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Review

Application of high-performance liquid chromatography to the study of biogenic amine-related enzymes

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ABSTRACT

The application of high-performance liquid chromatography to the study of biogenic amine-related enzymes is reviewed. Biogenic amines include catecholamines (dopamine, norepinephrine and epinephrine), indoleamines (serotonin and melatonin), imidazoleamines (histamine), polyamines (putrescine, spermidine and spermine) and acetylcholine. Three particular aspects are covered. The first aspect is the assay of enzyme activities of biogenic amine-related enzymes, such as tyrosine hydroxylase, tryptophan hydroxylase, aromatic L-amino acid decarboxylase, dopamine β-hydroxylase and phenylethanolamine N-methyltransferase. The introduction of highly sensitive assays of biogenic amines with electrochemical detection or fluorescence detection have made possible the non-isotopic assay of these activities, replacing the previously used radioisotopic methods. The second aspect is the purification of these enzymes. Since biogenic amine-synthesizing enzymes are generally unstable, rapid and efficient purification of these enzymes is very useful. The third aspect is the assay of biogenic amines (for example, acetylcholine and polyamines) using post-column derivatization with biogenic amine oxidases and electrochemical detection.

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1. INTRODUCTION

Biogenic amines include catecholamines (dopamine, norepinephrine and epinephrine), indoleamines (serotonin and melatonin), imidazoleamines (histamine) and polyamines (putrescine, spermidine and spermine) (Fig. 1). Acetylcholine is also included in the biogenic amines. Biogenic amines are neurotransmitters, hormones or biomodulators, with a wide range of potent biological activities, and therefore they are important in health and disease states.

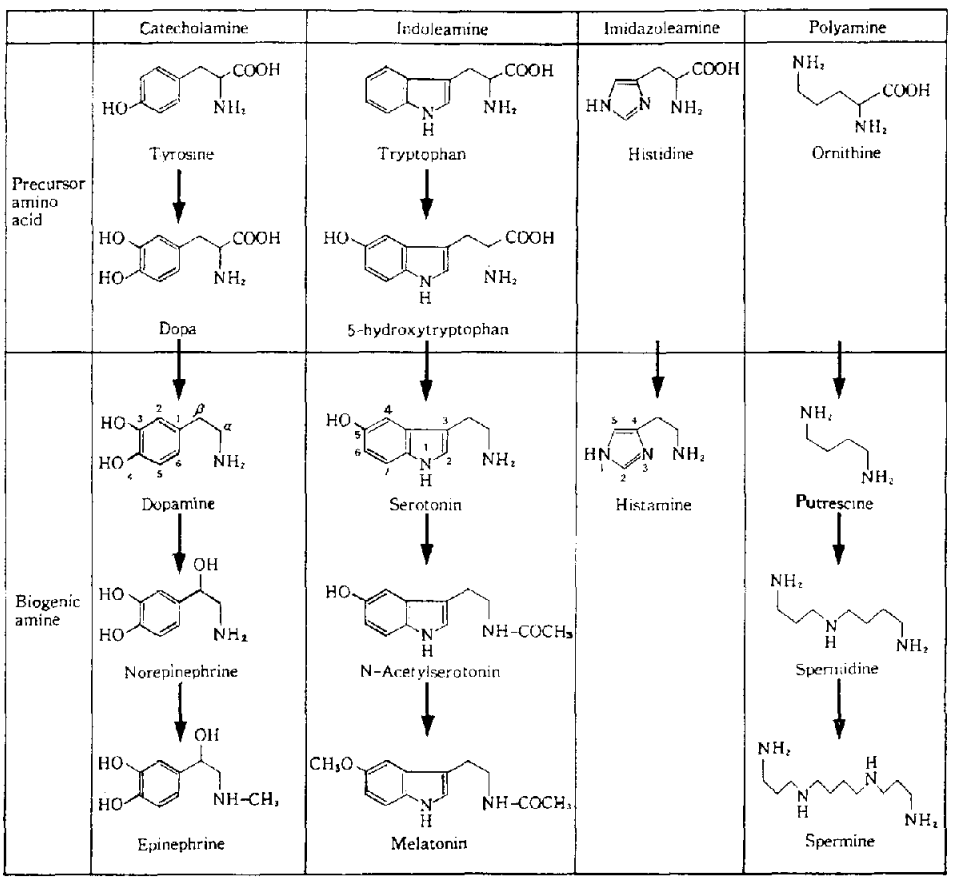

Fig. 1. Biosynthetic pathway of biogenic amines from their precursor amino acids.

As shown in Fig. 1, biogenic amines are synthesized from the precursor amino acids: tyrosine (for catecholamines), tryptophan (for indoleamines), histidine (for imidazoleamines) and ornithine (for polyamines).

There are three particular applications of high-performance liquid chromatography (HPLC) to the study of biogenic amine-related enzymes.

The first application is the assay of enzyme activities [1] of biogenic amine-related enzymes. The activities of biogenic amine-synthesizing enzymes are generally low and were originally measured only by sensitive radioisotopic assays using radiolabelled substrates. However, since 1975 [2–4], the introduction of highly sensitive HPLC assays of biogenic amines with electrochemical detection (ED) or fluorescence detection (FD) has made possible the non-isotopic assay of these enzyme activities [5,6].

The second application of HPLC to biogenic amine-related enzymes is their purification. Biogenic amine-synthesizing enzymes are generally unstable, but their rapid and efficient purification is now possible by HPLC.

The third application is the assay of biogenic amines by HPLC–ED using post-column derivatization with biogenic amine oxidases, such as immobilized polyamine oxidase.

2. ENZYMES RELATED TO BIOGENIC AMINES

The formation of biogenic amines from amino acids (except acetylcholine) is catalysed by aromatic L-amino acid decarboxylase (AADC, for catecholamines and indoleamines), histidine decarboxylase (for imidazoleamines) or ornithine decarboxylase (for polyamines). In the case of the biosynthesis of catecholamines or indoleamines, tyrosine or tryptophan is first transformed by tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH) to the corresponding hydroxyamino acid, 3,4-dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan, which is a better substrate and is preferentially decarboxylated by AADC to dopamine or serotonin.

In the case of catecholamines, dopamine is further transformed to norepinephrine by dopamine β-hydroxylase (DBH), and then to epinephrine by phenylethanolamine N-methyltransferase (PNMT). In the case of indoleamines, serotonin is the biologically active amine, but only in the pineal gland is serotonin further N-acetylated by N-acetyltransferase to N-acetylserotonin, and finally O-methylated to melatonin by hydroxyindole O-methyltransferase.

In the case of imidazoleamines, histidine is directly decarboxylated by histidine decarboxylase to histamine, which is the bioactive amine.

The first polyamine, putrescine, is directly formed from ornithine by ornithine decarboxylase, which is converted into spermidine and then into spermine by spermidine synthetase and spermine synthetase, respectively.

