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RAPID AND SENSITIVE METHOD FOR MEASURING NOREPINEPHRINE, DOPAMINE, 5-HYDROXYTRYPTAMINE AND THEIR MAJOR METABOLITES IN RAT BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

DIFFERENTIAL EFFECT OF PROBENECID, HALOPERIDOL AND YOHIMBINE ON THE CONCENTRATIONS OF BIOGENIC AMINES AND METABOLITES IN VARIOUS REGIONS OF RAT BRAIN

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SUMMARY

A rapid and sensitive method has been developed for the simultaneous determination of norepinephrine, dopamine, 5-hydroxytryptamine and their respective metabolites 3-methoxy-4-hydroxyphenylglycol, homovanillic acid, 3,4-dihydroxyphenylacetic acid, and 5-hydroxyindoleacetic acid in discrete brain regions of rats. The supernatants of tissue homogenates were injected directly into a reversed-phase liquid chromatography system, coupled with electrochemical detection. Each of these compounds gave a linear response over the range 5.5–200 ng/ml cerebellar homogenate (0.11–4.0 ng on column). Analytical recoveries of these compounds, added to the homogenate, were essentially complete when compared with standards dissolved in perchloric acid. The average between-assay coefficients of variation for all these compounds were lower than 6.9% over the range 5.5–200 ng/ml. The within-assay coefficients of variation were lower than 9.7%, measured at 5.5 or 23.6 ng/ml. With the present test parameters and mobile phase conditions, all compounds were readily oxidized at 0.8 V *vs.* a Ag/AgCl reference electrode. The method was applied to an analysis of the differential activity of biogenic amines in the rat striatum, hypothalamus, and hippocampus, produced by probenecid, haloperidol and yohimbine.

INTRODUCTION

Advances in the understanding of the biochemistry, physiology and pharma-

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cology of catecholaminergic and serotonergic systems during recent years have been made possible mainly through the development of sensitive assay techniques. A variety of assays has become available for the measurement of biogenic amines, including paper chromatographic, spectrofluorometric, gas chromatographic, gas chromatographic–mass fragmentographic, radiometric, and radioenzymatic methods. Despite this diversity, each of these procedures has disadvantages or inadequacies that relate to at least one of the following aspects: complexity of sample preparation, speed, sensitivity, flexibility, and specificity. Therefore, these procedures are being rapidly replaced by methods which utilize high-performance liquid chromatography (HPLC), coupled with electrochemical detection[1–22].

While available HPLC techniques undoubtedly offer a high degree of sensitivity and selectivity for the determination of biogenic amines, few methods have been described which permit the simultaneous determination of both catecholamines and indoleamines without long sample preparation time[5,12,17,22]. In addition, since most assays detect only the parent compounds but not their metabolites, assessment of the dynamic state of the pools of these neurotransmitters is often impossible. In fact, most currently employed HPLC procedures do not possess sufficiently high resolution to permit the measurement of catecholamines, indoles, and their metabolites in the same sample, unless these are first isolated and purified by column chromatography, alumina adsorption, or liquid liquid extraction before injection into the HPLC system.

In the present study, the simple and sensitive HPLC method that we published earlier[17,22] has been further extended and refined to provide a technique for the simultaneous determination of norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT), and their respective metabolites 3-methoxy-4-hydroxyphenylglycol (MHPG), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) in acidic supernatants, obtained from discrete regions of the rat brain.

MATERIALS AND METHODS

Materials

Materials and their sources were as follows: NE bitartrate (A-9512), DA hydrochloride (H-8502), 5-HT creatinine sulfate complex (H-7752), MHPG hemipiperazine salt (H-1377), MHPG sulfate potassium salt (H-8759), 5-HIAA (H-8876), 3,4-dihydroxybenzylamine hydrobromide (DHBA, D-7012), HVA (No. 1252), DOPAC (D-9128), haloperidol (H-1512), probenecid (P-8761), and yohimbine (Y-3152) were from Sigma (St. Louis, MO, U.S.A.); citric acid (B-10081) and disodium hydrogen orthophosphate (No. 10249) from BDH, (Toronto, Canada); disodium ethylenediaminetetraacetic acid (EDTA, S-311), 70% perchloric acid (ACS reagent, A-229), sodium hydroxide (S-318), potassium chloride (P-217), and calcium chloride (C-75) from Fisher (Fairlawn, NJ, U.S.A.); octyl sodium sulfate (No. 10577) from Eastman-Kodak (Rochester, NY, U.S.A.); sodium hydrogen carbonate (No. 25839) from AnalaR (Poole, U.K.); sodium chloride (No. 13875) and lactic acid (No. 511954) from J. T. Baker (Phillipsburg, NJ, U.S.A.); methanol (HPLC grade) from Caledon Labs. (Georgetown, Canada). Water was deionized and glass-distilled.

