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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF DOPAMINE IN SUBREGIONS OF RAT BRAIN

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SUMMARY

A quantitative gas chromatographic–mass spectrometric assay was developed for the determination of dopamine in subregions of rat brain. Five tissue punches weighing approximately 1.7 mg each were taken from the nucleus accumbens and four neostriatal regions differing in anterior–posterior level. The dopamine extracted from the tissue was treated with pentafluoropropionic anhydride (PFP) and quantitatively formed dopamine-$(PFP)_3$. The derivatizing procedure took 15 min and the retention time for dopamine-$(PFP)_3$ and its deuterated analogue [1,1,2,2-$^2H_4$]dopamine-$(PFP)_3$) was 120 sec. Selective ion monitoring was utilized to monitor the gas chromatographic effluent. Ions were generated by electron impact ionization. The assay was able to measure concentrations of 1 nanogram dopamine per milligram protein. An anterior–posterior gradient of dopamine was observed in the striatum. This assay should be useful in studies examining the effects of experimental manipulations on dopamine content in relatively small areas of brain tissue.

INTRODUCTION

Much experimental effort has been expended to evaluate the physiological and behavioral functions of brain neuronal systems utilizing dopamine (DA) as a neurotransmitter. Studies using fluorescence histochemistry (Ungerstedt[1], Lindvall and Björklund[2]) have shown the major brain DA systems to originate from cell bodies in midbrain which project their axons to terminate in several forebrain areas, particularly neostriatum (caudate and putamen and nucleus accumbens/olfactory tubercle. This projection is topographically organized, with different cell groups in the midbrain synapsing upon different structures and portions of structures in forebrain (Fallon and Moore[3]).

In order to evaluate the effects of experimental manipulations (lesions, stimulation, drugs, etc.) on subcomponents of the DA projection, it is necessary to be able to

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determine DA levels in small regions of the forebrain terminal areas. Several analytical methods have been developed for the determination of dopamine in small (less then 5 mg) samples of rat brain tissue. Enzymatic isotopic methods of Brownstein *et al.*[4] have reported dopamine levels of 96.6 ± 6.5 ng/mg protein in nucleus caudatus and 88.4 ± 10.3 ng/mg protein in caudate-putamen obtained from stainless steel punches. Tassin *et al.*[5] measured a progressively decreasing content of endogeneous dopamine (100–40 ng/mg protein) in microdiscs from the anterior to posterior neostriatum.

Recently, mass spectrographic methods have become available for dopamine assays. Koslow *et al.*[6] reported a gas chromatographic–mass spectrometric (GC–MS) method for dopamine determination using alpha-methyldopamine as internal standard and measured an anterior–posterior gradient in punched samples of the nucleus caudate-putamen. Freed *et al.*[7] have used a direct inlet probe chemical ionization method with [$^2H_2$]dopamine as internal standard and determined an average value for whole rat corpus striatum of 9.49 ± 0.46 ng/mg tissue. Other GC–MS methods, with [$^2H_3$]dopamine as internal standard, have reported 8.11 ± 0.15 ng/mg tissue in each pair of striata (Wiesel[8]), and 12.73 ± 1.50 ng/mg tissue using one whole rat caudate nucleus tissue for each sample (Kilts *et al.*[9]).

This paper describes a GC–MS assay for dopamine in subregions of rat neostriatum. In this study [1,1,2,2-$^2H_4$]dopamine was used as the internal standard with pentafluoropropionic anhydride (PFP) as the derivatizing agent.

EXPERIMENTAL

Materials

Analytical grade [1,1,2,2-$^2H_4$]dopamine obtained from Merck & Co., Rahway, NJ, U.S.A. was used as the internal stanard. This solution was made by dissolving the isotope (final concentration of 91.9 ng [$^2H_4$]dopamine per 175 μl of solution) in 100 ml of deionized water with 10% trichloroacetic acid (Sigma, St. Louis, MO, U.S.A.) and 0.1% sodium metabisulfite (Fisher Scientific, Pittsburgh, PA, U.S.A.). Calibration plots were made with dopamine obtained from Sigma Chemical Company. The derivatizing agent used was PFP (Pierce, Rockford, IL, U.S.A.); however, other derivatizing agents such as trifluoroacetic anhydride (TFA) and heptafluoropropionic anhydride (both from Pirece) could be used, although the TFA derivative is somewhat less stable.