The biodegradation (inactivation) of biogenic amines is similar for each amine; mainly deamination by amine oxidase, methylation by O- or N-methyl-

transferase, or acetylation by N-acetyltransferase. Catecholamines and serotonin are oxidatively deaminated by monoamine oxidase type A and type B. Catecholamines are also inactivated by catechol O-methyltransferase. Imidazoleamine, *i.e.* histamine, is mainly inactivated by histamine N-methyltransferase to form N^{τ}-methylhistamine, which is then oxidized by monoamine oxidase. Histamine is also directly deaminated to imidazoleacetaldehyde by diamine oxidase (histaminase). Polyamines are either oxidized by polyamine oxidase or acetylated by N-acetyltransferase. N^1-Acetylspermidine or N^1-acetylspermine is oxidized back to putrescine or spermidine, respectively, by polyamine oxidase. Therefore, the reverse biosynthetic pathway exists in polyamines: from spermine via spermidine to putrescine.

3. ASSAY OF THE ACTIVITY OF BIOGENIC AMINE-RELATED ENZYMES BY HPLC

3.1. General analytical considerations

The activity of an enzyme can be measured either by the rate of disappearance of a substrate or by the rate of appearance of a product formed by the enzyme reaction. Various analytical methods are available for the assay of substrates or products [1]. HPLC was introduced around 1975 for the assay of the activity of biogenic amine-synthesizing enzymes: for example, for the assay of the activities of TPH [2] and DBH [3]. The main advantage is the high sensitivity (femtomole level) and high specificity of HPLC, especially HPLC–ED or HPLC–FD. Both techniques make it possible to measure the extremely low activities of biogenic amine-synthesizing enzymes, which was previously possible only by sensitive radioassays. Chromatographic–photometric measurements of a product in an enzyme reaction have been used for many years. However, the major drawback of the open-column (gravity flow) chromatographic method was the excessively long analysis time. The introduction of HPLC has reduced the time form several hours to several minutes. The combined use of an auto-sampling apparatus and an automatic data processor makes possible the unattended measurement of several tens of samples during several hours. The enzyme activities in microgram amounts of punched brain nuclei and microlitre amounts of body fluids such as blood (plasma, serum) or cerebrospinal fluid (CSF) can be easily measured by HPLC. For example, the very low activity of dopamine β-hydroxylase was found in CSF by HPLC-FD and HPLC–ED [4].

The volume of the incubation mixture for HPLC assay of enzyme activity should be kept as small as possible, to obtain high sensitivity. Since the sample injection volume for HPLC is generally up to 100 μl, the volume of an incubation mixture is frequently between 50 and 1000 μl. Recently, microbore columns (internal diameter less than 1–2 mm) have been used to increase the sensitivity of ED. The detection limit for catecholamine using microbore HPLC–ED is *ca.* 50 fg, whereas in conventional HPLC the detection limit is *ca.* 5 pg. When microbore HPLC–ED is used for enzyme assay, the volume of the incubation mixture can be as small as 10 μl.

The concentrations of substrate and cofactors should be optimal. Since saturation concentrations of substrates can seldom be used in the radioisotopic methods, this is an advantage of the HPLC methods. When a high concentration of a substrate interferes with the peak of the product to be assayed, the substrate may be removed in a preliminary separation step. Either solvent extraction or rapid column chromatography is frequently used to remove excess substrate and to isolate the product.

Since enzyme assay by HPLC is highly sensitive but not completeley specific, the choice of the blank (control) is important for the detection of the blank peak due to non-enzymic reaction. ED is highly sensitive for catecholamines and their metabolites, but less specific. FD, particularly with either derivatization with diphenylethylenediamine for dopamine, norepinephrine and epinephrine or the trihydroxyindole reaction for norepinephrine and epinephrine, is highly specific and even more sensitive than ED. The following blanks (controls) are generally used: (1) incubation with the inactive stereoisomer of the substrate, if the enzyme can recognize stereospecificity of the substrate (TH, TPH and AADC); (2) incubation with a specific enzyme inhibitor (3-iodo-L-tyrosine for TH and fusaric acid for DBH); (3) incubation without the enzyme and its addition after incubation; (4) incubation with the boiled, inactivated enzyme; and (5) incubation without a substrate and its addition after incubation.

The HPLC conditions should be chosen to give complete separation of the product of the enzyme reaction from any interfering substances. Bonded-phase columns are frequently used. If possible, the HPLC conditions should be adjusted so that the product to be assayed is eluted before the substrate, thus preventing the large peak of the substrate from interfering with the assay of the product.

ODS (octadecylsilyl) reversed-phase columns are frequently used for the assay of biogenic amine-related enzymes, and the column lifetime is generally several hundred samples. Use of a short guard column, which contains a stationary phase equivalent to that in the analytical column and is inserted between the injection valve and the analytical column, prolongs the life of the analytical column.

The internal standard should be eluted shortly before or after the product under investigation. The amount of product formed enzymically in the incubation mixture is usually calculated from the peak heights (or areas) by the equation:

$$\frac{R(\mathrm{E}) - R(\mathrm{B})}{R(\mathrm{B} + \mathrm{S}) - R(\mathrm{B})} \times \text{(amount of standard added to a blank incubation)}$$

where R is the ratio of peak heights of the product and the internal standard, $R(\mathrm{E})$ being that from the enzyme incubation, $R(\mathrm{B})$ that from the blank incubation, and $R(\mathrm{B} + \mathrm{S})$ that from the blank incubation plus the product added as standard.

3.2. Tyrosine hydroxylase

TH [tyrosine 3-monooxygenase; EC 1.14.16.2; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating)], which is a pteridine-requiring monooxigenase, catalyses the first step in the biosynthesis of catecholamine [7–9]:

L-tyrosine + O_2 + tetrahydropteridine → 3,4-dihydroxy-L-phenylalanine (L-DOPA) + quinonoid dihydropteridine + H_2O

TH couples *in vivo* with dihydropteridine reductase (Fig. 2). Dihydropteridine reductase regenerates tetrahydropteridine on reaction with NADH:

quinonoid dihydropteridine + NADH + H^+ → tetrahydropterine + NAD^+

Either NADH or NADPH is effective as an electron donor *in vitro*, but dihydropteridine reductase *in vivo* is the NADH form [10].

(6*R*)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin is the natural pteridine cofactor.

The low activity of TH has necessitated the use of the radioisotopic method [7–9]. Blank and Pike [11] first reported an assay for TH by HPLC–ED [12,13].

