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SHORT COMMUNICATIONS

Effect of 6-hydroxydopamine on dopa and tyrosine entry into brain

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The drug, 6-hydroxydopamine (OHDA), has become an extremely valuable tool in brain research [1–4]. This drug can selectively destroy catecholaminergic nerve fibers leaving other types of nerve fibers relatively intact. It is generally assumed that animals treated with the drug differ from normal animals only by the absence of catecholamine neurons. I have studied the entry of labeled tyrosine into various regions after injection into the carotid artery, comparing normal rats with those previously treated with 6-hydroxydopamine. This method of study was developed by Oldendorf [5] and gives information not available from conventional uptake studies employing brain slices and homogenates, where the interface between blood and brain cells has been obliterated. The Oldendorf technique compares removal of a ^{14}C-test substance with that of [^{3}H]H_2O (THO) during a single passage through the cerebral circulation. Only a very few studies on uptake *in vivo* of amino acids by brain have been published where the amino acid concentration presented to brain was controlled [5–7], and these did not consider possible regional differences. With the Oldendorf technique, it has been possible to study regional differences in tyrosine entry into the brain in normal and OHDA-treated rats.

Rats (300–400 g), anesthetized with pentobarbital, 45 mg/kg, were tied down on a frame which kept all four limbs extended. The neck was extended, an incision made in the neck, and the right common carotid artery dissected free by blunt dissection; cotton sutures were passed behind the artery to lift the vessel gently. During dissection, the tissues were kept moist with saline, and dissection and puncture of the artery were facilitated by use of a neurosurgical magnifying loupe ($2\frac{1}{2}\times$). The artery was punctured with a 27 gauge needle attached to a Hamilton syringe. Injection volumes were 100 μl, and two different solutions were used for injecting the radioactive compounds. First, the radioactive materials were added to serum of another rat and injected. Subsequently, the radioactive compounds were suspended in the following artificial solution which was found to give the same extraction index as rat serum and was more convenient to use: (the data given in this paper all derive from use of the following "artificial plasma") NaCl 120 mM, NaH_2PO_4 2.5, KCl 4.5, $MgCl_2$ 2.5, $CaCl_2$ 1.6, Tris–HCl 20, glycine 0.2, L-glutamate 0.2, L-alanine 0.2, and L-threonine 0.4 mM, to which was added bovine albumin (final concentrations) 0.075%, glucose 1 mg/ml, and brought to pH 7.4. The amino acids were added to approximate the concentration of the most abundant amino acids in rat serum [8] to insure that the radioactive tracers were accompanied by some amino acids when they passed through the cerebral circulation. In some experiments, DL[^{14}C]dopa was added to the injectate in place of [^{14}C]tyrosine, but the same concentration and radioactivity were used. In other experiments, additional non-radioactive L-tyrosine was added to the solution to obtain a final concentration of 2 mM. The injection was made rapidly and after 15 sec the rat was decapitated without removal of the needle. Approximately 0.5 ml of blood from the decapitated head was allowed to run into a heparinized tube and the head was then dropped into a bath of dry ice and acetone. Radioactivity of the plasma from the severed head was checked to insure that the injectate had passed through the cerebral circulation and recirculation had not occurred. Animals whose plasma radioactivity exceeded 500 dis./min/ml (either isotope) were excluded from the study because it was not certain that the radioactivity in their brain represented extraction from a single pass through the cerebral circulation. Four of thirty otherwise satisfactory experiments were excluded for this reason.