Instrumentation

MS was accomplished using a Finnigan 4000 GC/MS quadrupole mass analyzer spectrometer with a Model 6000 automated data system. GC was performed on a 1.8 m × 2 mm I.D. glass column with 3% OV-17 on Gas-Chrom Q, 100–120 mesh. The column temperature was maintained at 160°C, injection port at 175°C, jet separator at 190°C and the ion source at 250°C. The voltage of the electron multiplier was 1800 volts with an ionization potential of 70 eV. The ions were measured at *m/e* 428, 428.1 and 431, 431.1 for dopamine and [$^2H_4$]dopamine, respectively. A Varian Cary 118 ultra-violet–visible spectrophotometer was used for the protein assay with a fixed wavelength of 725 nm.

Sample Collection

Adult male CFE albino rats (Charles River, 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 at AP 10.0 (brain atlas of Pellegrino and Cushman[10]). 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. Punches were made with stainless steel tubing of 1.35 mm I.D. The punched samples were transferred to plastic test tubes containing 175 μl of the standard solution, immediately homogenized by sonication (Heat Systems Model W-220F) for 5–10 sec and the tubes placed in ice. These tubes were then centrifuged for 10 min at 500 *g* to precipitate the protein after which 125 μl of the supernatant was removed, placed in 1.5 ml micro test tubes and shaken for 10 min with 150 μl of isooctane. The micro tubes were centrifuged at 15,000 *g* (Eppendorf micro centrifuge) for 4 min and the organic layer discarded. The samples were placed in a desiccator and nitrogen was passed into the desiccator for 10 min. The samples were then vacuum-dried and derivatized with 20 μl of derivatizing agent (50% ethyl acetate and 50% PFP). All samples were run immediately using 1 μl injection. Retention time of dopamine and [$^2H_4$]dopamine was 120 sec. The tissue precipitate was analyzed for protein as described by Lowry *et al.*[11] and the tissue concentration of dopamine is reported on the basis of milligrams protein content.

To determine the relative intensity of the ions to be monitored, microgram samples of the hydrochloride salts of dopamine and $^2H_4$-dopamine were weighed (Kahn electrobalance) in various ratios and the mixtures analyzed by measuring the intensity of ions 428 and 431. A graph of intensity ratio (as measured by the area of each selected ion monitoring (SIM) signal) *versus* the ratio of amounts of dopamine and [$^2H_4$]dopamine was constructed (Fig. 1). A least-squares fit ($R = 0.997$) of the data was performed, resulting in the following equation:

$$\text{ng DA} = 0.749 \frac{\text{Area DA}}{\text{Area D4DA}} + 0.041 \text{ ng D4DA} \quad (1)$$

Eqn. 1 was used to calculate the dopamine content of the tissue samples.

RESULTS AND DISCUSSION

Ion intensities

A graph of the ion intensity ratio of dopamine and [$^2H_4$]dopamine *versus* the molar ratio of the two compounds should result in a slope of unity if each structure generates ions of equal intensity. Even on a weight basis the slope should be within 2.1% of unity. As the graph indicates, however, the deuterated dopamine generates an ion of significantly lower intensity at *m/e* 431 than did the dopamine at *m/e* 428. This can be understood by examining Fig. 2 where the fragmentation leading to these ions is depicted for a general catecholamine structure. It can be seen that the ions are

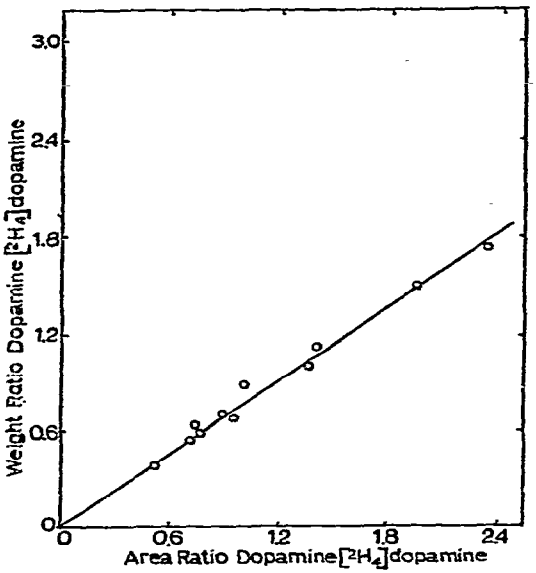

Fig. 1. Graph of intensity ratio *versus* area ratio for dopamine and [$^2H_4$]dopamine.