We have combined the simple and specific isolation of enzymically formed DOPA by our double-column procedure (the top column is of Amberlite CG-50 and the bottom column of alumina) with the highly sensitive assay for DOPA by HPLC–ED. α-Methyldopa is added as an internal standard to each sample after TH incubation. The combined use of the double-column system and HPLC permits nearly complete isolation of DOPA, and thus the blank values are very low. However, the Amberlite CG-50 column can be omitted in routine TH assays, and only the alumina column need be used. The only interfering substance is endoge-

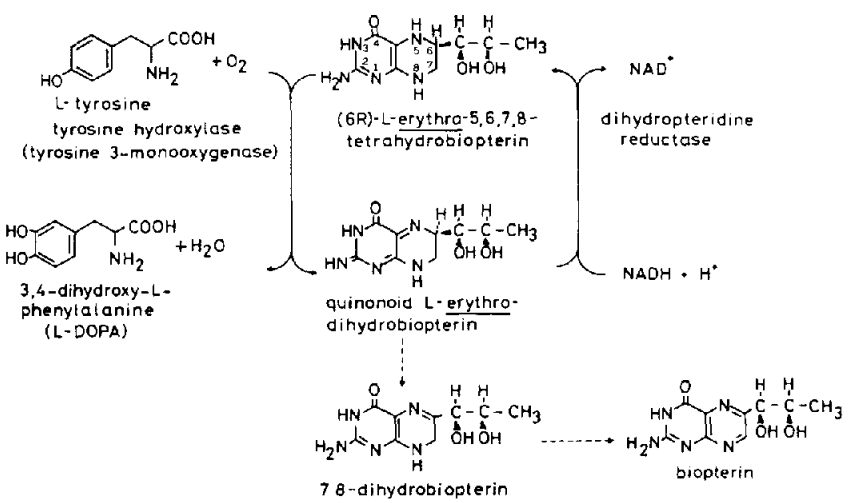

Fig. 2. Reaction of tyrosine hydroxylase (TH).

nous DOPA in crude tissues and non-enzymically formed DOPA from both L- and D-tyrosine, and this blank value can be completely cancelled by use of a control with D-tyrosine (as substrate) plus 3-iodo-L-tyrosine (an enzyme inhibitor). The use of α-methyldopa as an internal standard makes the assay very accurate. TH activity in less than 1 mg of a brain nucleus can be assayed by this method. Inhibition of AADC (*e.g.*, by *p*-bromobenzyloxyamine, NSD-1055) is not required at the optimal pH for TH (6.0).

Naoi *et al.* [14] devised a simple assay procedure for TH that requires minimal sample preparation using HPLC with coulometric ED. The incubation mixture was deproteinized, and the supernatant was directly applied to the chromatographic apparatus connected to a coulometric electrochemical detector. The voltage of a conditioning cell was set at +250 mV and those of the first and second electrode of an analytical cell were set at +50 mV and −300 mV, respectively, and the output of the second electrode was monitored. A reversed-phase column (250 mm × 4.6 mm I.D.) (mobile phase, 90 m*M* sodium acetate, 35 m*M* citric acid, 130 μ*M* disodium EDTA and 230 μ*M* sodium *n*-octanesulphonate, containing 10.5% methanol) was connected to a precolumn (50 mm × 4.6 mm I.D.).

The standard incubation mixture (0.10 ml) contains (final concentrations in parentheses), in order of addition: 0.02 ml of 1 *M* sodium acetate buffer (0.2 *M*), 0.01 ml of 1 mg/ml catalase (10 μg), 0.01 ml of 10 m*M* ferrous ammonium sulphate (1 m*M*), 0.03 ml of water plus TH, 0.01 ml of 1 *M* mercaptoethanol (0.1 *M*) plus 10 m*M* 6-methyltetrahydropterin or 10 m*M* (6*R*)-L-*erythro*-tetrahydrobiopterin (0.1 m*M*), and 0.02 ml of 1 m*M* L-tyrosine (0.2 m*M*). For the blank reaction, 0.02 ml of 1 m*M* D-tyrosine (0.2 m*M*) and 0.01 ml of 1 m*M* 3-iodo-L-tyrosine (0.1 m*M*) are used instead of L-tyrosine. L-DOPA (50 pmol) is added to another blank incubation as a standard. The reaction is started by the addition of L-tyrosine, is carried out in an open test-tube at 30°C for 10 min (the reaction is linear for 15 min), and is stopped by adding 0.30 ml of 0.05 *M* perchloric acid containing 50 pmol of L-α-methyldopa as an internal standard.

In the TH reaction mixture, the enzymically formed quinonoid dihydropteridine is reduced to tetrahydropteridine either by a coupled enzymic reaction with dihydropteridine reductase and NAD(P)H, or by chemical reduction with mercaptoethanol, dithiothreitol or ascorbic acid. The order of TH activity is ascorbic acid > mercaptoethanol > dithiothreitol. Ascorbic acid gives the highest TH activity, but frequently interferes with the DOPA peak in HPLC. Therefore, mercaptoethanol is most frequently used for chemical reduction of quinonoid dihydropteridine and also to protect the tetrahydropteridine cofactor in the TH incubation mixture.

The natural pteridine cofactor is (6*R*)-L-*erythro*-tetrahydrobiopterin. The use of this naturally occurring cofactor is preferable, especially for kinetic studies, but (6*RS*)-L-*erythro*-biopterin gives kinetic results similar to those obtained with the (6*R*) form. It should be noted that oxygen at the level found in air is inhibitory *in vitro* with (6*R*)- or (6*RS*)-tetrahydrobiopterin as cofactor, but not with unnatural

(6*S*)-tetrahydrobiopterin and 6-methyl- or 6,7-dimethyltetrahydropterin. For most routine assays of TH activity, either (6*RS*)-6-methyltetrahydropterin or (6*RS*)-6,7-dimethyltetrahydropterin is used. (6*RS*)-Methyltetrahydropterin is structurally similar to the natural tetrahydrobiopterin and gives higher activity than (6*RS*)-6,7-dimethyltetrahydropterin (Fig. 3).

An example of the HPLC assay of TH activity in human brain homogenate (grey matter and white matter) is shown in Fig. 4. It is shown that the TH activity with (6*R*)-tetrahydropterin as cofactor in the grey matter is higher than that in the white matter: 4.68 ± 0.17 and 0.38 ± 0.02 pmol/min per mg total protein for grey and white matter homogenate, respectively [14].

TH assay can also be performed by using HPLC–FD, either by detection of native fluorescence of DOPA at 314 nm with excitation at 281 nm [15] or by pre-column derivatization with diphenylethylenediamine [16]. In the latter method [16], DOPA was isolated from the reaction mixture with small cartridge columns of alumina and a cation exchange, and converted into the corresponding fluorescent compound by reaction with diphenylethylenediamine. The fluorescent derivative was separated by reversed-phase chromatography.

3.3. Tryptophan hydroxylase

TPH (tryptophan 5-monooxygenase; EC 1.14.16.4) catalyses the formation of L-5-hydroxytryptophan from L-tryptophan, the initial step in the biosynthesis of serotonin in serotonergic neurons and of melatonin in the pineal gland. The enzyme was found in the brain of various mammals, such as rats, rabbits, guinea-pigs and dogs, by various assay procedures: radioisotopic methods [17,18] and a fluorometric method [19]. We developed a highly sensitive HPLC–FD assay for

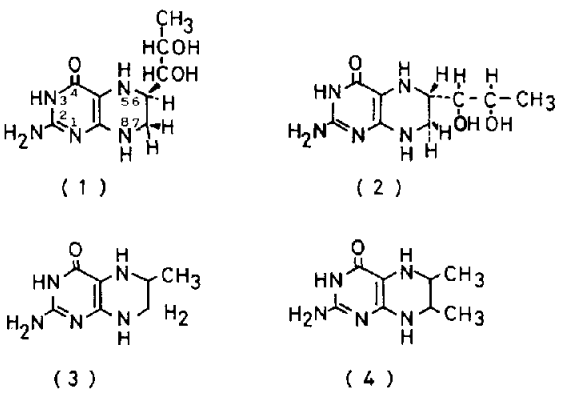

Fig. 3. Structures of the pteridine cofactors used for tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) assays: (1) (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BPH_4), the natural pteridine cofactor; (2) (6*S*)-L-*erythro*-tetrahydrobiopterin, the unnatural form; (3) (6*RS*)-methyl-5,6,7,8-tetrahydropterin, a synthetic cofactor; (4) (6*RS*)-6,7-dimethyl-5,6,7,8-tetrahydropterin, a synthetic cofactor.

