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IMPROVED LIQUID CHROMATOGRAPHIC DETERMINATION OF DOPAMINE-β-HYDROXYLASE ACTIVITY IN TISSUES AND PLASMA

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SUMMARY

We describe a new assay for dopamine-β-hydroxylase (DβH) activity in human and rat plasma and rat tissues using reversed-phase high-performance liquid chromatography with electrochemical detection. Human and rat plasma DβH activity was measured directly, without extraction of the enzyme. The DβH from rat tissues was extracted on Concanavalin A-Sepharose before the assay to avoid interference from the presence of tissue catecholamines. Dopamine, the natural substrate of DβH, was utilized at optimal (enzyme-saturating) concentration. The reaction product, norepinephrine, was isolated on Dowex AG 50W-X4 (H^+ form) column. An internal standard, [^{3}H]norepinephrine, was included to correct for the loss of norepinephrine during the procedure. This method allows for the first time the determination of DβH activity in small volumes of rat and human plasma (5—20 μl) and tissues. The procedure can be easily set up in any laboratory equipped with a high-performance liquid chromatograph, an electrochemical detector, and a liquid scintillation counter.

INTRODUCTION

Until recently, the determination of dopamine-β-hydroxylase (DβH) activity in biological samples has been dependent on relatively difficult methods with low sensitivity. Although several new methods were developed, the main difficulty remained the precise measurement of the reaction product in the presence of high substrate concentrations. Since the majority of assays kept the enzyme reaction zero order with respect to substrate, the large amount of substrate usually interfered with the assay. For this reason, additional enzymatic

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[1–3], derivatization [4, 5] or chromatographic [6] steps have been included to isolate the reaction product. However, these procedures can result in great variations of the results owing to loss of the reaction product. Another disadvantage is that the natural substrate of DβH, dopamine, cannot be used in the tissue DβH assay since the high norepinephrine content in tissues also interferes with the assay.

Recently, high-performance liquid chromatography (HPLC) has been used to develop a specific and sensitive methodology to measure DβH activity in biological samples [7–11]. Another recent advance has been the elimination of interference from tissue norepinephrine by the extraction of the tissue DβH before its assay [12].

This paper describes a new assay, which further validates HPLC with electrochemical detection (ED) for the measurement of DβH activity and extends its use to tissue samples by the previously reported DβH extraction method. In this new assay, the natural substrate of DβH, dopamine, was used under saturating conditions. The reaction product, norepinephrine, was isolated on miniature Dowex AG 50W-X4 columns and then extracted on alumina. An internal standard, [^{3}H]norepinephrine, was included to correct for the loss of norepinephrine during the isolation and extraction steps. The method is highly specific and sensitive, which makes it especially suitable for the analysis of DβH activity in small volumes of human or rat plasma (5–20 μl) or rat tissues.

EXPERIMENTAL

Chemicals

All reagents were of the highest purity. Buffers and aqueous solutions were prepared in double-distilled, deionized water. Catecholamines (CA), sodium metabisulphite, and N-ethylmaleimide were obtained from Calbiochem (La Jolla, CA, U.S.A.); Tris (free base) from Schwarz/Mann (Orangeburg, NY, U.S.A.); alumina from Woelm Pharma (Eschwege, F.R.G.); Dowex AG 50W-X4 (H^+ form, 200–400 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.); dopamine-β-hydroxylase (from bovine adrenals), catalase (from bovine liver, twice crystallized), fumaric acid, fusaric acid, ascorbic acid, and pargyline from Sigma (St. Louis, MO, U.S.A.); [^{3}H]norepinephrine (DL-[7-H(N)]-norepinephrine, specific activity, 11.8 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.); Concanavalin A (Con A)-Sepharose from Pharmacia (Uppsala, Sweden); other chemicals from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Chromatography

A Waters M-45 pump was used to deliver the mobile phase through a μBondapak C_{18} chromatographic column (30 cm × 3.9 mm; 5 μm; Waters Assoc., Milford, MA, U.S.A.). The column eluent was monitored by a glassy-carbon electrode with an LC 4A amperometric detector. The oxidation potential was set at +0.72 V vs. an Ag/AgCl electrode. The oxidation current was registered by a Chromatopac C-R1B data processor (Shimadzu, Kyoto, Japan). The chromatographic system was equipped with an automatic sample injector (WISP 710B, Waters Assoc.). The solvent system consisted of 0.1 *M* sodium phosphate

(pH 4.5) buffer containing 4% acetonitrile, 1 m*M* EDTA and 4 m*M* sodium heptanesulphonate, filtered through a Millipore membrane filter (pore size 0.45 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum before use. The flow-rate was 1 ml/min and the temperature was ambient.