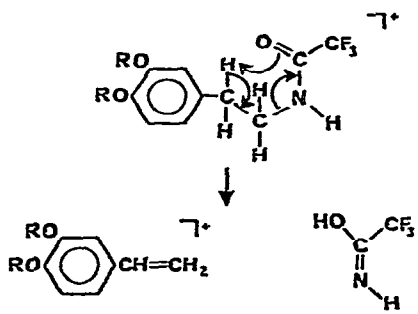

Fig. 2. McLafferty rearrangement of general catecholamine structure leading to ions monitored in SIM of catecholamines.

formed through a McLafferty rearrangement[12] in which a hydrogen or deuterium atom migrates from the beta carbon to the carbonyl oxygen through a six membered transition state. Thus the difference in ion intensities is due to a deuterium isotope effect. The deuterium isotope effect arises from several contributing factors. The most important of these are (1) the difference in zero-point energy between a bond to hydrogen and the corresponding bond to deuterium, which is on the order of 1.2–1.5 kcal/mole, and (2) the reduced velocity of passage over the potential energy barrier of a reaction involving displacement or loss of deuterium in place of hydrogen[13]. The first effect, that of lower zero-point energy, is less important in a reaction of the type in Fig. 2 because a new bond with the leaving atom is being formed as the old one is broken, cancelling the zero point effect to some extent.

When energy is readily available, such as at high temperature or after ionization with 70 eV electrons, the isotope effect should approach the square root of the

mass ratio, in this case 1.4[13]. When the data of Fig. 1 are calculated on a molar ratio basis, the isotope effect is found to be 1.37, which is in good agreement with the square root of the mass ratio.

Dopamine assay

Fragmentation patterns of dopamine-$(PFP)_3$ have been previously described by Koslow *et al.*[14] and Gelpi *et al.*[15]. As stated earlier an additional mass fragmentogram at one tenth of an a.m.u. higher mass was monitored for the dopamine and the internal standard to increase the signal to noise ratio. When the areas for each pair were calculated, errors in the data were easily checked and precision was improved over measuring 428 and 431 only. In Fig. 3, a mass fragmentogram from one of the samples obtained from rat striatum is shown. Assurance of specificity was obtained by the fact that the chromatographic retention time of the endogenous compound was identical to that of its deuterated analog.

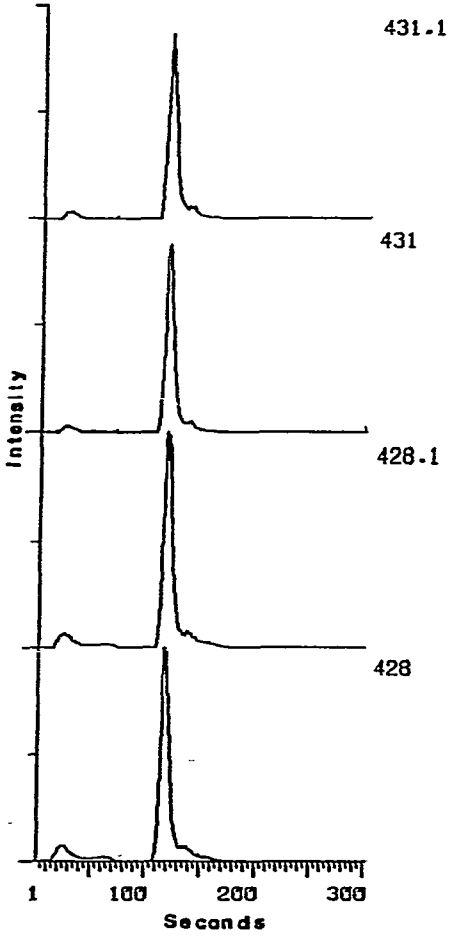

Fig. 3. Selected ion monitoring of ions 428 from dopamine and 431 from deuterated dopamine. The signal was also monitored at 428.1 and 431.1 to improve the signal to noise ratios.

In Fig. 4, the tissue punch placement can be seen. The first punch was a 1 mm-thick tissue slice containing the nucleus accumbens and a portion of olfactory tubercle; the next four tissue punches were taken from the four successive 1 mm-thick tissue slices in the striatum. Fig. 5 is a plot of ng dopamine/mg protein from the five contiguous sections of tissue; the anterior–posterior striatal gradient observed in this study agrees with Koslow *et al.*[6] and Tassin *et al.*[5].