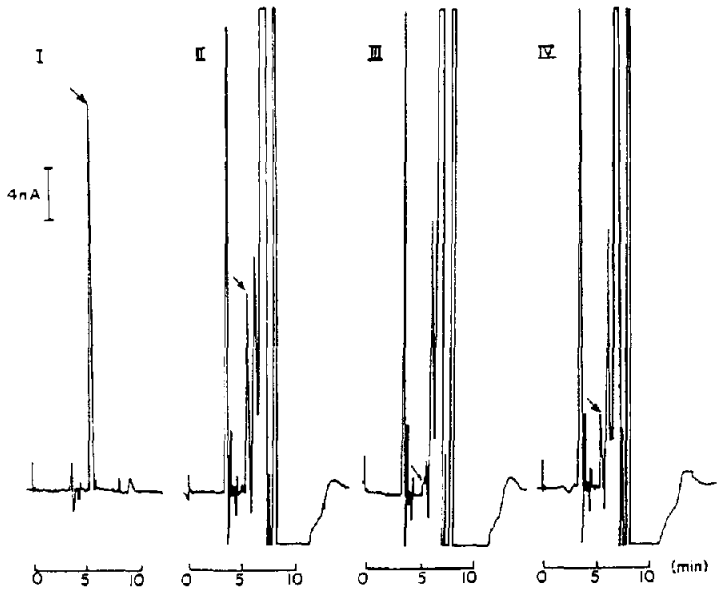

Fig. 4. Chromatograms of the reaction mixture of grey matter homogenates with L- or D-tyrosine. Grey (350 μg of protein) and white matter (450 μg of protein) homogenates were incubated at pH 6.0 with 0.2 m*M* L- or D-tyrosine at 37°C for 10 min. The filtered sample (1 μl) was applied to the HPLC apparatus, and the HPLC conditions were as described in the text. (I) Standard L-DOPA (1 pmol); (II) reaction product of grey matter homogenates in the complete reaction mixture; (III) grey matter homogenates after incubation in the reaction mixture minus L-tyrosine; (IV) reaction product of white matter homogenates (Reprinted from ref. 14 with permission.)

TPH activity based on the determination of 5-hydroxytryptophan enzymically formed from L-trytophan. Detection limits of 20 pg (*ca.* 100 fmol) of 5-hydroxytryptophan were obtained by measuring the native fluorescence, and we were able to detect low but significant activity of the enzyme in various regions of the human brains after autopsy and to examine the kinetic properties of the human brain ezyme.

The incubation mixture (total volume 150 μl) contained (in final concentrations) 120 m*M* Tris–acetate buffer (pH 7.5), 1 m*M* dithiothreitol, 1 m*M* NSD-1055 (*p*-bromobenzyloxyamine, an aromatic L-amino acid decarboxylase inhibitor), 0.3 m*M* 6-methyl-5,6,7,8-tetrahydroperin (or at various concentrations for kinetic studies), 0.4 m*M* L-trytophan (or at various concentrations for kinetic studies), 25 μg of catalase and 20 μl of homogenate as an enzyme sample. For the control, D-tryptophan was used instead of L-tryptophan. Incubation was carried out in air at 37°C for 25 min, and the reaction was stopped by adding 15 μl of 60% perchloric acid. The mixture was centrifuged at 1400 *g* for 10 min, and 30 μl of the supernatant were injected into the reversed-phase HPLC column. The fluorescence was monitored at 345 nm with excitation at 294 nm. The carrier

buffer for HPLC was 10 m*M* potassium phosphate buffer (pH 5.0) containing 5% methanol with a flow-rate of 0.8 ml/min. The peak height of 5-hydroxytryptophan was measured and converted into picomoles from the standard curve of 5-hydroxytryptophan in the same column.

In this HPLC–FD system, 5-hydroxytryptophan, tryptophan and serotonin are completely separated. Since 5-hydroxytryptophan is eluted prior to tryptophan, the large amount of the substrate tryptophan compared with the small amount of 5-hydroxytryptophan formed by the enzyme reaction does not interfere with the assay of 5-hydroxytryptophan. The detection limit is *ca.* 20 pg (100 fmol), and the peak height showed good linearity with the amount of 5-hydroxytryptophan. The chromatographic pattern of TPH reaction with human raphe nucleus of brain as enzyme is shown in Fig. 5. The first unidentified peak appeared just in front of the 5-hydroxytryptophan peak, but did not interfere with it. This peak was observed with the human brain homogenate enzyme form but not with rat brain homogenate. Since this peak was observed both in control and in experiment, it is probably derived from human brain tissue. The control incubation with D-tryptophan as substrate showed a very small peak at the position of 5-hydroxytryptophan, whereas experimental incubation with L-tryptophan as substrate gave a large peak at this position. A small peak of serotonin was also observed both in control and in experiment, indicating that it was derived from endogenous serotonin in the brain tissue. By this new assay, the enzyme activity was first demonstrated in autopsy human brain tissues. The enzyme

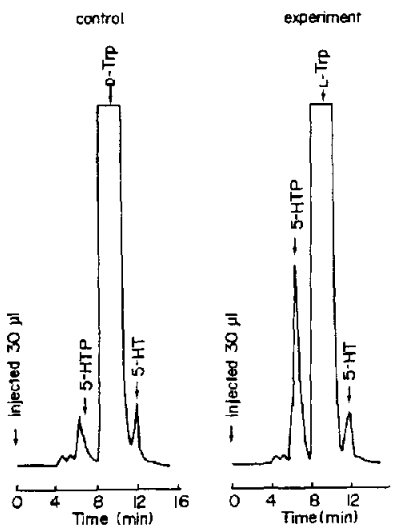

Fig. 5. HPLC–FD elution pattern of TPH incubation mixtures with the homogenate of human raphe nucleus as enzyme. Left (control): blank incubation with D-tryptophan. Right (experiment): experimental incubation wsith L-tryptophan. Peaks: 5-HTP = 5-hydroxytryptophan; Trp = tryptophan; 5-HT = serotonin (Reprinted from ref. 20 with permission.)

in the human brain was found to be very unstable. The optimum pH was 7.8. Relatively high activity was observed in raphe nucleus, locus caeruleus and hypothalamus. The human brain enzyme was more unstable than the rat brain enzyme. This may be why the enzyme activity in the autopsy human brain cannot be detected. The human brains in this study were removed within 2–4 h *post mortem*. The regional distribution was parallel with that of serotonergic neurons. Thus raphe nucleus with serotonergic cell bodies has the highest activity, and the value is *ca.* 1–2% of that in rat brain (*ca.* 90 nmol/min/g tissue).

