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FLUORIMETRIC ASSAY FOR ORNITHINE DECARBOXYLASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A highly sensitive method for the assay of ornithine decarboxylase in sample solutions prepared from rat tissue homogenate is described which employs high-performance liquid chromatography with fluorescence detection. Putrescine formed from ornithine under the optimal conditions for the enzyme reaction is treated by Cellex P column chromatography for clean-up and converted into the fluorescamine derivative in the presence of cupric ion which inhibits the reaction of interfering amines with fluorescamine. The derivative is separated by reversed-phase chromatography on LiChrosorb RP-18 with linear gradient elution. The lower limit of detection for putrescine formed enzymatically is 5 pmol.

INTRODUCTION

Ornithine decarboxylase (ODC; E.C. 4.1.1.17), one of the enzymes in the polyamine biosynthetic pathway in mammalian tissues, catalyzes the decarboxylation of ornithine to putrescine (Put), and appears to perform an important regulatory function in cell division and growth[1–4]. The activity of ODC in preparations from tissue homogenates is normally very low and so has been assayed radiochemically using [1-^{14}C]ornithine as substrate, the amount of [^{14}C]carbon dioxide evolved enzymatically being measured[5–8]. The method is very sensitive, although any loss of the carbon dioxide formed should be carefully avoided, but the radioactive substrate is expensive and not easy to prepare. The assay of ODC may be achieved by estimation of the Put formed enzymatically. In this case, methods for the determination of di- and polyamines (Put, cadaverine, spermidine and spermine) in biological samples are applicable if their sensitivities are sufficiently high. Many methods have been reported [*e.g.*, enzymatic and immunological methods, ion-exchange chromatography, gas chromatography, gas chromatography–mass spectrometry, thin-layer chromatography and high-performance liquid chromatography (HPLC)], and have been reviewed by Seiler[9] and Bachrach[10]. Among these, HPLC with fluorescence detection seems to be suitable for selective and rapid determination of Put at the picomole level in the presence of many biogenic amines other than the polyamines; here the polyamines are converted into the corresponding 5-dimethylaminonaphthalene-1-sul-

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phonyl[11–13] or fluorescamine[14,15] derivatives before the HPLC separations. HPLC of the fluorescamine derivatives was improved previously in our laboratory to give a simple, simultaneous and highly sensitive determination of the amines in serum[16].

Recently, we found that the reaction of many biogenic amines other than Put, cadaverine and 1,6-hexanediamine (Hda) with fluorescamine is inhibited considerably by cupric ion, which may serve to minimize the interference from those amines. This paper describes a highly sensitive method for the assay of ODC in sample solutions prepared from tissue homogenates. The method is based on the determination of Put, formed from the substrate under the optimum conditions for the enzyme reaction, as its fluorescamine derivative by means of the previously improved HPLC[16] with some modifications and utilizing the above observation on Cu^{2+}. An ODC preparation from rat intestinal mucosa homogenate was employed to establish the assay procedure, and Hda was used as an internal standard.

EXPERIMENTAL

Materials

All solvents and chemicals were of reagent grade.

ODC was prepared from rat intestinal mucosa as follows. Sprague–Dawley male rats (8–10 weeks old) were killed by cervical dislocation. The intestines were quickly removed and placed in saline. The intestinal mucosa was scraped off with a glass-slide, placed in about five volumes of homogenation buffer (20 m*M* phosphate buffer (pH 7.3), containing 0.1 m*M* dithiothreitol, 10 m*M* β-mercaptoethanol and 0.1 m*M* disodium ethylenediaminetetraacetate) and then homogenized with an Omega electric homogenizer at 1200 *g* for 15 min. After centrifugation at 20,000 *g* for 60 min, the supernatant was subjected to protein fractionation with ammonium sulphate. The fraction precipitating between 20 and 80% saturation with ammonium sulphate was collected, dissolved in 10 ml of the buffer and dialized against 2 l of the buffer for 14 h. The above subsequent operations were carried out at 0–4°C. The protein concentration was adjusted to 5 mg per 0.3 ml or less with the buffer, and measured by the method of Lowry *et al.*[17] using bovine serum albumin as a standard protein.

High-performance liquid chromatography

A Mitsumi liquid chromatograph was equipped with a 7120 syringe-loading sample injector, a Hitachi gradient device and a Hitachi 203 spectrofluorimeter fitted with a Hitachi flow-cell unit (cell volume, 20 μl) operating at the emission wavelength of 490 nm and the excitation wavelength of 390 nm. The column (150 × 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size 5 μm; Japan Merck, Tokyo, Japan). The column temperature was 30 ± 0.5°C. A linear gradient elution was carried out between 45 and 80% methanol containing 35 m*M* sodium benzenesulphonate, 0.1 *M* ammonium chloride and 7 m*M* acetate buffer (pH 4.0) during 25 min at a constant flow-rate of 1 ml/min.