Sample preparation

Human blood was obtained from healthy subjects into heparinized tubes and was centrifuged at 10 000 *g* for 10 min. Plasma samples were stored at −70°C until the DβH assay (usually within one week).

Heparinized rat plasma was obtained from male Sprague—Dawley rats (250–300 g) after decapitation of the animals, and the samples were kept frozen at −70°C until used (usually within one week). Rat tissues were rinsed free of blood, and rapidly frozen on dry ice.

Tissue DβH was extracted according to previously published methods [12, 13]. Tissues were homogenized in sodium acetate buffer containing 0.5% Triton X (pH 5.0; adrenals and vas deferens, 1 ml of buffer; heart, three volumes of buffer). The tissue homogenates were shaken for 20 min, then centrifuged at 15 000 *g* for 20 min. Aliquots of 300 μl of supernates were transferred into Eppendorf centrifuge tubes, then 200 mg of Con-A Sepharose were added and the volume was adjusted to 1 ml with the addition of 500 μl of sodium acetate buffer (pH 5.0). The samples were shaken at 4°C for 20 min, then centrifuged, and the supernates were aspirated. The pellet was resuspended in 1 ml of cold distilled water and centrifuged. The wash of pellet with distilled water was repeated three times, and after the last centrifugation the pellet was resuspended in 550 μl of sodium acetate buffer (pH 5.0).

DβH assay

DβH activity was measured under optimal conditions [9] using the same concentrations of reagents as those used in previous studies [7, 9]. In a final volume of 1 ml, each incubation tube contained the sample (300 μl of resuspended pellet after extraction of tissue DβH or 5—20 μl of human or rat plasma diluted to 300 μl with distilled water), 100 μl of 2 *M* sodium acetate buffer (pH 5.0), 150 μl of 0.2 *M* N-ethylmaleimide, 50 μl of 200 μ*M* copper sulphate (for tissues) or 50 μl of 100 μ*M* copper sulphate (for plasma), 25 μl of catalase (25 000 U), 25 μl of 40 m*M* pargyline, 50 μl of 0.2 μ*M* ascorbic acid, 50 μl of 0.2 *M* fumaric acid, 50 μl of 0.4 *M* dopamine (DA) and 200 μl of distilled water. Each sample was repeated in two parallel sets of incubation and there were also incubates that contained 50 μl of 2 m*M* fusaric acid. The tubes containing the reaction mixture were placed into a moving water-bath and were incubated at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 95% ethanol and 100 μl of 10 m*M* fusaric acid. After the addition of 100 μl of [^{3}H]norepinephrine (30 000 cpm in 100 μl of 0.1 *M* hydrochloric acid), 100 μl of 10% sodium metabisulphite and 100 μl of 0.2 *M* EDTA, the tubes were kept at 4°C for 30 min.

Measurement of norepinephrine

The norepinephrine content of incubates was determined by reversed-phase HPLC–ED after the separation of norepinephrine with miniature Dowex AG

50W-X4 columns (H^+ form, 200—400 mesh, 55 × 4 mm I.D.) [12]. The samples were centrifuged at 10 000 *g* for 10 min, then the supernates were passed through the Dowex columns. The columns were washed with 6 ml of distilled water and 6.5 ml of 1 *M* hydrochloric acid, then norepinephrine was eluted with 3.0 ml of 1 *M* hydrochloric acid. Aliquots of 1 ml of eluates were transferred to another set of tubes, and the pH was adjusted to above 8.0 by the addition of 1 ml of 2 *M* Tris buffer containing 1% EDTA (pH 8.6). Norepinephrine was adsorbed on 20 mg of acid-washed, heat-activated alumina [14]. After repeated washing of the alumina with distilled water, the norepinephrine was desorbed by the addition of 600 μl of 0.5 *M* acetic acid containing 0.1% EDTA. Aliquots of 300 μl of eluates were transferred into plastic scintillation vials, and the isotope content was determined in a liquid scintillation counter. Another set of aliquots of 50 μl of eluates were analysed by reversed-phase HPLC—ED.

Calculations

A reference external standard containing 1 ng of norepinephrine was injected into the HPLC system before each set of samples. The chromatographic peak of norepinephrine in samples was identified on the basis of its retention time. The concentration of norepinephrine in samples was calculated from its peak height and converted into 100% recovery based on recovery determined for the isotope content of samples.

