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Note

Rapid and sensitive assay of tyrosine 3-monooxygenase activity by high-performance liquid chromatography using the native fluorescence of DOPA

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Recent studies on the biosynthesis of catecholamines in the nervous system and chromaffin cells of the adrenal medulla have called for a sensitive assay of tyrosine 3-monooxygenase (tyrosine hydroxylase, E.C. 1.14.16.2) activity, *i.e.*, the first and probably rate-limiting step of the pathway [for a review, see ref. 1]. Radiochemical methods, using either ^{14}C- or ^{3}H-labelled L-tyrosine as the substrate, have generally been considered the most accurate[2], but high-performance liquid chromatography (HPLC) with electrochemical detection has more recently been found convenient for the assay of dihydroxyphenylalanine (DOPA)[3,4]. Owing to the inherent problems of lifetime and maintenance of the electrochemical detector[5], we have based our detection of biogenic amines on measurement of their native fluorescence using a sensitive spectrofluorimeter equipped with a 20-μl flow-through cell[6–9]. Although the native fluorescence of DOPA has generally been considered too weak for the assay of tyrosine 3-monooxygenase activity in biological material[10], we have found it very useful in combination with HPLC. In the present study it is shown that the assay of DOPA by HPLC and fluorescence detection gives a sensitivity comparable to that of the electrochemical detector[3,4] and, owing to its simpler experimental approach, our method can more easily be applied to automated analyses to meet the special requirements of multiple analyses. Further, the method requires no particular maintenance, as is the case with the electrochemical detector. Finally, the published HPLC procedures for the assay of tyrosine hydroxylase activity have been found to be unsatisfactory in one or more of the following respects: (1) the procedures are laborious and time consuming; (2) they cannot be applied to crude biological materials owing to interference from endogenous substances of that material, notably catecholamines; and (3) they require preliminary clean-up of the sample before HPLC analysis can be performed.

EXPERIMENTAL

Materials

L-Tyrosine was obtained from Koch-Light (Colnbrook, Great Britain) and benzyloxyamine (*o*-benzylhydroxylamine hydrochloride), 6,7-dimethyltetrahydropterin and 2-(N-morpholinoethane)sulfonic acid (MES) from Sigma (St. Louis, MO, U.S.A.). Other reagents (analytical-reagent grade) were supplied by E. Merck (Darmstadt, G.F.R.).

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Subcellular fractions of bovine adrenal homogenates were prepared by centrifugation in 0.25 *M* sucrose at 0–5°C[11].

HPLC analysis

The high-performance liquid chromatograph with a fluorescence detector and integrator (Hewlett-Packard Model 3380A) has previously been described in detail[6–8]. The chromatographic separation was achieved at ambient temperature on a sulphonated fluorocarbon polymer coated on a pellicular silica support (Zipax SCX from DuPont, Wilmington, DE, U.S.A., dry-packed in a 50.0 cm × 3 mm I.D. stainless-steel tube) with a theoretical plate number of about 1000/m. A short precolumn (40 × 2 mm I.D. stainless-steel tube) packed with pellicular silica (HC Pellosil from Whatman, Maidstone, Great Britain) was used to protect the cation-exchange column. The mobile phase, consisting of 10 m*M* acetate buffer (pH 3.70) with 1% (v/v) of propanol, was pumped at a flow-rate of 1.5 ml/min (890 p.s.i.).

Assay of tyrosine 3-monooxygenase activity

Tyrosine 3-monooxygenase activity was assayed at 30°C as described by Nagatsu[2], with the following modifications. The total volume of the incubation mixture was 100 μl, containing 40 m*M* MES buffer (pH 6.0), 0.3 m*M* L-tyrosine and 0.1 m*M* benzyloxyamine in order to inhibit any aromatic-L-amino-acid decarboxylase (DOPA decarboxylase, E.C. 4.1.1.28) activity present in the crude enzyme preparation[12].

The reaction was stopped after 10–20 min by the addition of an equal volume of ice–cold ethanol, containing glacial acetic acid to pH 4.1. This mixture was allowed to stand for 30 min in an ice–water bath, after which it was centrifuged at 10,000 rpm for 5 min (Eppendorf Model 5412 Microfuge). Samples (10–50 μl) of the supernatant were injected directly into the liquid chromatograph.

Preparation of standards for HPLC analyses

Standards of L-tyrosine and DOPA were dissolved in 10 m*M* acetate buffer (pH 3.70) (mobile phase solvent), and their concentrations were determined spectrophotometrically using the known molar absorptivities, *i.e.*, $\varepsilon = 1.42$ mmol l^{-1} cm^{-1} at 274.5 nm[13] and $\varepsilon = 2.63$ mmol l^{-1} cm^{-1} at 280.0 nm[14] for tyrosine and DOPA, respectively.

Protein determination

Protein was determined according to the procedure described by Bradford[15].

RESULTS

Fluorescence properties of L-tyrosine and DOPA

The uncorrected fluorescence excitation and emission spectra of DOPA and L-tyrosine were obtained by injection of the compounds directly into the flow-through cell of the spectrofluorimeter. The spectrum of DOPA ($\lambda_{ex} = 281$ nm and $\lambda_{em} = 314$ nm) and of tyrosine ($\lambda_{ex} = 274$ nm and $\lambda_{em} = 304$ nm) were obtained with very little contribution from the solvent.

