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DETERMINATION OF DOPAMINE, HOMOVANILLIC ACID AND 3,4-DIHYDROXYPHENYLACETIC ACID IN RAT BRAIN STRIATUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Two procedures using liquid chromatography with electrochemical detection are described for the determination of dopamine (DA) and its two acidic metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in subregions of rat striatum and nucleus accumbens. A strong cation-exchange column was used for DA analysis and a C_{18} reversed-phase column was used for the analysis of the metabolites. Effects of pH, temperature and percentage of methanol on the retention time of HVA and DOPAC were studied. Levels of these compounds in the subregions of rat striatum and nucleus accumbens are reported.

INTRODUCTION

High-performance liquid chromatography with electrochemical detection is now a frequently used and well established technique for the determination of catecholamines, related compounds and their metabolites in biological samples. These compounds include dopamine (DA), norepinephrine (NE), epinephrine (EPI) and their acid metabolites homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC), and vanillylmandelic acid (VMA). Because of very simple procedures of sample preparation, high sensitivity and selectivity, this method is well suited for the measurement of low levels of these compounds in biolog-

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ical samples. Various methods have been reported for the determination of catecholamines and their metabolites in plasma [1—3], urine [4--8], and tissue [3, 9—12] samples. DA and NE have been determined in whole brain and different parts of the rat brain by analyzing tissues weighing 0.5—500 mg [10, 11]. Wagner et al. [9] determined DA, NE, EPI, and DOPAC in rat brain, heart, kidney, etc., with tissues weighing 40—500 mg. Oke et al. [12] studied the effect of neonatal 6-hydroxydopamine treatment on levels of DA and NE in discrete rat brain regions. Freed and Asmus [3] determined DA, NE, and DOPAC in rat brain samples with approximately 35 mg of the tissue. In the present paper sample size is 1—2 mg.

The sample preparation generally involves two steps: separation of proteins and high-molecular-weight compounds by precipitation followed by the isolation of catecholamines and their metabolites from other organic compounds present in the samples by adsorption or extraction. Isolation of catechols by selective adsorption on alumina is the most frequently used method. The alumina is then washed, compounds are eluted and injected on the liquid chromatography (LC) column. Felice and Kissinger [6] determined HVA in urine samples by combining the techniques of solvent extraction, thin-layer chromatography (TLC) and LC. Sasa and Blank [10] reported a butanol extraction for the determination of DA, NE, and 5-hydroxytryptamine (5-HT) in rat brain samples. Soldin and Hill [7] determined HVA and VMA in urine using anion-exchange resin for the clean-up of the samples.

In the present paper we report simple procedures for the analysis of dopamine and for the analysis of the acid metabolites of dopamine, HVA, and DOPAC, which are easy to perform and require a minimum number of steps. For the determination of DA, the tissue samples, punched from slices of rat brain striatum and weighing 1—2 mg, were homogenized in trichloroacetic acid (TCA) solution and the proteins were precipitated by centrifugation. Other acidic or neutral organic compounds were extracted into isooctane and the organic layer was aspirated. The aqueous layer was directly injected on the LC column. For the DOPAC and HVA analysis, proteins were precipitated similarly and the acids were extracted into ethyl acetate. The organic layer was dried under vacuum, the residue dissolved in the mobile phase and injected on the LC column.

EXPERIMENTAL

Materials

Dopamine hydrochloride and DOPAC were purchased from Sigma (St. Louis, MO, U.S.A.). HVA, 3,4-dihydroxycinnamic acid (DHCA) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were from Aldrich (Milwaukee, WI, U.S.A.). Other chemicals used were all analytical reagent grade and were used without further purification.

Liquid chromatograph

A Waters Model 6000A solvent delivery system was used with a Chromatronix Model HPSV sampling-valve injector and a thin-layer amperometric detector. The potentiostat/amplifier was built in our laboratory. The detector

consisted of a carbon paste working electrode, Ag/AgCl reference electrode, and a platinum wire as an auxiliary electrode. The carbon paste working electrode was stable for 4—5 weeks in dopamine analysis, where only an aqueous solvent was used. In the HVA and DOPAC analysis, the working electrode was repacked every week. Apparently, the electrode life was shortened by the introduction of methanol into the solvent system.

