules were removed by ultrafiltration, it was unnecessary to measure the endogenous concentration of L-tyrosine, which may affect the TH activity determinations in radiochemical assays, where the specific activity of the substrate must be known

The rate of DOPA formation was linear for 10 min (Fig. 2), which resulted in short incubation times. The amount of DOPA increased linearly with protein concentration up to 1 mg (Fig. 3). The reproducibility of the standard assay with 0.8 mg of enzyme protein and a 10-min incubation was 4% (n = 6), expressed as a coefficient of variation This accuracy describes well the properties of HPLC with radiochemical detection to assay enzyme activities. The counting efficiency was 61% for ^{14}C under the described chromatographic conditions Hence, HPLC with radiochemical detection offers about the same sensitivity as conventional liquid scintillation counting. This makes it possible to quantitate 100 dpm per injection, corresponding to 20 pmol of product using [^{14}C]tyrosine (specific acitivity 2.7 pCi/pmol)

The Michaelis—Menten constant (K_M) for bovine adrenal L-tyrosine was found to be 275 ± 62 μM (n = 3), and a V_{max} value of 1.60 ± 0.70 nmol/min/mg protein was calculated Previously reported K_M values for bovine adrenal TH vary from 50 to 170 μM, depending on the degree of purity, the amount of cofactor and the assay method [15—19].

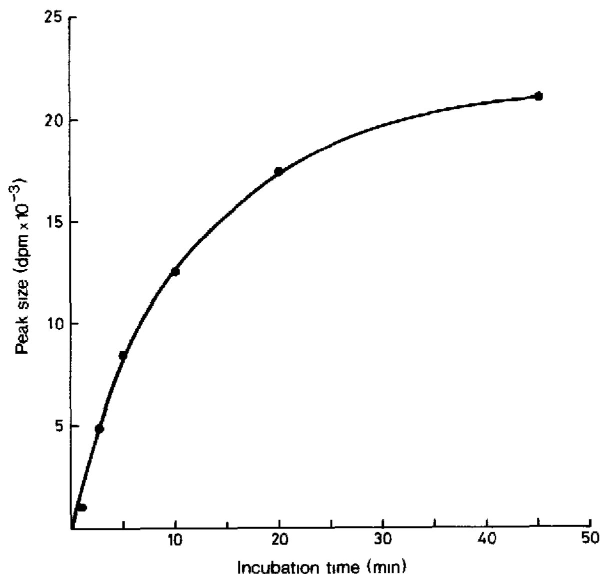

Fig 2 Time course of enzymatic [^{14}C]DOPA formation from 70 μM [^{14}C]tyrosine in the standard incubation mixture containing 0 8 mg of bovine adrenal tyrosine hydroxylase preparation

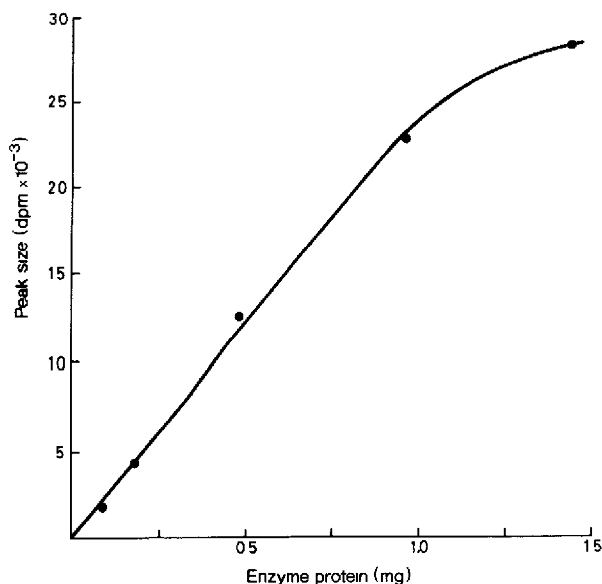

Fig 3 [^{14}C]DOPA formation as a function of enzyme concentration Standard reaction mixtures containing increasing amounts of enzym protein were incubated for 10 min at 37° C

TH can be activated with Fe^{2+} [16] and, consequently, in several assays, Fe^{2+} has been added to boost the TH activity (e.g refs 1, 3, 7 and 18). However, in inhibition studies the catechol analogues may form complexes with Fe^{2+} [20] and thus produce false information One has also to keep in mind that crude enzyme preparations contain other endogenous compounds, such as catecholamines and biopterin, which may interfere with TH assay.

The TH assay method described here does not have these drawbacks It is sensitive enough to study TH activity in adrenals, striatum and other tissues in a well defined incubation mixture. The HPLC—radiochemical assay is more costly than the HPLC—electrochemical assay, but it allows interference-free reliable handling of about 40 samples per day, making it useful for rapid screening and inhibition studies

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