3.4. Aromatic L-amino acid decarboxylase

AADC (EC 4.1.1.28) catalyses the decarboxylation of various aromatic L-amino acids, including L-DOPA and L-5-hydroxytryptophan [21]. Thus the enzyme is responsible for the formation of dopamine and serotonin. Among a wide range of naturally occurring and synthetic aromatic L-amino acids, L-DOPA and 5-hydroxytryptophan are good substrates. The value of V_{max} for L-DOPA is *ca.* 10 times that for L-5-hydroxytryptophan.

Various methods are available for AADC assay [22]. The most frequently used assay has been the radioisotopic method, with L-[1-^{14}C]DOPA or L-[1-^{14}C]-5-hydroxytryptophan as substrate, in which $^{14}CO_2$ formed in the enzymic decarboxylation is measured.

We developed HPLC methods using either L-DOPA [23] or L-5-hydroxytryptophan [24] as a substrate to measure enzymically formed dopamine or serotonin.

3.4.1. HPLC–ED using L-DOPA as substrate

L-DOPA is used as a substrate, and the product, dopamine, is measured by HPLC–ED. A large amount of the substrate, L-DOPA, which interferes with the dopamine peak in HPLC, is removed by preliminary chromatography on Amberlite CG-50. The standard incubation mixture contains (total volume 400 μl): 30 m*M* sodium phosphate buffer (pH 7.2), 0.3 m*M* EDTA, 0.17 m*M* ascorbic acid, 0.01 m*M* pyridoxal phosphate, 1.0 m*M* L-DOPA (or D-DOPA for the blank), 0.1 m*M* pargyline hydrochloride (a monoamine oxidase inhibitor) and the enzyme. Incubation is done at 37°C for 20 min, and the reaction is stopped by adding 80 μl of 3 *M* trichloroacetic acid. After 10 min, 1.87 ml of water and 50 μl of 0.01 *M* hydrochloric acid containing 50 pmol of dihydroxybenzylamine as an internal standard are added, and the mixture is centrifuged at 1600 *g* for 10 min. The supernatant liquid is passed through a column (packed volume 0.5 ml) of Amberlite CG-50-Na$^+$ equilibrated with 0.1 *M* potassium phosphate buffer (pH 6.5). The resin is washed with 2.5 ml of the buffer and 200 μl of 1 *M* hydrochloric acid, and the dopamine adsorbed is eluted with 700 μl of 1 *M* hydrochloric acid. A 100-μl volume of the elute is injected into the chromatograph equipped with an electrochemical detector. The order of elution under these conditions is dihydroxybenzylamine, DOPA and dopamine.

3.4.2. HPLC ED using L-5-hydroxytryptophan as substrate

L-5-Hydroxytryptophan is used as a substrate, and the product, serotonin, is measured by HPLC. Although the value of V_{max} for AADC with L-5-hydroxytryptophan as substrate is *ca.* 10% of that with L-DOPA as substrate, the AADC reaction proceeds linearly at 37°C for 150 min with L-5-hydroxytryptophan as substrate, but only for 20 min with L-DOPA as substrate. Therefore, this method is also highly sensitive with longer incubation.

The standard incubation mixture contains (total volume 400 μl, final pH 8.3): 30 m*M* sodium phosphate buffer (pH 9.0), 0.01 m*M* pyridoxal phosphate, 1.0 m*M* L-5-hydroxytryptophan (or D-5-hydroxytryptophan for the blank), 0.1 m*M* pargyline hydrochloride and the enzyme. Incubation is done at 37°C for 20–120 min, and the reaction is stopped by adding 80 μl of 3 *M* trichloroacetic acid. After 10 min, 1.82 ml of water and 100 μl of 0.01 *M* HCl containing 100–500 pmol of N-methyldopamine as an internal standard are added, and the mixture is centrifuged at 1600 *g* for 10 min. The supernatant liquid is passed through a column (packed volume 0.5 ml) of Amberlite CG-50 (Na^+) equilibrated with 0.1 *M* potassium phosphate buffer (pH 6.5). The resin is washed twice with 4.5 ml of the buffer and with 200 μl of 1 *M* hydrochloric acid. The serotonin is eluted with 1.4 ml of 1 *M* hydrochloric acid. A 100-μl aliquot of the eluate is analysed by HPLC ED. *N*-Methyldopamine is used as an internal standard. Under these conditions the order of elution is *N*-methyldopamine, serotonin and 5-hydroxytryptophan.

3.5. Dopamine β-hydroxylase

DBH (EC 1.14.17.1) [25], which catalyses the conversion of dopamine into norpinephrine, is an ascorbate-requiring, copper-containing monooxygenase and is localized in peripheral and central noradrenergic neurons and chromaffin cells of the adrenal medulla. Besides the formation of norepinephrine from dopamine, DBH generally catalyses the conversion of various phenylethylamines into the corresponding phenylethanolamines. DBH is secreted into the blood from peripheral sympathetic nerve endings and the adrenal medulla, and probably into CSF from the brain, together with norepinephrine. Therefore DBH is present in serum or plasma and CSF [4]. Since DBH is an intraneuronal enzyme specific for noradrenergic neurons, the assay of its activity in blood or CSF to estimate the neuronal function has gained much attention.

The assay of DBH activity in crude tissue preparations, such as homogenate or blood, is difficult, mainly because of the presence of endogenous inhibitors [26] and the low activity of the enzyme. Endogenous inhibitors are mostly thiol compounds [26] and can be eliminated by use of thiol-blocking agents such as *N*-ethylmaleimide or Cu^{2+} [26].

Numerous methods for the assay of DBH activity by use of HPLC–FD [3,27,28] and HPLC–ED [29–32] have been reported.

Dopamine is the main natural substrate, and the product, norepinephrine, is

sensitively measured by HPLC–ED [29–32]. However, since dopamine is unstable and easily oxidized, tyramine is frequently used as a substrate. The product, octopamine, is measured by HPLC–FD (post-column derivatization using *o*-phthalaldehyde [3]) or by HPLC–ED after pre-column conversion into *p*-hydroxybenzaldehyde [33]. This assay of *p*-hydroxybenzaldehyde formed from the product, octopamine, by HPLC–ED [33] may be the simplest and the most sensitive of all HPLC methods.

