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Note

Rapid and sensitive assay of ornithine decarboxylase activity by high-performance liquid chromatography of the *o*-phthalaldehyde derivative of putrescine

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Ornithine decarboxylase (ODC; L-ornithine decarboxylase, EC 4.1.1.17) is the first and rate-limiting enzyme in polyamine biosynthesis in animal tissues [1]. The enzyme catalyses the decarboxylation of ornithine to yield carbon dioxide and the diamine putrescine, which is further metabolized to the polyamines spermidine and spermine. Although the specific functions of polyamines in animal cells are only partially understood, their ubiquitous distribution, high concentration in cells and increased concentration found in rapidly growing tissues have stimulated many investigations on these compounds and the enzymes involved in their biosynthesis [2]. ODC is involved in the regulation of cell growth and maturation as well as in a number of other cellular metabolic processes [2]. More recently, strong experimental evidence demonstrated that elevated levels of ODC and polyamines play an essential role in tumour promotion [3].

Several methods have been reported for the assay of ODC activity. The conventional and most commonly used method is based on the assay of $^{14}CO_2$ liberated when [1-^{14}C]ornithine is decarboxylated by ODC [4]. Using [^{14}C]ornithine labelled in the 2- or 5-position, Clark [5] measured radioactive putrescine by its chromatographic separation from ornithine on cation-exchange paper. This method has been modified [6] using 0.1 *M* aqueous ammonia to elute ornithine from Whatman P81 cation-exchange paper, leaving putrescine quantitatively behind. An alternative method was described [7] in which ^{3}H-labelled ornithine

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of high specific activity is the substrate, and [^{3}H]putrescine is separated from the substrate on small columns of Bio-Rex 70, a weak cation-exchanger.

The purpose of the present study was to develop a rapid, sensitive and inexpensive assay of ODC activity in which putrescine is assayed by reversed-phase high-performance liquid chromatography (HPLC) after pre-column derivatization with *o*-phthalaldehyde (OPA). The fluorescence detection has a sensitivity comparable with that of the radiochemical assays [4–7], and owing to its simpler experimental approach and lower cost, the method is more suited to the special requirements of multiple analyses. Furthermore, the endogenous content of putrescine in the biological materials tested is obtained at the same time.

EXPERIMENTAL

Materials

L-Ornithine, L-putrescine and OPA were obtained from Sigma (St. Louis, MO, U.S.A.) and 2-mercaptoethanol from Koch-Light (Kolnbrook, U.K.). Other reagents (analytical-reagent grade) were purchased from E. Merck (Darmstadt, F.R.G.).

Animals and induction of ornithine decarboxylase in rat liver

Male Wistar rats (purchased from Møllegaard, Denmark) of 100 g body weight were used. The animals were fed a standard pellet diet (R3 from Ewos, Södertälje, Sweden). The animals were daily given 60 mg of clofibrate (ethyl-*p*-chlorophenoxyisobutyrate) per 100 g of body weight by subcutaneous injection [8] to induce ODC formation in the liver [9].

Preparation of the cytosolic fraction of rat liver homogenates

ODC activity and endogenous putrescine content were assayed in post-microsomal supernatants [10] prepared by homogenization of 1.0 g of liver tissue in a medium (4 ml) consisting of 0.25 *M* sucrose, 10 m*M* sodium phosphate buffer (pH 7.2), 0.2 m*M* pyridoxal 5-phosphate, 5 m*M* dithiothreitol and 0.4 m*M* sodium EDTA. Samples were stored at –80 °C.