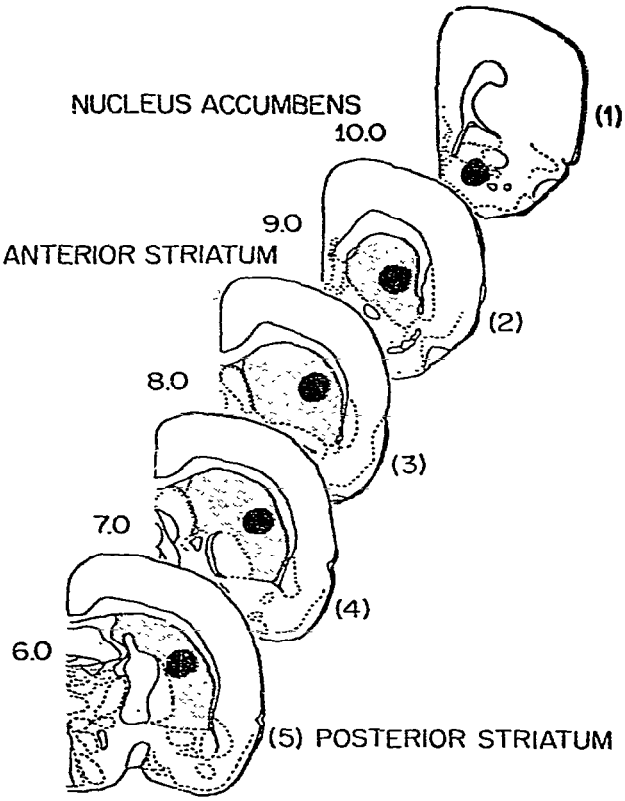

Fig. 4. Tissue punch placement. The first punch was from a 1-mm thick tissue slice containing portions of nucleus accumbens and olfactory tubercle; the next four punches were taken from successive 1-mm thick tissue slices in the striatum (shaded structure). The numbers on the left refer to the anterior–posterior axis coordinates in the brain atlas of Pellegrino and Cushman[10]. Punch diameter, 1.35 mm.

Dopamine loss in the interval between sacrifice and sonication does not seem to be a major problem. Moleman *et al.*[16] found very little change in dopamine levels in dissected and undissected rat striata a few minutes after killing the rat and removing the brain. A 15% decrease in dopamine level was observed in dissected tissue 20 min after removal of the brain although this effect was not statistically significant. In our samples the tissue was sonicated within 5 min after sacrifice.

Protein assay

The average weight of representative tissue samples in the combined punches

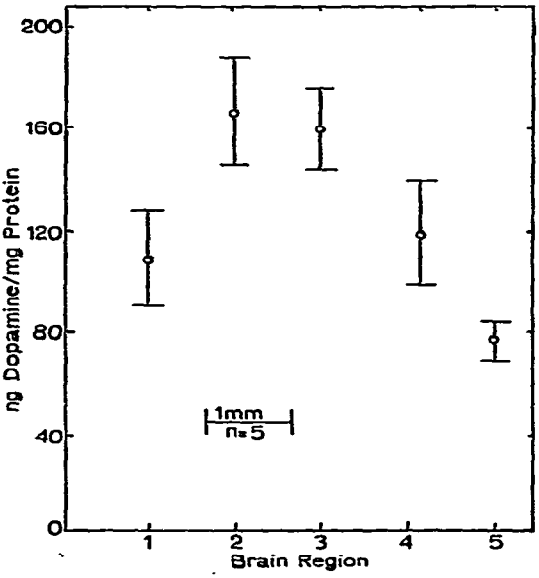

Fig. 5. Average (± S.D.) values of ng dopamine/mg protein in each brain region. Region 1 is the nucleus accumbens, regions 2–5 are the striatum with regions 2 and 5 the anterior and posterior striatal regions, respectively.

(one punch from the right hemisphere and one punch from the left hemisphere) from each brain section was 1.72 mg tissue (wet weight). An average of 0.224 mg protein/mg tissue was obtained in this study.

CONCLUSION

This method is useful for determination of dopamine in different DA terminal areas of rat brain. As found by others[5,6] an anterior–posterior gradient of DA content was observed in the neostriata of the rat brains in this study. A large number of samples can be run on a daily basis (*e.g.*, 35–50 samples from 7–10 rats). The GC–MS method is sensitive and specific. The limit of detection was 1 ng dopamine per mg protein. The assay described in this paper has been found useful in testing the hypothesis that the dopaminergic innervations of subregions of the striatum are functionally different[17].

The results of this assay demonstrate that levels of dopamine observed depend on the region of tissue sampled. This observation has important implications in functional studies of brain dopamine systems. For example, we have found that the degree of correlation between levels of dopamine in striatum and various behaviors is enhanced if dopamine in subregions of striatum is considered rather than total striatal dopamine[17].

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