Dopamine analysis

Chromatographic conditions. A stainless-steel column (1 m × 4.6 mm I.D.) packed with Zipax strong cation-exchange resin (DuPont, Wilmington, DE, U.S.A.), 30—40 μm particle size, was used. The working electrode potential was maintained at +0.61 V vs. the reference electrode. The flow-rate was 0.6 ml/min and the volume injected per sample 10 μl. Analysis was performed at ambient column temperatures and with isocratic elution using citrate—acetate buffer of pH 5.2.

Reagents. Citrate—acetate buffer was prepared by dissolving 8.2 g of anhydrous sodium acetate, 2.1 ml of glacial acetic acid, 4.8 g of sodium hydroxide, 10.5 g of citric acid monohydrate, and 0.37 g of disodium salt of EDTA in 1 l of distilled water. The eluent was degassed by heating the reservoir at 50°C during the analysis.

Stock solutions of dopamine hydrochloride and DHBA were prepared in 0.01 *M* hydrochloric acid containing 0.1% sodium metabisulphite. Standard solutions containing DA free base (0.2—2 ng per 10 μl) and DHBA free base (1.03 ng per 10 μl) were prepared from these stock solutions for standard curves. Hydrochloric acid was deaerated for 15—20 min by passing nitrogen before use. All the solutions were stored at 4°C and stock solutions were prepared fresh every 2 weeks.

For brain tissue analysis a solution of 10% TCA containing 0.1% sodium metabisulphite and 0.95 ng per 10 μl DHBA (free base), as internal standard, was used as the extracting solution.

Sample preparation. Adult male CFE albino rats (Charles River, Wilmington, MA, U.S.A.) were used in all experiments. Animals were killed with a guillotine and the brains quickly removed from the skull and frozen in powdered dry ice. The brains were kept frozen on the stage of a Super Histofreeze (Scientific Products) and five contiguous 1 mm thick sections were cut using a sliding microtome. The landmark for the first section was the nucleus accumbens of AP 10.0 (brain atlas of Pellegrino and Cushman) [13]. As soon as they were cut, the brain slices were transferred to a cold plate and two tissue punches were taken from each slice. These punches were from symmetrical locations in the left and right hemispheres and were combined for further analysis. Fig. 1 illustrates the locations of the samples. Punches were made with stainless-steel tubing of 1.35 mm I.D. The punched samples were transferred to plastic test-tubes containing 250 μl of the extracting solution, immediately homogenized by sonication (Heat Systems Model W-220F) for 5—10 sec and the tubes were placed in ice. These tubes were then centrifuged for 10 min at 2500 *g* to precipitate the protein after which 200 μl of the supernatant were transferred to 1.5-ml micro-test-tubes containing 150 μl of isooctane and shaken for 10 min. The micro-test-tubes were then centrifuged at 15,000 *g* (Eppendorf micro

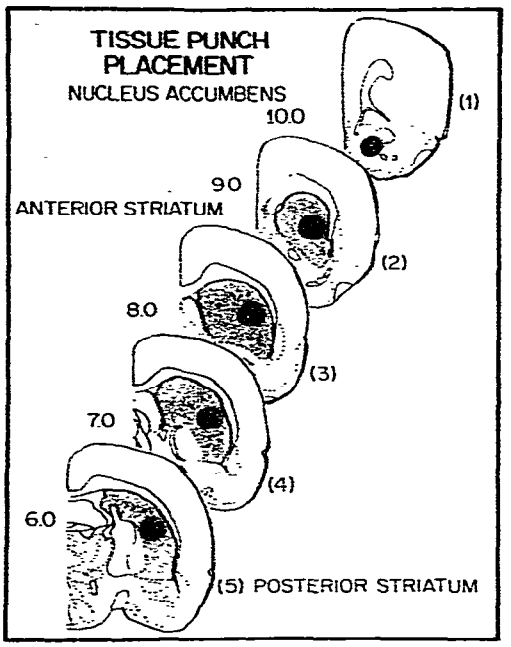


Fig. 1. Tissue punch placement. The first punch was from a 1 mm thick tissue slice containing portions of nucleus accumbens and olfactory tubercle; the next four punches were taken from successive 1 mm thick tissue slices in the striatum (shaded structure). The numbers on the left refer to the anterior—posterior axis coordinates in the brain atlas of Pellegrino and Cushman [13]. Punch diameter, 1.35 mm.

centrifuge) for 4 min and the organic layer was removed by aspiration. Aliquots of 10 μl of the aqueous phase were directly injected into the LC column.