Assay of ODC activity

ODC activity was assayed at 37 °C as previously described [11,12], but with the following modifications. The total volume of the incubation mixture was 250 μl, containing 50 m*M* sodium phosphate buffer (pH 7.2), 0.4 m*M* L-ornithine, 0.2 m*M* pyridoxal phosphate, 5 m*M* dithiothreitol and 140 μl of enzyme sample. A 40-μl volume of the reaction mixture was withdrawn at 30-min intervals, and the reaction stopped by perchloric acid at a final concentration of 10% (w/v). After 30 min at 0 °C, the precipitated protein was pelleted by centrifugation at 15 000 *g* for 5 min (Eppendorf Model 5412 Microfuge). The supernatant was neutralized by potassium hydrogen carbonate–potassium hydroxide and centri-

fuged under the same conditions. Samples of the supernatant were derivatized and analysed by HPLC (see below).

Precolumn derivatization of putrescine for HPLC analysis

Samples (20 μl) of the neutralized assay mixture were thoroughly mixed with an equal volume of OPA reagent [13], which contained 50 μg of OPA, 1 ml of methanol, 10 ml of 50 m*M* sodium borate buffer (pH 9.5) and 40 μl of mercaptoethanol (to stabilize the reagent 4 μl of mercaptoethanol were added every third day). After a reaction period of 25 s, 400 μl of the chromatographic solvent (see below) were added to the mixture to stabilize the fluorescent derivative of putrescine (see Results).

High-performance liquid chromatography

Separation of putrescine was performed by HPLC using a ConstaMetric III pump from Laboratory Data Control (Riviera Beach, FL, U.S.A.), a Rheodyne loop injector (Berkeley, CA, U.S.A.) and a filter fluorescence detector with standard filters for OPA (Fluorichrom from Varian Assoc., Walnut Creek, CA, U.S.A.) connected to a Model 3390A reporting integrator from Hewlett-Packard (Avondale, PA, U.S.A.). The column used was an Ultrasphere C_{18} column (15 cm $\times$ 4.6 mm I.D., particle size 5 μm) from Beckman Instruments (Palo Alto, CA, U.S.A.) equipped with a Pelliguard guard column (3 cm $\times$ 2 mm I.D.) packed with pellicular C_8 of 40-μm particle size from Supelco (Bellefonte, PA, U.S.A.). The mobile phase was methanol–0.05 *M* sodium acetate buffer pH 5.9 (70.5:29.5, v/v) and was delivered at a flow-rate of 1.2 ml/min at ambient temperature.

RESULTS

Properties of the OPA derivative of putrescine

The resulting OPA derivative of putrescine was found to be rather labile under the conditions selected for the coupling reaction. The half-life of the derivative was estimated to be ca. 85 s in the dark, and even shorter when exposed to light. To overcome this instability, 400 μl of the HPLC solvent (see above) were added by vigorous shaking at a fixed time (25 s) after mixing samples and OPA reagent, and the mixture was protected from light. Under these conditions the half-life of the putrescine derivative increased to ca. 3 h, and the time of injection was less critical.

Fluorescence properties of the OPA derivative of putrescine

The uncorrected fluorescence excitation and emission spectra of the putrescine derivative of OPA exhibited maxima at 338 nm (λ_{ex}) and 425 nm (λ_{em}). In the standard procedure a filter fluorescence detector was used with excitation at ca. 360 nm (cut-off filter plus band filter) and emission at 420–540 nm (two band filters).