Procedure

The incubation mixture consisted of 0.3 ml of the ODC preparation, 0.1 ml of 2 m*M* pyridoxal phosphate, 0.2 ml of 12.5 m*M* dithiothreitol, 0.2 ml of 0.1 *M* phosphate buffer (pH 7.3) and 0.2 ml of 10 m*M* ornithine. It was incubated at 37°C

for 1 h, then 0.1 ml each of 2.0 nmol/ml Hda (as an internal standard) and 3 *M* perchloric acid (to stop the enzyme reaction) were successively added. The resulting mixture was centrifuged at 1200 *g* for 10 min. The supernatant was neutralized with *ca.* 0.2 ml of 1.5 *M* potassium hydroxide, mixed with 0.5 ml of chloroform and 0.2 ml of methanol and then centrifuged at 1200 *g* for 5 min. The aqueous layer (*ca.* 1.6 ml) was applied to a Cellex P column (15 × 0.5 cm I.D.) prepared as described previously[16], and the column was washed successively with 2 ml of 0.01 *M* phosphate buffer (pH 6.0), 1.0 ml of water and 1.0 ml of 0.1 *M* sodium chloride. Then, both Put and Hda were eluted from the column with 1 ml of 0.5 *M* sodium chloride. To the eluate, 0.5 ml of 0.4 *M* borate buffer (pH 9.2) and 0.2 ml of 20 m*M* cupric acetate were added, followed by 0.5 ml of 1.0 m*M* fluorescamine solution in anhydrous acetone with vigorous mixing. After adding 1.0 ml of 0.3 *M* succinic acid, the fluorescamine derivatives were extracted with 1 ml of diethyl ether by shaking for *ca.* 2 min on a Vortex mixer. The mixture was allowed to stand for 2 min. The ether layer was then transferred to a test-tube and mixed with 0.15 ml of 0.4 *M* borate buffer (pH 10). An aliquot (100 μl) of the resulting aqueous layer was injected into the chromatograph.

For the blank, the same procedure was carried out except that incubation was omitted. The amount of Put formed was obtained from the ratio of the net peak height due to Put to that due to the internal standard. ODC activity was expressed as pmol of Put formed per 30 min per mg of protein.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram obtained according to the procedure. Put is completely separated from Hda (internal standard). The small peak 1 is due to the substrate ornithine remaining even after the treatments with the Cellex P column and cupric ion. When the former treatment is omitted, a large peak due to the substrate, which masks that due to Put, is observed. The recoveries of Put and Hda

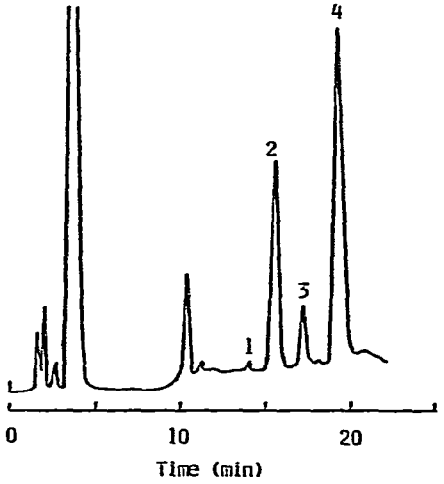

Fig. 1. Chromatogram obtained according to the procedure. Peaks: 1 = ornithine; 2 = putrescine; 3 = cadaverine; 4 = 1,6-hexanediamine (internal standard).

from the Cellex P column were both 96%. Cupric ion has a useful inhibitory effect on the derivatization of the interfering amines with fluorescamine, but not on that of Put, cadaverine and Hda under the present conditions (Table I).

TABLE I

INHIBITORY EFFECT OF CUPRIC ION ON THE REACTION OF BIOLOGICAL AMINES WITH FLUORESCAMINE

To 10 nmol of the amine in a mixture of 1.0 ml of 0.5 *M* sodium chloride and 0.5 ml of 0.4 *M* borate buffer (pH 9.2) mixed with 0.2 ml of 20 m*M* cupric acetate solution or water, 0.5 ml of 1 m*M* fluorescamine solution in acetone were added. The fluorescence intensity was measured with a Hitachi MPF-4 spectrofluorimeter in a 1 × 1 cm cell at 480 nm with the excitation at 394 nm.

Amine	*Inhibition (%)*	*Amine*	*Inhibition (%)*
Tryptamine	100	Lysine	54
Histidine	100	Isoleucine	40
Histamine	98	Spermidine	25
Phenylalanine	98	Agmatine	55
Leucine	95	1,3-Diaminopropane	80
Spermine	95	Cadaverine	0
Ornithine	87	1,6-Hexanediamine	0
Arginine	87	Putrescine	0

Extraction of the fluorescamine derivatives with diethyl ether and the back-extraction of the extract into the borate buffer serve to concentrate the derivatives approximately ten-fold. This gives a sufficient sensitivity to the assay of a low ODC activity. The lower limit of detection for Put formed enzymatically is 5 pmol. The sensitivity is comparable to that of the radiochemical method.