Quantitation. Six standard solutions containing 0.2, 0.4, 0.81, 1.21, 1.61, and 2.02 ng DA per 10 μl with 1.03 ng DHBA per 10 μl were prepared in deaerated 0.01 *M* hydrochloric acid. Standard solutions were run every day before the analysis and curves were prepared by plotting the peak height ratios versus the ratio of the amounts (in ng) of the compound and the internal standard. Dopamine concentrations in the samples as nanogram DA per mg protein are calculated by the following equation:

$$\frac{\text{ng DA}}{\text{mg protein}} = \frac{\dfrac{\text{PH}_{\text{DA}}}{\text{PH}_{\text{DHBA}}} \times \dfrac{0.95 \text{ ng DHBA}}{10\ \mu\text{l}} \times 250\ \mu\text{l}}{\text{slope of standard curve} \times \text{mg protein}}$$

where PH is peak height.

HVA and DOPAC analysis

Chromatographic conditions. A stainless-steel reversed-phase column (25 cm × 2.1 mm I.D.) Zorbax ODS (DuPont), 6—8 μm particle size was used. The potential of the working electrode was set at +0.85 V vs. Ag/AgCl reference electrode. Column temperature was 25°C and the flow-rate 0.4 ml/min. Phos-

phate (0.1 *M*)—citrate (0.05 *M*) buffer pH 4.3 with 10% methanol was used as the mobile phase. The sample loop was 100 μl.

Reagents. For the preparation of phosphate—citrate buffer 14.2 g of disodium hydrogen phosphate and 10.5 g of citric acid monohydrate were dissolved in 850 ml of distilled water and the pH was adjusted to 4.3 by 6 *M* hydrochloric acid. After adjusting the pH 100 ml of methanol were added and the volume was made up to a liter with distilled water. The solvent was degassed by heating the reservoir at 50°C during the analysis.

Stock solutions of HVA, DOPAC, and DHCA were prepared in deaerated phosphate—citrate buffer pH 4.3 (no methanol) which contained 0.1% sodium metabisulphite. Standard solutions containing HVA, DOPAC (0.5—2.5 ng per 100 μl) and DHCA (2.0 ng per 100 μl) were prepared every day for the standard curves. Stock solutions were prepared every two weeks. All the solutions were stored at 4°C.

The extracting solution for brain tissue analysis was prepared as described for dopamine analysis with 2.0 ng per 100 μl of DHCA as internal standard.

Sample preparation. The initial procedure of taking punches was similar to that described for dopamine assay. Tissue punches were homogenized in 250 μl of extracting solution and the tubes were centrifuged for 10 min at 2500 *g*. Aliquots of 200 μl of the supernatant were then transferred to small micro-test-tubes which contained 300 μl of ethyl acetate. These tubes were shaken for 20 min and then 250-μl aliquots of the organic layer were transferred to another micro-test-tube and dried in a vacuum desiccator. The samples were reconstituted in 250 μl of phosphate—citrate buffer pH 4.3 (no methanol) and 100 μl of the sample injected into the LC column.

The precipitate obtained after tissue homogenization and centrifugation was analyzed for protein by the method of Lowry et al. [14] in all analyses.

Quantitation. Five standard solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 ng HVA and DOPAC each per 100 μl with 2 ng DHCA per 100 μl were prepared every day in the phosphate—citrate buffer. Standard solutions were run before the analysis and the curves were prepared as described for dopamine. The concentration of DOPAC and HVA in each sample is calculated as follows:

$$\frac{\text{ng DOPAC or HVA}}{\text{mg protein}} = \frac{\dfrac{PH_{\text{DOPAC or HVA}}}{PH_{\text{DHCA}}} \times \dfrac{2 \text{ ng DHCA}}{100\ \mu l} \times 250\ \mu l}{\text{slope of standard curve}_{\text{DOPAC or HVA}} \times \text{mg protein}}$$

Recoveries of HVA, DOPAC, and the internal standard were examined as a function of extraction time with ethyl acetate. Samples for extraction were prepared by taking non-striatal tissue samples in ten tubes shown to contain no endogenous HVA and DOPAC, and adding known amounts of HVA, DOPAC and internal standard in 10% TCA containing 0.1% sodium metabisulphite. The samples were extracted for periods of 10, 20, and 60 min. Standard solutions were prepared in buffer containing equal amounts of the three compounds as in the samples. Recoveries were calculated by comparing the peak heights of the standard and the samples. The recoveries with the standard