The incubation mixture contains (total volume 1.0 ml, each final concentration in parenthesis): 500 μl of the enzyme solution (for CSF, 500 μl), 100 μl of 2 *M* sodium acetate buffer, pH 5.0 (0.2 *M*), 150 μl of 0.2 *M* N-ethylmaleimide (30 m*M*), 50 μl of 100 μ*M* cupric sulphate (5 μ*M*), 25 μl of aqueous solution (20 mg/ml) of catalase (25 000 U, 500 μg), 25 μl of 40 m*M* pargyline hydrochloride (1 m*M*), 50 μl of 0.2 *M* ascorbic acid (10 m*M*), 50 μl of 0.2 *M* sodium fumarate (10 m*M*), and 50 μl of 0.4 *M* tyramine hydrochloride or dopamine hydrochloride (20 m*M*). The blank incubation also contains 50 μl of 2 m*M* fusaric acid (a specific inhibitor of DBH). The reaction is started by the addition of tyramine or dopamine. Acetate buffer plus N-ethylmaleimide plus cupric sulphate (cocktail A), and catalase plus pargyline plus ascorbic acid plus fumarate (cocktail B), can be mixed together; 500 μl of an enzyme preparation, 300 μl of cocktail A and 150 μl of cocktail B, are mixed in that order, and finally the reaction is started by adding 50 μl of tyramine or dopamine solution.

Endogenous inhibitors in the enzyme preparation are completely inactivated by addition of N-ethylmaleimide and a small amount of Cu^{2+}.

Incubation is performed at 37°C for 45 min. When dopamine is used as the substrate, norepinephrine formed enzymically from dopamine is isolated by a double-column procedure, the first column being of Dowex-50 (H^+ form) and the second of alumina, and is finally measured by HPLC–ED [32]. Epinephrine is used as an internal standard. The order of elution under the HPLC conditions [32] [ODS column; mobile phase, 0.1 *M* potassium phosphate buffer, pH 3.0, containing pentanesulphonic acid (20 mg/100 ml of buffer)] is dopamine, norepinephrine and epinephrine.

Figs. 6 and 7 show chromatograms obtained by HPLC–ED with dopamine [32] and tyramine [33] as substrate.

The norepinephrine formed can be measured with very high sensitivity by HPLC–ED [32]. A typical chromatographic pattern of the DBH reaction with human CSF as enzyme is shown in Fig. 6 The test-mixture incubation (B) formed a significant amount of norepinephrine, in contrast to the very small amount formed in the blank incubation (A). The peak for norepinephrine in the blank incubation may be due mainly to its non-enzymic formation. An amount of 100 pmol of norepinephrine is added to a blank incubation to act as a standard for calculating the amount of norepinephrine (C) from the peak height.

A typical HPLC–ED pattern of the DBH reaction with tyramine as substrate [33] is shown in Fig. 7. The peak of *p*-hydroxybenzaldehyde (peak 1) derived from

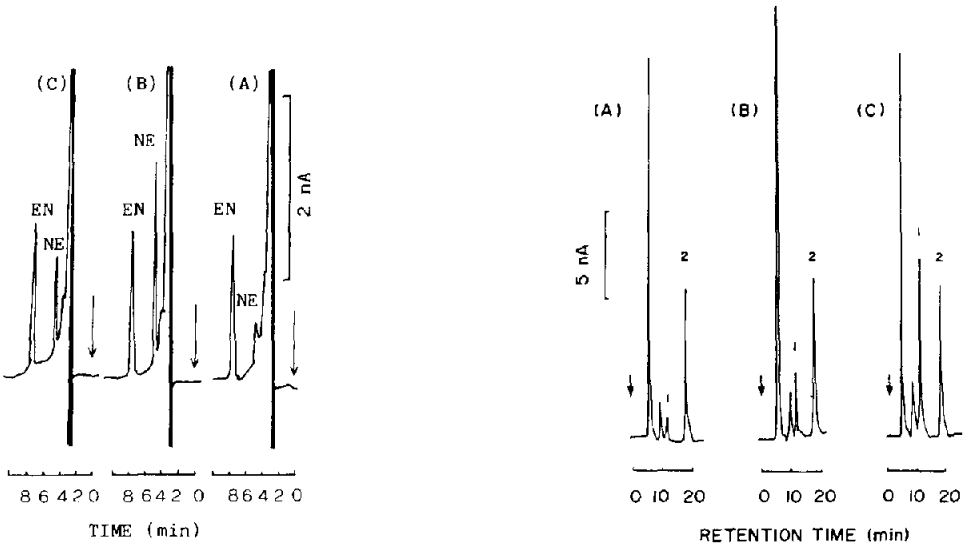

Fig. 6. Elution pattern of DBH incubation mixture with human CSF as enzyme in HPLC. A standard incubation mixture containing 500 μl of CSF was used. (A) Fusaric acid blank incubation. (B) Test incubation. (C) Norepinephrine (NE) (500 pmol) is added to the fusaric acid blank incubation. Epinephrine (EN) (500 pmol) is added to each sample after incubation, as an internal standard. DBH activity is calculated to be 13.3 nmol/min/l of CSF. Column, 10-μm ODS (350 mm × 4.0 mm I.D.); mobile phase, 0.1 *M* potassium phosphate buffer (pH 3.0) containing pentanesulphonic acid (20 mg per 100 ml of buffer); flow-rate, 6.0 ml/min; detection, ED at +0.08 V *vs.* Ag/AgCl. (Reprinted from ref. 32 with permission.)

Fig. 7. Chromatographic patterns using DBH incubation mixture of human child CSF as enzyme. (A) Fusaric acid blank incubation. (B) Experimental incubation. (C) 50 pmol of octopamine were added to the fusaric acid blank. Isovanillin (50 pmol) was added to each sample as internal standard. DBH activity was calculated to be 12.0 nmol/min/l of CSF. Peaks: 1 = *p*-hydroxybenzaldehyde; 2 = isovanillin. (Reprinted from ref. 33 with permission.)

enzymically formed octopamine in the experimental incubation (B) was 3.3 times higher than that in the blank incubation (A). The peak in the chromatogram of the blank incubation may be due to *p*-hydroxybenzaldehyde, indicating that octopamine is present in commercial tyramine hydrochloride.

DBH activity can also be measured by HPLC–FD. Dopamine, the main natural substrate, is used, and the product, norepinephrine, is measured by HPLC–FD, by native fluorescence [27], by post-column derivatization with trihydroxyindole reaction [28] or by pre-column derivatization with diphenylethylenediamine reaction [29]. Epinephrine or N-methyldopamine is used as internal standard.

3.6. Phenylethanolamine N-methyltransferase

Phenylethanolamine N-methyltransferase (PNMT), also referred to as noradrenaline N-methyltransferase (EC 2.1.1.28), is the enzyme that catalyses the for-

mation of epinephrine from norepinephrine [34]. The enzyme activity is high in the adrenal gland, and is also detected in specific brain regions of rats by a highly sensitive radioassay [35]. PNMT activity is also demonstrated in human brain regions by using radioassay [36]. Previous investigations of PNMT activity in brain were performed only by radioassays. The recently developed HPLC–ED method provides a rapid, sensitive and accurate technique for measuring PNMT activity, and permits the measurement of PNMT activity in all rat and human brain regions [37,38].

PNMT activity is assayed by using norepinephrine as a substrate. The enzymically formed epinephrine is determined by HPLC–ED. Commercially available norepinephrine contains *ca.* 0.3% of contaminating epinephrine, which is removed by recrystallization to reduce the blank value. The enzymically formed epinephrine is absorbed on an aluminium oxide column, eluted with 0.5 *M* hydrochloric acid, separated by revered-phase ion-pair HPLC and detected electrochemically. 3,4-Dihydroxybenzylamine is added to the incubation mixture after the reaction, as an internal standard. This assay is very sensitive, and 0.5 pmol of epinephrine formed enzymically can be detected.