A linear relationship was obtained between the ratio of the peak height of Put to that of the internal standard and the amount of Put added in the range of 5–200 pmol to the assay mixture without incubation. The recovery of Put added to the incubated mixture in amounts of 15–200 pmol was 95 ± 4% (mean ± S.D.).

Substantially all of the ODC (*ca.* 90%) in the supernatant of the intestinal mucosa homogenate is precipitated between 20 and 80% saturation with ammonium sulphate. This fraction was used as the ODC preparation.

ODC is most active at pH 7.0–7.5 in 10–80 m*M* phosphate buffer (Fig. 2); 20 m*M* phosphate buffer (pH 7.3) was used. A maximum and constant activity is obtained in the presence of 0.5–3 m*M* ornithine with the Michaelis constant (K_m) at 0.1 m*M* (Fig. 3); 2 m*M* ornithine was employed in the incubation mixture as saturating concentration for the enzyme reaction. Pyridoxal phosphate, the coenzyme of ODC, at a concentration above 0.1 m*M* in the incubation mixture results in an activity of ODC approximately eight times higher than that in its absence; a concentration of 0.2 m*M* was used in our assay procedure. Dithiothreitol stabilizes the enzyme at a concentration greater than 2 m*M* in the incubation mixture; 2.5 m*M* was used as the optimum. Hda (internal standard) has a weak inhibitory effect on the amount of Put formed when added to the incubation mixture. It was therefore added at the end of the enzyme reaction. The enzyme activity is linear with the time up to at least 60 min, when the mixture containing 5 mg or less protein is incubated at 37°C (Fig. 4).

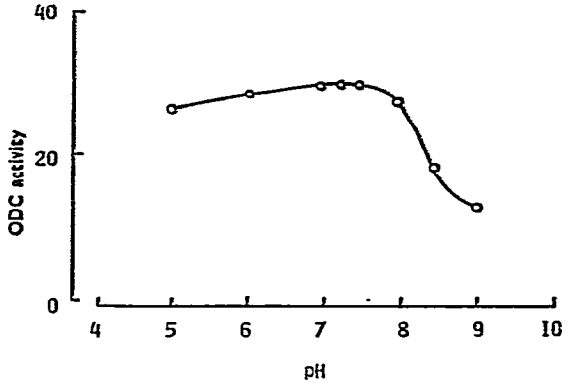

Fig. 2. Effect of pH on ODC activity.

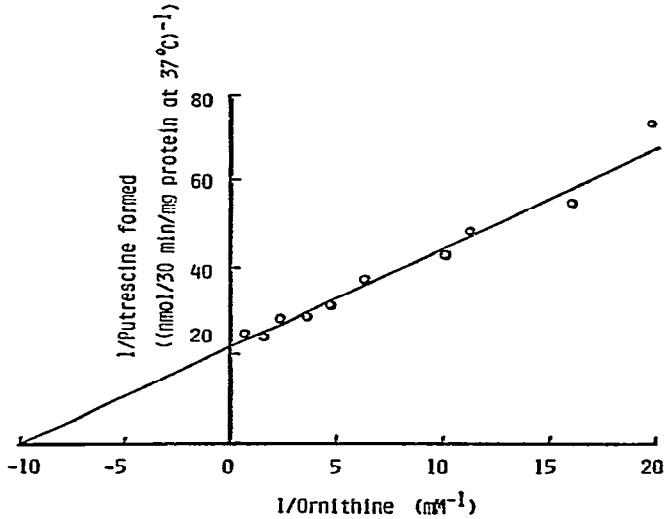

Fig. 3. Lineweaver–Burk plot of the dependence of ODC activity on ornithine concentration.

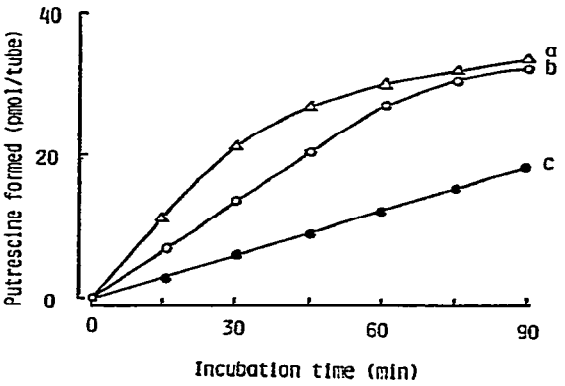

Fig. 4. Effect of incubation time and protein concentration on the amount of putrescine formed. Amount of protein in the incubation mixture: (a) 8; (b) 5; (c) 3 mg.

The precision was established with respect to repeatability. The standard deviation was 1.3 ($n = 20$) for a mean activity of 32 pmol per 30 min per mg protein.

This method also permits the assay of ODC in preparations obtained from other rat tissues, *e.g.*, liver, lung, prostate and brain. This study provides the first HPLC method for the assay of ODC. The method is very sensitive and can be used in place of the radiochemical method.

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