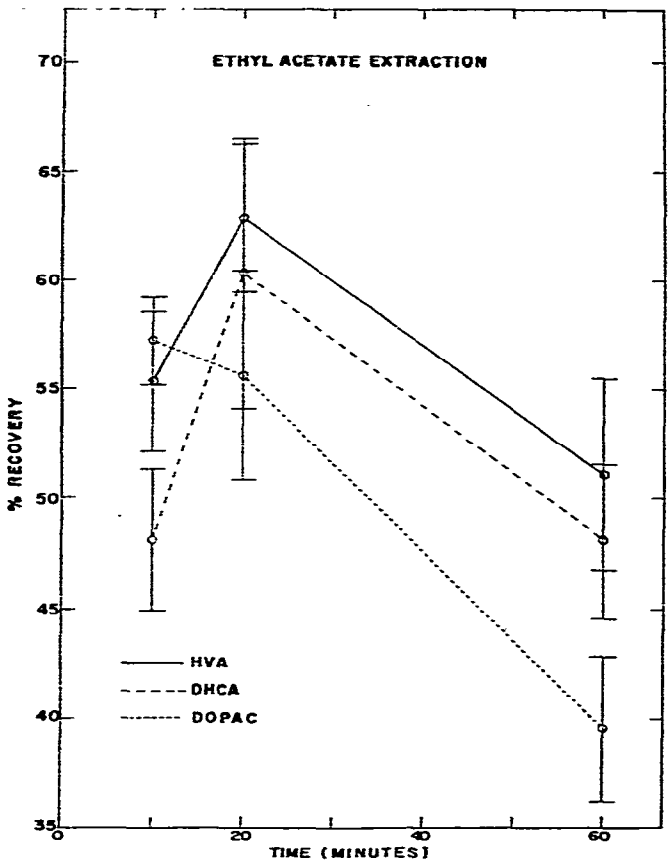


Fig. 2. Recoveries of HVA, DOPAC and the internal standard as a function of extraction time with ethyl acetate (n = 6—9).

deviations are given in Fig. 2. Samples extracted with ethyl acetate for 20 min gave the best recoveries. Longer time periods (1 h) gave low recoveries due to the loss by oxidation. Note that maximum recovery is 67% based on volumes transferred.

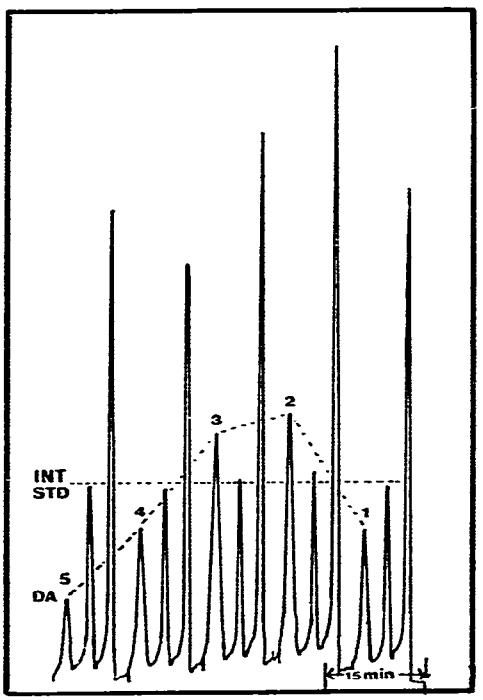
RESULTS AND DISCUSSION

Dopamine

Fig. 3 shows a chromatogram of five samples (from five different regions) for one rat. Retention times for DHBA and DA are 7 min and 10 min, respectively. The total analysis time per sample was 12 min.

HVA and DOPAC

Fig. 4 shows a chromatogram of a brain sample. The first peak after the solvent front is dopamine which is extracted into the ethyl acetate and has a retention time of 4 min. Approximately 2% of the total dopamine is extracted.



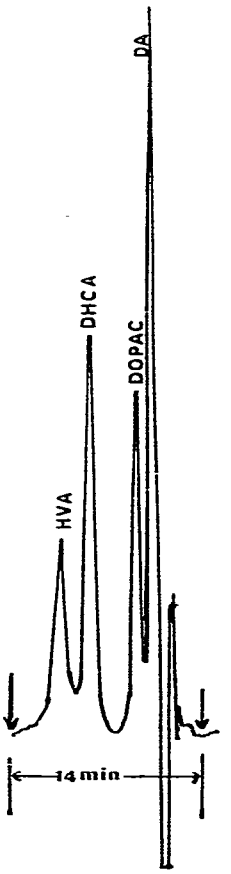


Fig. 3. A chromatogram of five samples (from five different regions) for one rat. Column, Zipax SCX (1 m × 4.6 mm I.D.); mobile phase, citrate—acetate buffer pH 5.2, flow-rate 0.6 ml/min; column temperature, 25°C.