Chromatographic conditions

A sulphonated fluorocarbon polymer coated on pellicular silica support was selected for the separation of L-tyrosine and DOPA using 10 m*M* sodium acetate buffer (pH 3.70) with 1% (v/v) of propanol as the mobile phase. Fig. 1a shows that the chromatographic conditions selected allow the complete separation of DOPA (retention time, t_R = 1.03 min) from L-tyrosine (t_R = 1.55 min), and this separation was obtained even in the presence of a 100-fold higher concentration of L-tyrosine. Noradrenaline and adrenaline were eluted in this system with long retention times of *ca.* 10 and 40 min, respectively.

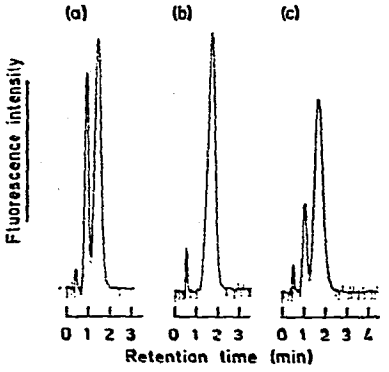

Fig. 1. (a) Chromatogram of 384 pmol of DOPA (t_R = 1.03 min) and 1.92 nmol of L-tyrosine (t_R = 1.55 min). (b) and (c) chromatograms of an acidic ethanol extract of an incubation mixture in the assay of tyrosine 3-monooxygenase activity of bovine adrenal medulla microsomes; (b) zero-time control with a single peak of L-tyrosine (t_R = 1.55 min), and (c) the formation of DOPA (t_R = 1.03 min) following a reaction period of 20 min. Volumes of 20 μl of the diluted (twice) incubation mixture were injected into the liquid chromatograph; λ_{ex} = 281 nm and λ_{em} = 314 nm.

A linear relationship was obtained between the amount of DOPA injected and the integrated peak area (r = 0.99) or the peak height (data not shown). The limit of detection was about 5 pmol of DOPA (signal-to-noise ratio = 3).

Assay of tyrosine hydroxylase activity in subcellular fractions of the bovine adrenal medulla

The useful application of HPLC to the assay of DOPA in crude biological material is shown by the assay of tyrosine 3-monooxygenase (tyrosine hydroxylase E.C. 1.14.16.2) activity in subcellular fractions of the bovine adrenal medulla. From Fig. 1c it can be seen that about 3.48 nmol min^{-1} of DOPA per milligram of protein is formed when the microsomal fraction is the enzyme source. All subcellular fractions revealed tyrosine 3-monooxygenase activity, but the specific activity was found to be highest in the microsomal fraction (data not shown).

DISCUSSION

HPLC with electrochemical detection has recently been introduced for the rapid and sensitive assay of DOPA formed in the tyrosine 3-monooxygenase (tyrosine

hydroxylase, E.C. 1.14.16.2) reaction[3,4]. However, owing to the non-selectivity of this detector, working with crude extracts of biological materials, as well as the rapid deterioration of the electrode and problems with its maintenance[5], an alternative method for the detection of biogenic amines has been developed in this laboratory[6–9]. In this study it was found that the native fluorescence of DOPA is sufficiently strong for its detection by HPLC at acidic pH, in good agreement with our recent finding for catecholamines[6–8].

The main advantages of HPLC with fluorometric detection are its sensitivity, higher selectivity, reliability and precision[6–9]. With DOPA, the limit of detection is about 5 pmol at a signal-to-noise ratio of 3, which is comparable to that of electrochemical detection with a reported lower limit of about 5 pmol of DOPA formed enzymically[4]. Fluorescence detection is, however, more specific than electrochemical detection, as DOPA has unique fluorescence properties in the UV region. As already stressed[6–9], the fluorimetric detector has no problems with maintenance and is particularly useful in combination with an automatic injector. As in most HPLC techniques, the present method for the assay of DOPA has a high precision (better than $\pm 1\%$ relative standard deviation). The short time required for analysis and the simple procedure generally followed make the method very convenient for routine analysis and automation.

The chromatographic system selected is a modification of that described by Blank and Pike[3]. Using the original perchlorate solvent system of Blank and Pike[3] and a flow-rate of 1.5 ml/min, we found considerable interference in the assay of DOPA ($t_R = 1.01$ min) by catecholamines [noradrenaline (NA) and adrenaline (A)] present in the biological material being analysed [t_R (NA) = 0.89 min and t_R (A) = 2.13 min]. However, by using the acetate buffer as the solvent system, the retention times were 1.03 min (DOPA), *ca.* 10 min (NA) and *ca.* 40 min (A). Further, the acetate solvent system also increases the lifetime of the column.

It should be mentioned that the sensitivity of this HPLC assay of DOPA may be increased even further, *e.g.*, by using a flow cell of slightly larger volume and surface area, with only a slight loss in chromatographic resolution. Finally, the cost of this method, in terms of reagents and work, is lower than that for any assay of tyrosine 3-monooxygenase activity published so far.

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