The standard incubation mixture (250 μl) consists of the following components (final concentrations in parentheses): 10 μl of 0.01 *M* pargyline (a monoamine oxidase inhibitor) in 0.01 *M* hydrochloric acid (0.4 m*M*), 50 μl of 0.5 *M* Tris–HCl buffer, pH 8.0 (0.1 *M*), 15 μl of 0.3 m*M* S-adenosylmethionine (18 μ*M*), 20 μl of 0.2 m*M* norepinephrine (16 μ*M*), 100 μl of 0.32 *M* sucrose (128 m*M*) containing homogenized tissues as enzyme, and water. The blank reaction mixture is incubated either without enzyme or with enzyme heated for 5 min at 90°C. Epinephrine (15 or 30 pmol) is added to an enzyme-free blank incubation as a standard.

Epinephrine formed by PNMT can also be measured by HPLC FD, with norepinephrine as substrate, by the post-column trihydroxyindole reaction [39]. Reversed-phase ion-pair chromatography permits complete separation of epinephrine from excess norepinephrine as substrate, which cannot be achieved by cation-exchange column chromatography. α-Methylnorepinephrine (α-methyl-NE) is used as an internal standard in this HPLC–FD method. A post-column trihydroxyindole reaction is required, but this HPLC FD method is more specific and sensitive than the HPLC–ED method [39]. PNMT activity can also be measured by HPLC–FD using pre-column derivatization with diphenylethylenediamine [40]. Although the analysis time for HPLC–FD assays is longer than that for HPLC–ED methods, owing to the time needed for the pre-column (the diphenylethylenediamine method [40]) or post-column (the trihydroxyindole reaction [39]), this assay is more specific than the HPLC–ED method.

4. PURIFICATION OF BIOGENIC AMINE-RELATED ENZYMES BY HPLC

4.1. General

In the past several years, HPLC has become one of the most important and valuable techniques for enzyme purification. It offers the advantages of greater speed and of an improved quality of separation of proteins. Several techniques have been developed for separation and isolation of large peptides and proteins by HPLC in the size-exclusion or gel-permeation (GP), affinity (AF), ion-exchange (IE) and reversed-phase (RP) modes.

The proper combination of chromatographic conditions must be chosen. This selection includes such variables as the support, the eluting solvents and the flow-rate.

Gel permeation chromatography (GP-HPLC) is a rapid method with high resolution for separating enzymes by size, and it also has the added benefit of providing estimates of the molecular masses of enzymes. However, commercially available column supports for GP-HPLC all exhibit a considerable degree of non-specific retention of enzyme proteins, which limits their applicability for the determination of molecular masses. For purification of enzyme proteins by GP-HPLC, the temperature must be kept low and the mobile phase should not decrease the enzyme activity or decompose the enzyme molecule. The use of GP-HPLC has generally been restricted to the final step of enzyme purification. The SWG column used by Kato *et al.* [41] is a preparative column of large diameter. Samples up to 100 mg can be applied to the column without loss of separation efficiency, and the recoveries of enzymic activity are nearly 100%. Ion-exchange HPLC (IE-HPLC) is a valuable and widely used technique for the purification of enzymes. A major limitation of conventional gel-type ion-exchange media was the lack of mechanical strength. However, strong ion-exchange supports suitable for HPLC are now commercially available. Recoveries of enzyme activity from IE-HPLC columns are generally equivalent to those obtained with conventional IE columns. However, the short separation time by HPLC will also be useful in recovering labile enzyme proteins without loss of enzyme activity.

Hydrophobic chromatography with HPLC, *e.g.* phenyl-5PW, is also useful for enzyme purification. The column is previously equilibrated with a high concentration of ammonium sulphate and elution is carried out by a gradient of decreasing concentration of ammonium sulphate.

4.2. Purification of human AADC by HPLC

The enzymes related to biogenic amines have been purified mostly by conventional column chromatography, but purification by HPLC is efficient and rapid. A good example is the purification of AADC from human pheochromocytoma [42]. HPLC with gel permeation and hydrophobic columns was highly effective,

and the entire purification could be finished with three days. All the procedures were carried out at 4°C.

For the extraction step, the tissue stored frozen at −80°C was homogenized by a blender in 4 volumes of a solution consisting of 0.25 *M* sucrose, 1 m*M* dithiothreitol (DTT) and 0.1 m*M* EDTA. The homogenate was centrifuged at 100 000 *g* for 1 h. The supernatant was passed through a glass-wool column to remove floating lipids.

The next step was acid treatment. The pH of the supernatant was adjusted to 4.7 by adding 1 *M* acetic acid, and it was centrifuged at 30 000 *g* for 15 min. The precipitate was removed, and the pH of the supernatant that contained the enzyme was adjusted to 7.0 by the addition of 1 *M* Tris–HCl buffer (pH 7.2).

For DEAE-Sephacel column chromatography, the enzyme solution was diluted with a half-volume of buffer A, consisting of 20 m*M* Tris–HCl (pH 7.2), 8% sucrose and 1 m*M* dithiothreitol, and applied to a column (23.5 cm × 5.0 cm I.D.) of DEAE-Sephacel previously equilibrated with buffer A. The column was washed with buffer A, and the enzyme was eluted by a linear gradient of 0–0.4 *M* NaCl in buffer A. The active fractions were pooled.

For GP-HPLC, solid ammonium sulphate was added to the active fraction to 60% saturation, and the resulting precipitate was collected by centrifugation and dissolved in a small amount of buffer B, consisting of 50 m*M* potassium phosphate buffer (pH 6.8), 0.2 *M* NaCl, 0.1 m*M* pyridoxal phosphate, 1 m*M* dithiothreitol and 0.1 m*M* EDTA. The enzyme preparation was applied to two columns, connected in series of Toyo-Soda G-3000 SW equilibrated with buffer B. Elution was carried out with the same buffer at a flow-rate of 3 ml/min. The active fractions were pooled.

For hydrophobic HPLC with a Phenyl-5PW column, solid ammonium sulphate was added to the enzyme solution to 15% saturation. The mixture was applied to the Phenyl-5PW column, previously equilibrated with buffer C consisting of 50 m*M* potassium phosphate buffer (pH 6.8), 15% ammonium sulphate, 0.1 m*M* pyridoxal phosphate, 1 m*M* dithiothreitol and 0.1 m*M* EDTA. Then, the column was washed with buffer C containing 10% ammonium sulphate, and elution was carried out by a linear gradient of 10–0% ammonium sulphate at a flow-rate of 0.5 ml/min (Fig. 8). The procedure was repeated in a second Phenyl-5PW column.