The brain was dissected on a slab of dry ice [9]: cortex was represented by a slab of parietal cortex, trimmed free of underlying white matter, weighing 100–200 mg; olfactory cortex was taken from the orbital surface of the brain, and included olfactory tubercle, piriform cortex and olfactory tract as far caudally as the anterior commissure, according to the atlas of Konig and Klippel [10] and weighed 60–100 mg on each side; hypothalamus weighed 90–100 mg total; striatum weighed 80–90 mg and included the globus pallidus, but not septal nuclei; thalamus weighed 100–125 mg total; midbrain included the structures from subthalamus to the caudal portion of the inferior colliculus and weighed 80–100 mg. The striatum and cortex were taken separately from each hemisphere, while thalamus, hypothalamus and midbrain were taken as single structures. Each specimen was homogenized in 10 vol. of 10% trichloroacetic acid (TCA) and the supernatant used for liquid scintillation counting in a toluene–Triton X-100/PPO* scintillator solution [11]. The specimens were homogenized quickly to minimize evaporative loss of THO. Because double label counting required sufficient counts in order to obtain an accurate ratio of activities for the two isotopes, vials were counted for 20 min and any vials which failed to reach 10,000 counts in the ^{14}C-channel were recounted for 50 min. The cortical specimen contralateral to the side of injection is not included in the tables because its radioactivity was insufficient for proper analysis in almost all cases, typically being 5–15 per cent of the activity of the cortex on the side injected. By using equal amounts (2 to 2.5 μCi) of ^{3}H and ^{14}C in the injection mixture, it was not necessary to compute a "brain uptake index" as used by Oldendorf: the ratio of ^{14}C and ^{3}H found in the tissue indicated the relative extraction of the ^{14}C-amino acid. Two of the thirty experiments which had been felt to be technically satisfactory had to be discarded because there was very little radioactivity in the brain; the second of these rats was found to contain approximately 85 per cent of the injected radioactivity still in the carotid artery at the site of injection. For subsequent experiments, the injection site was dissected out, homogenized, and counted, but never found to contain more than 5 per cent of the injected radioactivity. Flow through the vessel appeared normal so long as the needle was left *in situ* after the injection. The fact that six of thirty apparently successful experiments had to be discarded indicates that this technique requires careful checking to insure reproducible results.

Uniformly labeled [^{14}C]L-tyrosine was obtained from New England Nuclear Corp. (382 mCi/m-mole), tritiated water (THO) from Mallinckrodt Chemical Corp. (500 mCi/m-mole), DL-dopa[3-^{14}C] (58 mCi/m-mole) came from Schwartz–Mann, and L-dopa[^{14}C] (uniformly labeled, 330

* PPO = 2,5-diphenyloxazole.

Table 1. Comparison of extraction of [^{14}C]tyrosine by brain of normal and 6-hydroxydopamine-treated rats*

Region	Controls (9)	OHDA (8)
Sensorimotor cortex	0.392 ± 0.016	0.360 ± 0.025
Olfactory cortex	0.412 ± 0.028	0.360 ± 0.019†
Hypothalamus	0.450 ± 0.025	0.379 ± 0.021†
Striatum	0.453 ± 0.029	0.364 ± 0.017‡
Thalamus	0.407 ± 0.015	0.366 ± 0.019§
Midbrain	0.422 ± 0.017	0.349 ± 0.021†
Opposite striatum	0.408 ± 0.025	0.375 ± 0.021†

* After injection of equal amounts of THO and [^{14}C]L-tyrosine into the carotid artery, rats were decapitated, their brain regions were rapidly dissected and the extraction of ^{14}C-amino acid relative to THO was determined from the ratio of the two isotopes found in homogenates of the brain regions. Values given are means ± S. E. M. and numbers in parentheses refer to the number of rats used. Significance tests on the difference between control and experimental values for each region were done by the Mann–Whitney test [13].

† Difference significant at the P 0.05 level.

‡ Difference significant at the P 0.01 level.

§ Difference significant at the $0.10 \leq P \leq 0.05$ level.

mCi/m-mole) was from New England Nuclear. Non-radioactive amino acids were from Sigma Chemical Co.

Because the ratio of two normally distributed quantities is not itself ordinarily normally distributed [12], it was felt most appropriate to analyze the data by non-parametric methods. The Mann–Whitney signed ranks test [13] was used for comparison between the control and OHDA-treated groups, whereas multiple comparisons between the means of several different groups were made by means of the rank sum multiple comparison method of McDonald and Thompson [14].

Drug treatment. The OHDA-treated rats were given two separate injections of 250 μg (as the base) of 6-hydroxydopamine hydrobromide (Regis Chemical Co.) stereo-tactically into the left lateral ventricle, while anesthetized with ether. The drug was dissolved in 0.9% saline containing 0.10% ascorbic acid. The injections were given 2 or 3 days apart and testing followed 7–10 days after the second injection. Control rats received two separate injections of the saline vehicle.