Fig. 4. Chromatogram of a brain sample for HVA and DOPAC analysis. Column, Zorbax ODS (25 cm × 2.1 mm I.D.); mobile phase, phosphate (0.1 *M*)—citrate (0.05 *M*) buffer pH 4.3, 10% methanol, flow-rate, 0.4 ml/min; column temperature, 25°C.

Chromatographic conditions

Effects of temperature, pH and concentration of methanol on the retention time of HVA and DOPAC were studied. Retention time of HVA decreases very significantly with increase in column temperature, concentration of methanol and the pH of the solvent, whereas only slight decrease in retention time is observed in case of DOPAC. Phosphate (0.1 *M*)—citrate (0.05 *M*) buffer pH 4.3 with 10% methanol and column temperature at 25°C were found to be suitable for the analysis of HVA and DOPAC. Under the conditions described here for the analysis the retention times of DOPAC, DHCA, and HVA were 5, 8, and 10 min, respectively. The time for each sample run was 14 min.

Detector stability

The detector was more stable in the aqueous solvent of the DA analysis than in the 10% methanol of the HVA and DOPAC analysis. In the DA analysis, the detector was checked each day by running standard solutions. The standard solutions were stable for two weeks and were used in calibration. Slopes of the standard curves changed less than 7% over a period of one month. In the HVA and DOPAC analysis, the slope changed 15% during a two-week period. Therefore for the data reported, the detector was repacked every week and standard solutions prepared every day.

The least detectable amounts were 250 pg for DA, 100 pg for HVA, and 66 pg for DOPAC. Both analysis procedures gave chromatograms without any interfering peaks from other compounds present in the samples.

Figs. 5 and 6 show levels of DA, HVA, and DOPAC in the five sections of rat brain. The first section is from the nucleus accumbens. The next four sections are from the striatum with sections 2 and 5 being the anterior and the posterior striatum, respectively. The gradient in these sections of striatum is very clear in

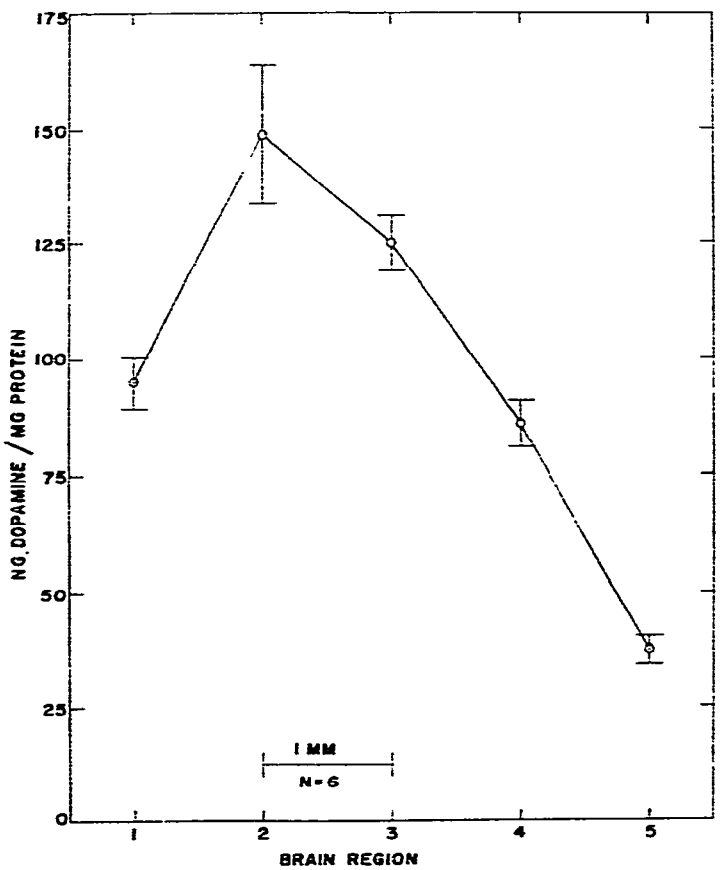


Fig. 5. Average (± S.D.) values of ng dopamine per mg protein in each brain region. Region 1 is the nucleus accumbens, regions 2—5 are the striatum with regions 2 and 5 the anterior and posterior striatal regions, respectively.