HPLC apparatus

HPLC determinations were performed with a Beckman Model 330 isocratic liquid chromatograph (Irvine, CA, U.S.A.), a Beckman Model 110A pump, a Bioanalytical Systems Model LC-4B amperometric detector (West Lafayette, IN, U.S.A.), and a Hewlett-Packard 3390A recording integrator (Palo Alto, CA, U.S.A.). A glassy-carbon working electrode was set at 0.8 V *vs.* a Ag/AgCl reference electrode. Sensitivity of the detector was maintained between 0.5 and 1.0 nA f.s., depending on the concentration. Separation by isocratic elution was performed on a 250 × 4.6 mm I.D. Altex Ultrasphere ODS column (C_{18} reversed-phase, particle size 5 μm) preceded by a guard column (Guard-Pak, C_{18}, Waters Assoc., Milford, MA, U.S.A.).

Mobile phase

The mobile phase was 15% (v/v) methanol in a solution (pH 4.2) of 32 m*M* citric acid, 12.5 m*M* disodium hydrogen orthophosphate, 0.5 m*M* octyl sodium sulfate and 0.05 m*M* EDTA. The mobile phase was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and then degassed under vacuum before use. A flow-rate of 1.2 ml/min (3000 p.s.i.) at ambient temperature was employed in the present study.

Preparation of standard solutions

Standard solutions of NE, DA, 5-HT, MHPG, MHPG-sulfate, DOPAC, HVA, 5-HIAA, and DHBA (each 0.1 mg/ml) were prepared in 0.17 *M* perchloric acid (PCA), which was previously filtered and degassed. These solutions were stored at −70°C and freshly prepared every 4 weeks.

Standard curve

Known amounts of NE, DA, 5-HT, MHPG, MHPG-sulfate, DOPAC, HVA, and 5-HIAA (in the range 5.5–200 ng) in 1.0-ml aliquots of pooled cerebellar homogenates (see *Animal study* below) were taken through the entire procedure, DHBA (125 ng) being added to each of these samples as an internal standard. The measured levels of endogenous compounds contained in aliquots of the same homogenates without these added exogenous compounds were subtracted from those of the corresponding standard samples. To construct the standard curve, the NE–DHBA, DA–DHBA, 5-HT–DHBA, MHPG–DHBA, MHPG-sulfate–DHBA, DOPAC–DHBA, HVA–DHBA and 5-HIAA–DHBA response ratios, corrected for the endogenous responses, were plotted against the amounts of each of these compounds injected into the HPLC system. Actual amounts of these compounds injected were in the range 0.11–4.0 ng, while the amount of DHBA was always 2.5 ng.

Assays of MHPG-sulfate

To a 0.5-ml aliquot of tissue supernatant, containing DHBA (62.5 ng), a 30-μl aliquot of 4 *M* PCA was added to adjust the molarity of PCA in the sample to 0.39. The acid-catalyzed hydrolysis of MHPG-sulfate was accomplished by heating these samples[13,19] in sealed glass tubes at 130°C for 12 min. A standard curve was prepared by addition of varying amounts of authentic MHPG-sulfate (12.5–100 ng) to 0.5-ml aliquots of pooled cerebellar supernatants. Standards were processed exactly like the tissue samples, as described above. After cooling to room tempera-

ture, the samples were filtered through a 0.45-μm filter, and 20 μl of each filtrate was injected into the HPLC system. Actual amounts of freed MHPG injected into the HPLC system were 0.47–3.77 ng (not corrected for recovery), while the amount of DHBA was 2.36 ng. An additional set of standards in which MHPG was dissolved in 0.5 ml of PCA was processed under identical conditions to investigate whether heat and/or acid decomposes MHPG under the described conditions of reaction.