Two methods were used to estimate the changes in catecholaamines produced by OHDA pretreatment. Aliquots of TCA homogenates of striatum and hypothalamus from control and experimental rats were shaken with ether to remove TCA and were then applied to 3.0 × 0.6 cm columns of Amberlite CG-50 resin, Na^+ form. Tyrosine was not retained by the resin and was measured by the method of Udenfriend [15]. Dopamine and norepinephrine were eluted from the resin with 2 N acetic acid and measured by the fluorimetric assay of Laverty and Taylor [16].

Additional rats received OHDA or vehicle injections but no carotid injections. They were anesthetized with pentobarbital, decapitated and their brains dissected and homogenized in 5 mM Tris–HCl, pH 7.0, containing 0.1% Triton X-100. Tyrosine hydroxylase was estimated by the procedure of Coyle [17] with these modifications: no pteridine reductase was added, pH of the KH_2PO_4 buffer was 6.2, and the final concentration of 6,7-dimethyltetrahydropteridine ($DMPH_4$) was 0.6 mM. Portions of the homogenates were assayed for tyrosine [15] and plasma tyrosine was also measured in blood obtained from the severed neck vessels.

The amine fraction was isolated by column chromatography after [^{14}C]dopa injections. Aliquots of the TCA homogenate were extracted with ether and sodium acetate buffer, 0.25 M, pH 6.0, was added to bring the pH to 6. The homogenates were now poured over Amberlite CG-50 columns, Na^+ form, 4 × 0.6 cm, buffered to pH 6.0, and were washed with 10 ml water. The amine fraction was eluted with 1.5 N HCl and aliquots were taken for liquid scintillation counting. In these experiments, sodium metabisulfite was added to the TCA prior to homogenizing the tissue to retard amine oxidation.

The absolute amount of THO or amino acid found in any region varied considerably, but the ratio between THO and ^{14}C-amino acid was much less variable than was the absolute number of dis./min found in each tissue. Table 1 compares the extraction of 1.3 μM tyrosine (relative to extraction of THO) for control and 6-hydroxydopamine-treated rats. Every region studied showed less extraction of labeled tyrosine in the 6-hydroxydopamine-treated group, and these differences were statistically significant in all regions except for cerebral cortex and thalamus. In addition to comparing the values for experimental and control groups for each brain region, different regions within each group were also compared. Analysis of variance and multiple comparison testing indicated that the tyrosine extraction index for striatum and hypothalamus was significantly different from that ($P \leq 0.05$) of the other brain regions among the control group, and that the small difference between striatal and hypothalamic extraction was not significant. The extraction values are similar for all regions in the 6-hydroxydopamine-treated animals, and analysis of variance indicated no significant difference between the brain regions of the drug-treated rats, in contrast to the controls.

Table 2 gives the brain extraction index for [^{14}C]tyrosine at two higher concentrations of [^{14}C]tyrosine and in the presence of a large excess of L-alanine (5 mM). The decreasing extraction with increasing tyrosine concentrations indicates a saturable process in all regions studied.

Table 2. Brain extraction index for [^{14}C]tyrosine at two concentrations of [^{14}C]tyrosine and in the presence of a large excess of L-alanine*

Region	[^{14}C]L-tyrosine, 100 μM (3)	[^{14}C]L-tyrosine, 2 mM (3)	[^{14}C]L-tyrosine, 1.3 μM, +5 mM L-alanine (3)
Sensorimotor cortex	0.206 ± 0.038	0.115 ± 0.032	0.126 ± 0.045
Olfactory cortex	0.187 ± 0.040	0.135 ± 0.020	0.123 ± 0.033
Hypothalamus	0.239 ± 0.031	0.185 ± 0.021	0.175 ± 0.049
Striatum	0.188 ± 0.029	0.169 ± 0.028	0.143 ± 0.030
Thalamus	0.215 ± 0.040	0.121 ± 0.019	0.128 ± 0.044
Midbrain	0.221 ± 0.033	0.158 ± 0.022	0.141 ± 0.025

* Normal rats were injected with equal amounts of [^{14}C]L-tyrosine and THO. The values given are means ± S. E. M.; the numbers in parentheses refer to the number of rats used. For details of procedure, see legend to Table 1.