A typical purification procedure of AADC from 98 g of human pheochromocytoma tissue is summarized in Table 1. This purification procedure was reproducible and rapid. The entire purification was finished within three days. The specific activity of the final preparation with L-DOPA as a substrate was 10.3 μmol/min/mg protein, with 33% recovery. A typical chromatogram of the last step is shown in Fig. 8. A single subunit with an M_r of 50 000 was observed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The M_r of the enzyme was estimated to be *ca.* 100 000 by GP chromatography, indicating the enzyme to be a dimer.

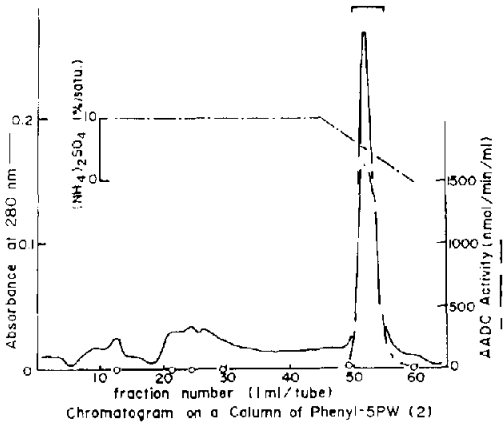

Fig. 8. Purification of AADC from human pheochromocytoma by double Phenyl-5PW HPLC. Elution was carried out with a linear gradient of ammonium sulphate, starting at fraction No. 46. The flow-rate was 0.5 ml/min, and a total of 4.9 ml of fractions was collected. (Reprinted from ref. 42 with permission.)

5. ASSAY OF BIOGENIC AMINES BY HPLC USING POST-COLUMN ED WITH IMMOBILIZED AMINE OXIDASE

The third application of HPLC to the study of biogenic amines is the assay of biogenic amines by HPLC using post-column ED with immobilized amine oxidase.

We developed a simple, sensitive assay of polyamines by HPLC ED after post-column reaction with immobilized polyamine oxidase [43,44]. Polyamines (putrescine, spermidine, spermine and cadaverine) are separated by isocratic ion-pair reversed-phase HPLC, then enzymically oxidized, with release of hydrogen

TABLE I

PURIFICATION OF AADC FROM HUMAN PHEOCHROMOCYTOMA

Step	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Homogenate	11000	44.8	0.004		
100 000 *g* supernatant	8460	27.7	0.003	1	100
pH 4.7 supernatant	6930	12.5	0.002	0.7	45
DEAE-Sephacel	221	27.0	0.12	40	98
G-3000 SW	20.8	17.9	0.86	287	65
Phenyl-5PW (1)	1.45	12.4	8.57	2860	45
Phenyl-5PW (2)	0.87	9.02	10.3	3430	33

[a] U = μmol dopamine formed per min.

peroxide, via the post-column reactor with immobilized polyamine oxidase partially purified from soybean; the hydrogen peroxide is detected by electrochemical oxidation on a platinum electrode. Since gradient elution can cause baseline drift in the detector, isocratic separations are mostly employed in HPLC–ED [45]. To separate polyamines by isocratic reversed-phase HPLC with a low salt eluent in a reasonably short time, we used a new, polymer-based, reversed-phase column (150 mm × 6.0 mm I.D.) containing polymer-based C_{18} Biophase III (BAS, Tokyo, Japan) that can withstand an alkaline mobile phase. The soybean polyamine oxidase (EC 1.4.3.6) is immobilized on amino propyl-derivatized controlled-pore glass-beads (Aminopropyl-CPG, 200–400 mesh, pore size 40 nm; Electro Nucleomics, Fairfield, NJ, U.S.A.) by a coupling reaction with glutaraldehyde. The enzyme has a wide substrate specificity and oxidizes putrescine, spermidine and spermine. The hydrogen peroxide produced can be measured electrochemically on a platinum electrode.

Fig. 9 shows chromatograms obtained for the standard solution and a sample of urine from a normal subject. Before HPLC, the urine was hydrolysed and pretreated by a Sep-Pak C_{18} column. A complete chromatographic analysis took 40 min. The mean analytical recoveries of putrescine, cadaverine, spermidine and spermine were 75.4 ± 1.6%, 96.5 ± 3.8%, 101.2 ± 5.2%, and 84.1 ± 5.3%, respectively. The dynamic ranges of peak responses were linear over two to three orders of magnitude. The lower limits of detection (at a signal-to-noise ratio of

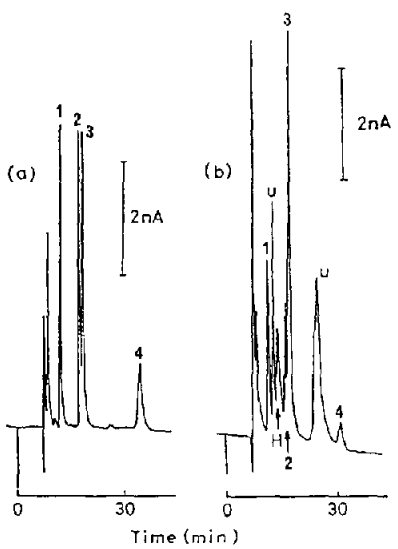

Fig. 9. Chromatograms obtained for: (a) a standard solution containing (1) putrescine (Put, 10 pmol), (2) cadaverine (Cad, 25 pmol), (3) spermidine (Spd, 30 pmol) and (4) spermine (Spm, 40 pmol); and (b) a urine sample containing (1) Put (7.5 nmol/mg of creatinine), (2) Cad (14.2 nmol/mg of creatinine), (3) Spd (57.3 nmol/mg of creatinine) and (4) Spm (16 nmol/mg of creatinine). Other peaks: H = histamine; U = unknown peak. (Reprinted from ref. 45 with permission.)

10) were 0.3, 0.5, 0.6 and 4 pmol injected for putrescine, cadaverine, spermidine and spermine, respectively. Putrescine and spermidine were increased significantly in patients with blood cancers and solid cancers [44]. Histamine can also be measured by this method [43,44].

Acetylcholine and choline are measured by HPLC–ED after post-column derivatization by acetylcholine esterase and choline oxidase [45]. We have applied this method to simultaneous measurement in a single chromatographic analysis of dopamine, norepinephrine, epinephrine and serotonin with a glassy carbon electrode, and choline and acetylcholine with a platinum electrode, by using boric acid gel to remove the interference of catecholamine to choline and acetylcholine [47].

We also applied this HPLC–ED method for choline and acetylcholine to the measurement of the enzyme activities of choline acetyltransferase [48] and acetylcholine esterase [49]. These assays for acetylcholine-related enzymes are highly sensitive.

6. SUMMARY

Applications of HPLC to the study of enzymes related to biogenic amines have been reviewed. HPLC is now frequently used for the assay of enzyme activity, especially with ED or FD. Examples of the assay of catecholamine- and serotonin-synthesizing enzymes, TH, TPH, AADC, DBH and PNMT, were given. The advantage is very high sensitivity. Catecholamine-related enzymes can be rapidly purified by gel-permeation or hydrophobic HPLC. The third application is the HPLC assay of biogenic amines using post-column oxidation by amine oxidase. An example is given for assay of polyamines. Hydrogen peroxidase formed from polyamine by immobilized soybean polyamine oxidase is measured electrochemically on a platinum electrode.

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