Preparation of drug solutions

Haloperidol. An amount of 2 mg of haloperidol was dissolved in 200 μl of 0.4% lactic acid. The solution was brought to a final concentration of 0.2 mg/ml in Ringer's solution, after adjusting the pH to 7.4 by addition of 1 *M* sodium hydroxide. The Ringer's solution (pH 7.4) contained 119.86 m*M* sodium chloride, 1.88 m*M* potassium chloride, 1.17 m*M* calcium chloride and 2.38 m*M* sodium hydrogen carbonate.

Probenecid. An amount of 200 mg of probenecid was dissolved in a minimum volume of 1 *M* sodium hydroxide. The solution was brought to a final concentration of 20 mg/ml in Ringer's solution, after adjusting the pH to 7.4 by addition of 1 *M* hydrochloric acid.

Yohimbine. An amount of 10 mg of yohimbine was dissolved in a minimum volume of distilled water. The solution was brought to a final concentration of 1 mg/ml in Ringer's solution, after adjusting the pH to 7.4 by addition of 0.1 *M* sodium hydroxide.

Animal study

Adult Sprague-Dawley rats (Charles River, Montreal, Canada), weighing 250–290 g when purchased were individually housed in an environmentally controlled room at 21–23°C and 40% relative humidity, with lighting on from 07:00 to 19:00 h, for one week prior to the experiment. Food and water were available *ad libitum*, except that food pellets were removed 16 h prior to sacrifice, while water continued to be available.

On the day of the experiment, rats were randomly assigned to receive an intraperitoneal injection of either haloperidol (2 mg/kg), probenecid (200 mg/kg), yohimbine (10 mg/kg) or Ringer's solution. After 90 min, the rats were sacrificed by decapitation and their brains were rapidly removed and placed on an ice-chilled petri dish. The striatum, hypothalamus, hippocampus, and cerebellum were dissected out as described by Glowinski and Iversen[23], frozen on dry ice and stored at −70°C until assayed. The cerebellum was saved to make a pooled cerebellum homogenate, used in the preparation of standard curves. For this purpose, the frozen cerebellum samples were thawed and homogenized in PCA (90 mg of tissue per ml of PCA), as described below.

The sample weights (mean ± S.E.M.) were 69 ± 1, 86 ± 3 and 108 ± 1 mg for striatum, hypothalamus, and hippocampus, respectively. Individual brain regions were placed in polypropylene tubes set in an ice-water bath, and were homogenized in 1.0 ml of PCA, containing 125 ng of DHBA as an internal standard, for 30 s with the use of a Polytron homogenizer (Brinkman Instrument, NY, U.S.A.) at a setting of 9. Homogenates were centrifuged at 4°C for 20 min at 31 550 *g*. The supernatants were separated and either immediately analyzed or stored at −70°C for later assay.

Supernatants were injected into the HPLC system in a volume of 20 μl, by means of a 50-μl Hamilton syringe.