Table 3. Brain extraction of [^{14}C]dopa*

Region	[^{14}C]DL-dopa, 1.1 μM (6)	[^{14}C]L-dopa, 1.5 μM (5)	[^{14}C]L-dopa, 1.5 μM, in OHDA-treated rats (4)
Sensorimotor cortex	0.241 ± 0.032	0.374 ± 0.036	0.359 ± 0.040
Olfactory cortex	0.263 ± 0.024	0.360 ± 0.038	0.371 ± 0.031
Hypothalamus	0.303 ± 0.030	0.406 ± 0.039	0.423 ± 0.026
Striatum	0.270 ± 0.028	0.387 ± 0.031	0.360 ± 0.019†
Thalamus	0.255 ± 0.027	0.369 ± 0.032	0.388 ± 0.029
Midbrain	0.230 ± 0.039	0.399 ± 0.030	0.405 ± 0.035

* Values are means ±S.E.M.; numbers in parentheses refer to the number of samples.
† Difference from control [^{14}C]L-dopa regional extraction significant at $0.10 \leq P \leq 0.05$.

In Table 3, extraction of [^{14}C]dopa is presented for control and OHDA-treated rats, with data presented for both racemic and L-dopa. In contrast to the effect seen with tyrosine, dopa extraction did not differ between the OHDA and control groups. As with tyrosine (Table 1), the extraction index for [^{14}C]dopa was significantly different between hypothalamus and striatum and all other regions studied. Analysis of variance indicates that this difference was significant at the $P \leq 0.05$ level. Table 4 gives the percentage of [^{14}C]dopa converted to amines in three brain regions for both control and OHDA-treated rats. Although this radioactivity may have been norepinephrine and epinephrine in addition to dopamine, it would all have derived ultimately from decarboxylation of [^{14}C]dopa. The table indicates a surprising finding: the percentage of radioactivity in the brain homogenate associated with the amine fraction did not differ between cortex, striatum and hypothalamus, nor did OHDA treatment alter this percentage in any of regions studied.

Table 5 provides data verifying the depletion of catecholamines by the OHDA treatment. Large decreases in tyrosine hydroxylase activity, dopamine and norepinephrine concentrations were observed. Tyrosine content of the brain regions was not decreased in the OHDA group, and actually increased in the striatum, although the increase is of marginal significance. Plasma tyrosine was not altered by the OHDA treatment (data not shown).

This study indicates a small but consistent regional difference in the extraction of low concentrations of tyrosine by brain. When tyrosine extraction was studied at a higher concentration, 100 μM, very close to the measured mean plasma tyrosine concentration of 92 μM, only hypothalamus had an extraction different from the other brain regions, but the number of rats studied was small (Table 2). The fact that OHDA pretreatment lowered tyrosine extraction in all brain regions and abolished the regional differences in tyrosine uptake seen in the controls suggests that these regional differences may be somehow related to catecholamine neurons. The amount of THO/g of tissue did not differ between cortex, striatum and hypothalamus, so that differences in extraction are not due to differences in THO distribution.

Table 4. Radioactivity of amine fraction after [^{14}C]L-dopa injection into carotid artery*

Region	Control (4)	OHDA (4)
Cortex	34.2 ± 5.9	37.6 ± 7.4
Striatum	32.0 ± 6.3	30.8 ± 7.0
Hypothalamus	38.4 ± 8.2	33.4 ± 6.8

* Values given represent the ^{14}C-radioactivity recovered in the amine fraction of the CG 50 column eluates, expressed as a percentage of the total radioactivity for each region. The concentration of [^{14}C]L-dopa was 1.5 μM. The number in parentheses refer to the number of samples.

Autoradiographic studies would be required to determine which cells retain radioactivity in this type of study, but since nerve cells do not directly contact the blood stream [18], it seems unlikely that the extraction measured is specific for neurons. It is known that OHDA is relatively selective in the doses used here (no effect on gamma amino butyric acid (GABA) or 5-hydropytryptamine (5-HT) concentrations [2, 4], but it is possible that glial cells related to catecholamine neurons might also be destroyed, or circulation in the brain might be altered. If it is estimated that 15 per cent of all striatal neurons, are catecholamine neurons [19], the decreased tyrosine extraction in the OHDA-treated rats (20 per cent less) is consistent with the suggestion that all neurons take up tyrosine approximately equally and that the decreased uptake indicates the proportion of cells lost. This does not agree with the postulate that specific neurons concentrate the precursor for their own transmitter [20–22]. Two points might be made: first, that some tyrosine hydroxylase activity remains after multiple OHDA injeections, indicating the persistence of some catecholamine neurons, and second, that the concept of specific uptake systems for different types of neurons derives from studies *in vitro*, where uptake from blood into brain is not relevant. Oldendorf's important work [6] indicated that substances such as GABA and glutamate, which are avidly taken up *in vitro* [23, 24], are poorly extracted from blood after carotid injections.