RESULTS

As illustrated in Fig. 1, tissue DβH activity was measured after adsorption of the enzyme on Con-A Sepharose. After incubation of tissue homogenates with Con A-Sepharose, the DβH—Con A-Sepharose complex was separated by centrifugation. Subsequent washing of the pellet resulted in a complete elimination of catecholamines, which made it possible to use the natural substrate, dopamine, for the DβH assay. The absence of catecholamines after the last washing of the pellet was confirmed by reversed-phase HPLC—ED.

The optimal conditions for the extraction of tissue DβH were determined by the addition of different concentrations of Con A-Sepharose to the adrenal, heart and vas deferens homogenates. At very low concentrations of the Con-A Sepharose (less than 20 mg per tube), the DβH activity recovered from these tissues appeared to be proportional to the Con A-Sepharose concentration. The DβH activity was the highest when the tissues were treated with more than 50 mg per tube of Con A-Sepharose. Any further increase in Con A-Sepharose concentrations (up to 300 mg) had no effect on the tissue DβH activity, suggesting that the extraction of the enzyme was already complete with 50 mg per tube of Con A-Sepharose. This was confirmed by the extraction of a similar amount of a commercially available DβH (from bovine adrenals, Sigma) with Con A-Sepharose, which indicated a high recovery of the enzyme after the extraction and washing steps (recovery of the DβH activity was between 84 and 96% in three different experiments).

The incubation mixture contained the previously standardized [7, 9] concentrations of the substrate, cofactor, substrate-stabilizing agents and other

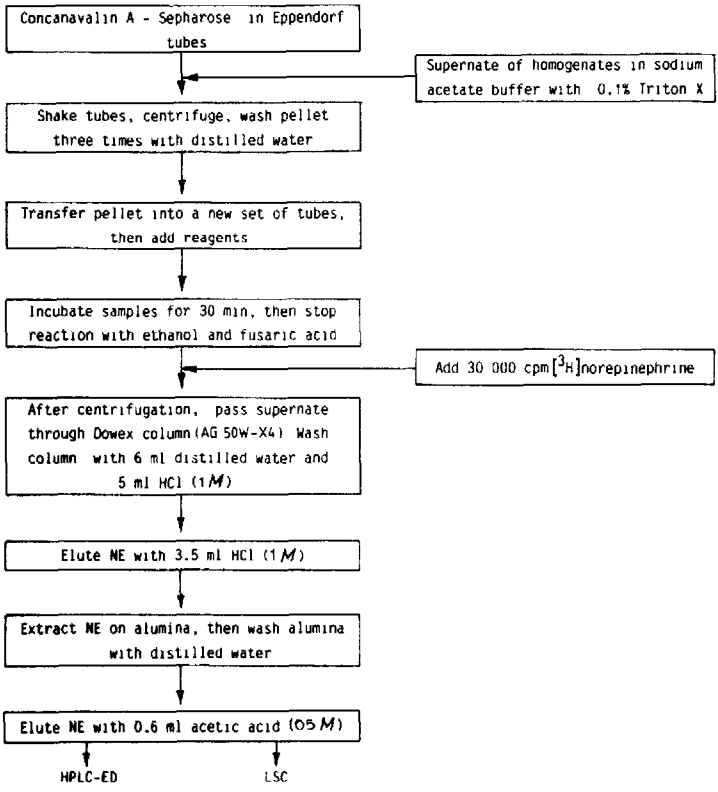

Fig. 1. Flow chart for the assay of DβH activity in rat tissues. Human and rat plasma were assayed similarly but the extraction step with Con A-Sepharose was omitted. The recovery of [^{3}H]norepinephrine was between 35 and 50%. NE = norepinephrine; LSC = liquid scintillation counting.

reagents. As shown in these previous studies [7, 9], dopamine, ascorbic acid or Cu^{2+} were not rate-limiting at those optimal concentrations, and the formation of norepinephrine had increased linearly up to 60 min. Although in our study the extraction of tissue DβH with Con A-Sepharose and the subsequent washing might eliminate not only catecholamines but also the DβH inhibitors that are present in tissues, we observed that in some experiments the omission of N-ethylmaleimide, an inhibitor of DβH inhibitory substances, from the incubation mixture resulted in a lowering of the DβH activity. This indicated that despite the extraction of the tissue DβH, the removal of endogenous tissue inhibitors by Con A-Sepharose was not complete.

After the incubation, the reaction product, norepinephrine, was isolated on Dowex AG 50W-X4 columns (Fig. 1). The addition of 30 000 cpm [^{3}H]norepinephrine proved to be a very reliable tool for the recovery calculation, since the elution of the radioactivity exactly overlapped with that of non-labelled norepinephrine. As shown in Fig. 2, epinephrine might not have been an ideal internal standard since its elution profile was different from that of norepinephrine.