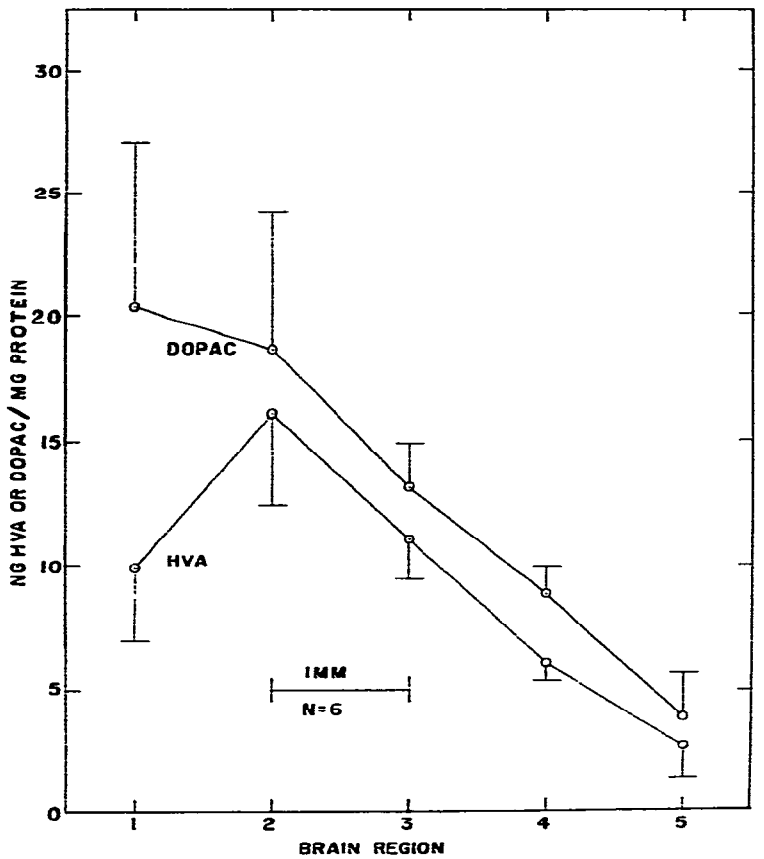


Fig. 6. Average (± S.D.) values of HVA and DOPAC (ng/mg protein) in the regions described in Fig. 5.

all cases. The dopamine content of the nucleus accumbens region 1 of Fig. 1, was found to be 95 ± 6 ng per mg protein. The anterior striatum, region 2, was found to have 149 ± 15 ng per mg protein. Regions 3, 4, and 5 from the striatum had 125 ± 6, 86 ± 5 and 37 ± 3 ng per mg protein respectively (n = 6). HVA and DOPAC were present in much lower concentration. For regions 1—5, HVA content was 9.9 ± 2.9, 16.1 ± 3.7, 11.0 ± 1.6, 6.1 ± 0.8, and 2.7 ± 1.4, respectively (n = 6). DOPAC content was 20.3 ± 6.7, 18.7 ± 5.5, 13.1 ± 1.8, 8.8 ± 1.1, and 3.9 ± 1.8, (n = 6) for the same regions. Tassin et al. [15] observed a similar gradient in dopamine levels (100—40 ng per mg protein) in these regions using a radioenzymatic method. Koslow et al. [16] reported a gas chromatography—mass spectrometry method for dopamine determination and also found an anterior—posterior gradient. Holdiness et al. [17] also showed the gradient in these regions with dopamine levels in the range of 165 to 75 ng per mg protein. The levels of DA reported here are in general agreement with the levels reported earlier.

The procedure described for DA analysis is simple and easy to perform. The method is rapid and 30—40 samples (6—8 rats) can be run in a routine day.

The procedure for HVA and DOPAC analysis also involves only a few simple steps and can be performed rapidly. 20—25 samples (4—5 rats) can be analyzed in a routine day.

The ability to determine levels of DA and its metabolites in small samples of brain tissue by the relatively simple and inexpensive technique described herein should facilitate functional studies of brain dopaminergic systems. Studies which have demonstrated surgically or drug-induced changes in whole striatal DA, HVA, and DOPAC [18—20] can now be extended to subregions of striatum and other areas. For example, we have found [21] that changes in behavior produced by lesions in striatum in rat correlate with resulting DA depletions in striatal subregions.

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