It is known that blood flow is greater, per g of tissue, for cortex than for hypothalamus or striatum in both cat [25] and rat [26] and that this difference is reduced by barbiturate anesthesia. The relative blood content of rat cortex is also greater than that of hypothalamus or striatum [27]. These differences would, if anything, produce greater tyrosine accumulation in cortical tissue than in striatum and hypothalamus. For this reason, and also because of the uniformity of regional THO content, the small but consistent regional differences in tyrosine extraction cannot be explained by regional differences in blood flow or vascularity. It is possible that the reduced extraction after OHDA treatment relates in some way to vascular changes. The data suggest a regional difference in dopa uptake, as well as for tyrosine, but the differences are not large, and the OHDA treatment had no clear effect on dopa extraction.

Recent work by Nicklas and Berl [28] indicates that changes in amino acid labeling after intracisternal injections of [1-^{14}C]acetate occur after OHDA treatment. OHDA treatment affects turnover, metabolism and uptake of amino acids in addition to its well known effect on catecholamines. Future research with the drug should control for these additional effects of OHDA.

The relatively uniform distribution of amine radioactivity after [^{14}C]dopa injection and the absence of any change in either dopa extraction or decarboxylation after OHDA treatment (Table 4) were surprising. The enzyme dopa decarboxylase is not uniformly distributed in the brain and its activity has been reported to decrease after

Table 5. Biochemical effects of 6-hydroxydopamine injections*

Region	Tyrosine hydroxylase activity (nmoles/g/hr)		Tyrosine (μg/g)		Dopamine (μg/g)		Norepinephrine (μg/g)	
	Control (4)	OHDA (4)	Control (5)	OHDA (4)	Control (4)	OHDA (4)	Control (4)	OHDA (4)
Cortex	21 ± 4.6	7.5 ± 3.0	15.3 ± 2.1	15.9 ± 3.0				
Striatum	585 ± 51	109.0 ± 48.0	15.1 ± 2.9	20.5 ± 2.7	6.45 ± 0.71	1.95 ± 0.42		
Hypothalamus	131 ± 19	27.0 ± 6.0	17.4 ± 3.8	16.4 ± 3.2			1.14 ± 0.29	0.27 ± 0.14

* Values are means ± S. E. M. and are corrected for recovery, except for tyrosine hydroxylase values. The numbers in parentheses refer to the number of samples.

OHDA [4]. The tissue distribution of endogenous catecholamines in brain is very non-uniform [29]. At this very short time after injection, much of the radioactivity studied is probably outside neurons. Only radioactive amine which is inside monoamine neurons can be stored and protected from degradation by monoamine oxidase. If rats were killed minutes or hours after intra-arterial injection of [^{14}C]dopa, the distribution of amine radioactivity would probably resemble more the distribution of endogenous amines, as was found by Romero *et al.* [30] who examined brain regions 1 or more hr after intraperitoneal injection of L-dopa, 100 mg/kg, in the rat. Large increases in brain dopa were found, but only small changes in the amines were found. Decarboxylation of dopa occurring in capillary and extra-neuronal tissues which lack storage granules may well explain the results of Table 4, but these decarboxylated products should not be able to increase total tissue amines unless a monoamine oxidase inhibitor is present.

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ADDENDUM

After submission of this work for publication, a detailed study of extraction of L-dopa labeled in both the 1- and 3-carbon atoms was published by Wade and Katzman [31]. This careful study also found little regional difference in the decarboxylation of dopa, using loss of the carbon label from [1-^{14}C]L-dopa as an indication of decarboxylation.