When increased amounts of rat or human plasma or rat tissue extracts were added to the incubation mixture, the formed norepinephrine was

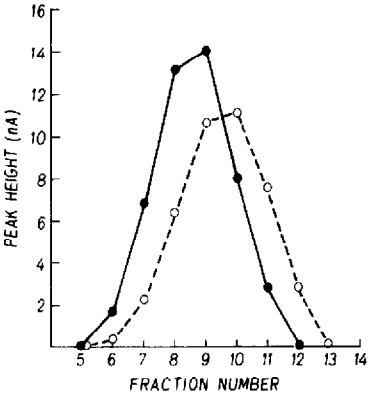

Fig. 2. Elution profile of Dowex AG 50W-X4 column (H^+ form, 200—400 mesh, 55 × 4 mm I.D.) for norepinephrine (•—•) and epinephrine (○- - -○). Norepinephrine and epinephrine (0.8 nmol each) were applied to the Dowex column and, after washing of the column with 6 ml of distilled water, catecholamines were eluted with 1 *M* hydrochloric acid. The eluate was collected in 1-ml fractions, and the catecholamine content of all fractions was determined by reversed-phase HPLC—ED.

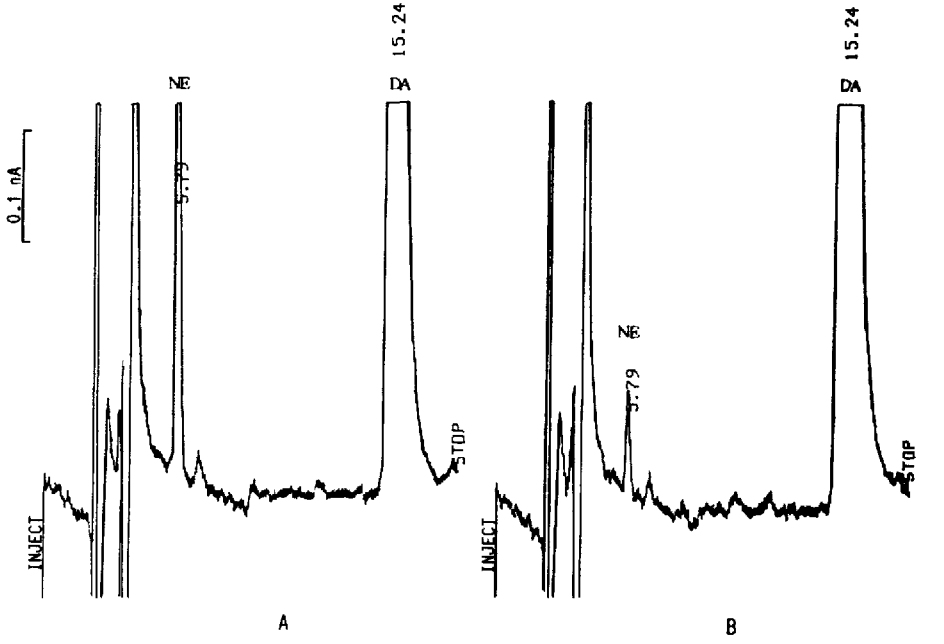

Fig. 3. Reversed-phase chromatogram of DβH incubation mixture containing 5 μl of human plasma (A). The addition of 0.1 m*M* fusaric acid diminished, although not completely, the formation of norepinephrine (B) Peaks: NE = norepinephrine; DA = dopamine.

proportionally increased. The relationship between norepinephrine and sample volume was linear between 5 and 20 μl for human plasma, between 20 and 80 μl for rat plasma, between 100 and 500 μl for whole adrenal homogenates (corresponding to 3–15 mg of whole adrenal tissue), between 50 and 400 μl

for vas deferens homogenates (corresponding to 4—32 mg tissue) and between 200 and 600 μl for heart homogenates (corresponding to 50—150 mg tissue), all tissues being extracted on 200 mg of Con A-Sepharose. The detection limit was 10 nmol/l per min for plasma or 10 pmol/g per min for tissues.

As illustrated in Fig. 3, the addition of fusaric acid to the incubation mixture decreased the norepinephrine formation, although the inhibition of norepinephrine synthesis was not complete. The incomplete inhibition by fusaric acid was apparent not only in human plasma (Fig. 3) but also in rat plasma and tissues. Whether this was due to incomplete inhibition of DβH by fusaric acid or to a previously proposed non-enzymatic norepinephrine formation [9] remains to be determined.