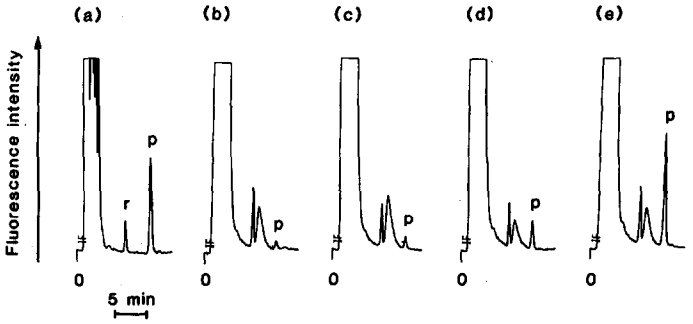

Fig. 1. Reversed-phase chromatographic separation of the fluorescent *o*-phthalaldehyde derivative of putrescine. (a) 21 pmol of putrescine (p) (t_R=11.6 min); r=unidentified compound of the derivatization reagent (t_R=7.6 min); (b,c) chromatograms of the incubation mixture in the assay of ODC activity of a cytosolic fraction from normal rat liver with incubation times of 0 and 90 min, respectively; (d,e) chromatograms of the incubation mixture in the assay of ODC activity of rat liver cytosol after induction of ODC by clofibrate (see Experimental) with incubation times of 0 and 90 min, respectively. The broad peak between the peaks labelled r and p is due to unidentified compounds in the rat liver cytosolic fractions. Volumes of 100 μl of the diluted incubation mixture, containing 5 μl of the cytosolic fraction, were injected into the liquid chromatograph.

Chromatographic conditions

At a flow-rate of 1.2 ml/min, the retention time (t_R) of the OPA derivative of putrescine was 11.6 min (Fig. 1) and the capacity factor (k') was 3.6. The capacity factor and retention time decreased with increasing methanol concentration, which also decreased the resolution of putrescine from OPA derivatives of other compounds in the cytosolic extracts of rat liver. Some of the non-polyamine fluorescence peaks, eluted as broad, unresolved peaks in the range 1.3 min $< t_R <$ 3.4 min and a peak at 7.6 min were assigned mainly to the chemicals of the derivatization mixture (Fig. 1a). The standard curves obtained in each analytical series were linear (r=0.99) in the range 0.5–50 pmol putrescine. The coefficient of variation of the retention time was less then 1%. The detection limit of putrescine was ca. 0.5 pmol, assuming a limiting value of 3 for the signal-to-noise ratio. Chromatograms of cytosolic extracts of rat liver revealed, in addition to the components derived from the derivatization mixture, a broad peak (t_R=8.6 min) of unidentified material in front of, but well separated from, the peak of putrescine (Fig. 1b–e).

A major advantage of the selected filter detector was a stable baseline even at high sensitivity.

Assay of ODC activity in rat liver cytosolic fractions

When the ODC activity was assayed in rat liver cytosolic fractions (Fig. 1b–e), the putrescine peak was found to vary over a wide concentration range depending on the level of ODC activity. The ODC activity increased 39-fold on induction by the tumour-promoting agent clofibrate, i.e. from 1.60 ± 0.4 pmol/min per mg of protein (mean ± S.D., n=8) in the control animals (Fig. 1b and c) to 62.6 ± 20.4 pmol/min per mg of protein in the clofibrate-treated animals (Fig. 1d and e).

The difference in the endogenous content of putrescine in the rat liver cytosolic fractions of the two groups of animals is also clearly seen (Fig. 1b and d).

DISCUSSION

The concentration of the fluorescent OPA derivative of putrescine was conveniently measured by HPLC in cytosolic fractions of rat liver. The method offers several advantages over those published previously [4–7]. No radioisotopes are required and analytical-grade reagents can be used. The method is also faster than, for example, the traditional assay of $^{14}CO_2$. Furthermore, the side-reaction by which $^{14}CO_2$ is formed from [1-^{14}C]ornithine by a transamination reaction is controlled. Compared with the methods using ^{14}C-labelled putrescine, it also has the added advantage of monitoring the endogenous putrescine level in the cellular extracts (Fig. 1b and d), which is often required, e.g. in studies of the induction of ODC and modulation of polyamine metabolism [9]. It is also more specific than the cation paper methods [6], because it also measures the labelled spermidine and spermine formed by the biotransformation of putrescine. HPLC offers a better resolution than the traditional column chromatography [7]. Although the OPA derivative of putrescine is unstable, its very slow decay under the delected experimental conditions ($t_{1/2}$ ca. 3 h) does not cause any problems in the assay.

The lower limit of detection was found to be ca. 0.5 pmol (applied to the column) at a signal-to-noise ratio of 3, which is ten times lower than the value of 5 pmol reported for the filter paper method of Djurhuus [6].

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