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Effect of carbidopa on the metabolism of L-dopa in the pigtail monkey*

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Carbidopa [L-2-hydrazine-α-methyl-β-(3,4-dihydroxy phenyl) propionic acid (MK-486)] is an inhibitor of extracerebral dopa decarboxylase which has been used in combination with L-dopa in the therapy of Parkinson's disease [1–3]. Inhibition of peripheral dopa decarboxylase allows greater quantities of L-dopa to reach the brain where it is then decarboxylated to dopamine, the active metabolite. We have examined the effect of carbidopa on the metabolism of [^{3}H]L-dopa administered intravenously to the pigtail monkey. In a preliminary study [4], the excretion of [^{3}H]dopamine in the urine was markedly reduced by carbidopa, while the urinary excretion of [^{3}H]homovanillic acid, a major dopamine metabolite, was not decreased. It was suggested that, in the presence of carbidopa, L-dopa is converted to homovanillic acid through a pathway which does not involve decarboxylation to dopamine but may involve a sequence of reactions which include *O*-methylation, transamination and oxidative decarboxylation. Recently, a shift in the metabolism of oral L-dopa from decarboxylation to transmination has been reported [5] in Parkinsonian patients treated with carbidopa. Since the sulfate conjugates of dopamine can represent major metabolites of L-dopa in Parkinsonian patients [6–9], the excretion of dopamine 3-*O*-sulfate and dopamine 4-*O*-sulfate was also determined in control and carbidopa-treated monkeys.

Decarboxylation of L-dopa was measured *in vivo* in the unanesthetized monkey in order to determine the effectiveness of carbidopa as an inhibitor of dopa decarboxylase under the conditions of this study. The procedure used by Hansson and Clark [10] for mice was modified for use in monkeys. A pigtail monkey (4.55 kg) was injected in the internal saphenous vein with 0.25 μCi (13.8 μg) of [^{14}C]carboxy-labeled L-dopa (3.56 mCi/m-mole). Exhaled $^{14}CO_2$ was trapped in a solution of 27% phenylethylamine, 27% methanol and 46% toluene at 10-min intervals for 2 hr. Radioactivity was determined in each of the 10-min fractions by liquid scintillation spectrometry [11]. In a separate experiment, more than 2 weeks later, carbidopa, 360 mg/kg, was administered orally by nasal gastric tube in a suspension of 0.5% methylcellulose (adjusted to pH 4.2) 1 hr prior to the injection of [^{14}C]L-dopa. The effect of carbidopa can be seen in Fig. 1. In the untreated monkey, 38 per cent of the radioactivity was exhaled in the first 60 min. During the same interval, only 11 per cent of the label was exhaled by monkeys receiving the decarboxylase inhibitor and, therefore, a 71 per cent inhibition of enzyme activity was achieved. Similar results were obtained when the experiment was repeated in a rhesus monkey (3.7 kg). Similar inhibition of dopa decarboxylase was also seen when pigtail monkeys were pretreated with the inhibitor 2 and 3 hr prior to [^{14}C]L-dopa.

The effect of carbidopa was then determined on the metabolism of [3-^{14}C]L-dopa (labeled on the β-carbon) administered intravenously to pigtail monkeys. A Foley catheter was placed in the urinary bladder of each of three female pigtail monkeys (4.2 to 5.7 kg) and urine was collected continuously. Each monkey was injected with 10–25 μCi (100–250 μg) of [3-^{14}C]L-dopa (sp. act. 20–40 mCi/m-mole) into the internal saphenous vein. Not less than 2 weeks later, the monkeys were treated with carbidopa as described above and the injection with [3-^{14}C]L-dopa was given 2.5 hr later. Carbidopa, 360 mg/kg, was given again 12 hr after the initial drug treatment. Each monkey received [^{14}C]L-dopa four times, twice as a control and twice after carbidopa pretreatment. Urine samples were collected for 24 hr after the injection of [3-^{14}C]L-dopa in bottles containing 200 mg sodium metabisulfite. The metabolites of L-dopa were measured with a liquid chromatography flow monitoring system described by Goodall and Alton [12]. The acidic metabolites, dihydroxyphenylacetic acid, homovanillic acid, vanillactic acid, dopamine 3-*O*-sulfate, dopamine 4-*O*-sulfate and methoxytyramine sulfate, were eluted from a 0.9 by 35 cm Dowex 1 anion exchange column. The effluent from the ion

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