As shown in Fig. 4, there was a large difference in plasma DβH activity between different normal human subjects. In a single individual, however, the plasma DβH activity was remarkably constant. Table I shows the values for DβH activity in rat and human plasma and rat tissues. A comparison of the

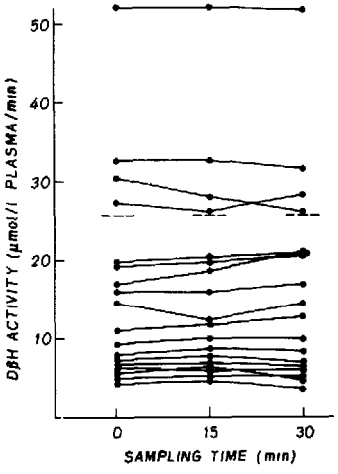

Fig. 4. DβH activity in human plasma. In all subjects (n = 18), the DβH activity was determined in three different plasma samples taken from an intravenous catheter at 0, 15 and 30 min. The dots and continuous lines represent the individual values, and the dashed line is the mean.

TABLE I

DβH ACTIVITY IN HUMAN AND RAT PLASMA AND RAT TISSUES

Values are means ± S.D.

Sample	DβH activity
Human plasma (n = 38)	15.1 ± 1.6 μmol/l of plasma per min
Rat plasma (n = 9)	1.4 ± 0.09 μmol/l of plasma per min
Rat tissue	
Adrenals (n = 8)	0.44 ± 0.04 nmol/pair per min
Heart (n = 8)	0.38 ± 0.06 nmol/g per min
Vas deferens (n = 12)	0.96 ± 0.09 nmol/g per min

values of parallel incubations (n = 9) of plasma or tissue samples indicated 7.5% or less intra-assay variation. The inter-assay variation was 8.5% or less.

DISCUSSION

Although the incubation conditions in our DβH experiments were similar to those reported by other investigators [7, 9], we introduced several modifications that simplify the procedure, improve its reproducibility and make it suitable for the determination of both tissue and plasma DβH activity.

Whenever dopamine is used as a substrate, norepinephrine must be eliminated from the samples before the assay. Because of the high sensitivity of the norepinephrine assay by the reversed-phase HPLC—ED method, we used only 5—20 μl of human or rat plasma for the DβH assay. The norepinephrine content in such a small amount of plasma did not interfere with the DβH assay since the amount of norepinephrine formed during the incubation was at least 100 times higher than the normal plasma norepinephrine content. However, analytical problems associated with the high tissue concentrations of norepinephrine made the direct assay unsuitable for the determination of tissue DβH activity. To circumvent this problem, we extracted the tissue DβH on Con A-Sepharose. We found, in agreement with other studies [12], that this procedure completely removed the catecholamines from the tissue homogenates. However, in our hands the use of Con A-Sepharose was not entirely satisfactory for the elimination of endogenous tissue inhibitors. Although this finding is different from those reported by other investigators [12], in our assay the addition of N-ethylmaleimide prevented the interference from those inhibitors.

According to previous studies, the DβH reaction can be kept zero order with dopamine concentrations greater than 20 mM. However, these high substrate concentrations do not allow the direct measurement of the reaction product, norepinephrine. For the isolation of norepinephrine, we used small columns of an ion-exchange resin (Dowex AG 50W-X4, H^+ form, 200–400 mesh, 55 × 4 mm I.D.). Unfortunately, these columns introduced an important source of variation due to a loss of an unknown amount of norepinephrine. Although some current methods use epinephrine as an internal standard for recovery calculations [7, 9], in our hands this internal standard did not provide an accurate and consistent correction. However, we were able to minimize this source of error with the addition of another internal standard, [^{3}H]norepinephrine. The finding that the elutions of labelled and non-labelled norepinephrine were the same but the elution of epinephrine was different made [^{3}H]norepinephrine a more reliable internal standard in our assay.

With the method described, the values for DβH are predictably higher than those reported using suboptimal (less than 20 mM) concentrations of the substrate [1, 15]. On the other hand, those previous studies which used optimal concentration of the substrate found DβH activity values in human [4, 10, 16, 17] and rat plasma [8], and rat tissues [18] similar to ours.

The method is currently used in our laboratory and it appears to be a useful tool in the evaluation of sympathetic function in both clinical and laboratory research. It is simple and rapid and requires no sophisticated equipment.

With the use of automated HPLC—ED and a liquid scintillation counter, 48 samples can be analysed overnight in a continuous unattended run. The results are reliable, reproducible and accurate.

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