RESULTS AND DISCUSSION

Chromatographic analyses

Representative chromatograms obtained for NE, DA, 5-HT, MHPG, DO-

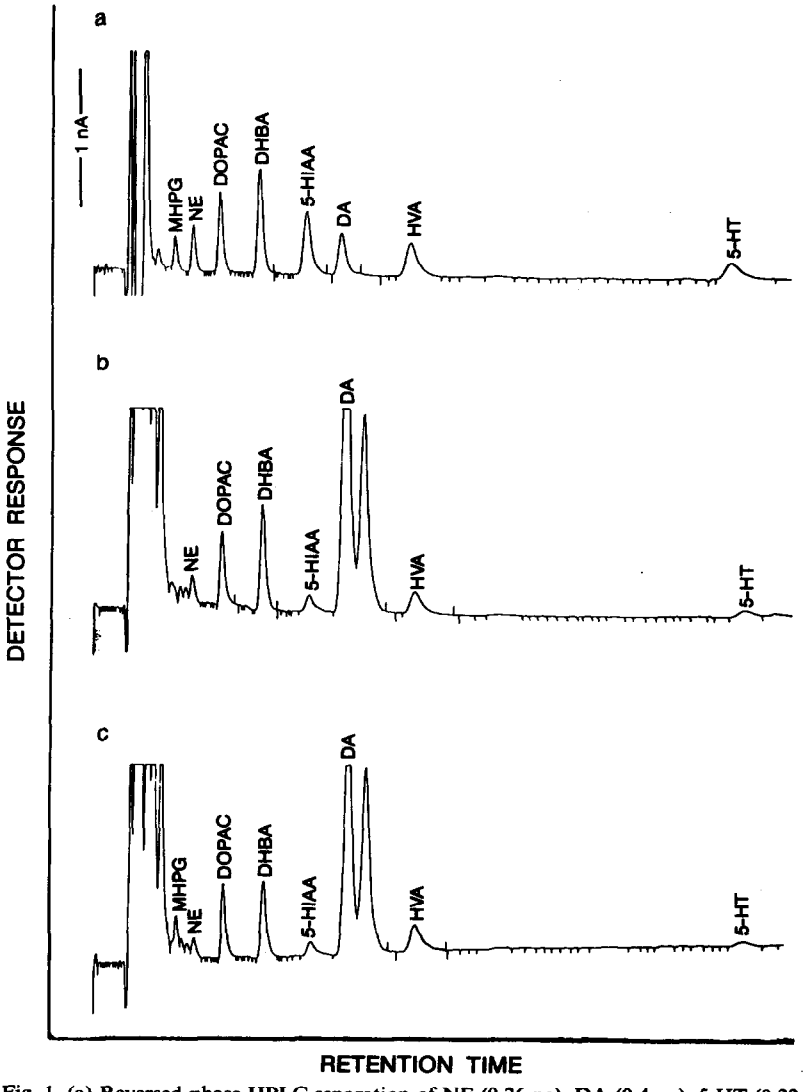

Fig. 1. (a) Reversed-phase HPLC separation of NE (0.26 ng), DA (0.4 ng), 5-HT (0.22 ng), MHPG (0.4 ng), DOPAC (0.5 ng), HVA (0.5 ng), 5-HIAA (0.5 ng), and their internal standard DHBA (2.5 ng) in 0.17 *M* PCA. (b) Representative chromatogram demonstrating the HPLC separation and detection of endogenous NE, DA, 5-HT, DOPAC, HVA, and 5-HIAA from the striatum of an untreated rat. (c) Representative chromatogram demonstrating the HPLC separation and detection of endogenous NE, DA, 5-HT, DOPAC, HVA, 5-HIAA, and MHPG from the striatum of an untreated rat after heat-catalyzed hydrolysis.

TABLE I

RECOVERY AND PRECISION OF THE HPLC DETERMINATION OF NE, DA, 5-HT, MHPG, MHPG-SULFATE, DOPAC, HVA AND 5-HIAA IN CEREBELLAR HOMOGENATES

Compounds	*Concentration range (ng/ml)*	*Recovery**,** *(%)*	*Between-assay C.V. (%)****	*Within-assay C.V. (%)*§
NE	6.5–106	100 ± 0.7	2.0	2.3
MHPG	10.0–162	99 ± 2	3.9	5.3
MHPG-Sulfate	23.6–189	92 ± 7	6.9	9.7
DA	10.0–162	96 ± 3	3.6	8.0
DOPAC	12.5–200	101 ± 3	2.3	7.5
HVA	12.5–200	103 ± 2	3.7	5.7
5-HT	5.5–91	98 ± 1	3.9	5.2
5-HIAA	12.5–200	99 ± 2	3.0	2.0

* Relative to value for PCA solution.
** Mean ± S.D. over the range as shown, ng/ml homogenates (n = 5).
*** Determinations over the range as shown, ng/ml homogenates (at each concentration, n = 5).
§ Determinations at the lowest concentrations, ng/ml homogenates (n = 5).

PAC, HVA, and 5-HIAA standards in PCA and for the same compounds occurring endogenously in rat striatum, as well as for freed MHPG after hydrolysis, are shown in Fig. 1a–c, respectively. No interfering endogenous compounds are apparent. In the present study, MHPG-sulfate that was not subjected to heat-catalyzed hydrolysis did not chromatograph.

A linear concentration response relation for each of these compounds was found in the range of 5.5 to 200 ng in 1.0 ml of cerebellar homogenate (0.11–4.0 ng on column). The correlation coefficients for all these compounds were higher than 0.9986. Analytical recoveries of each of these compounds, added to the cerebellar homogenates, were calculated by comparison of peak areas with results obtained with the corresponding samples in PCA, as well as with the peak area of internal standard DHBA. Each analyte was essentially completely recovered (Table I). The average between-assay and within-assay coefficients of variation for all these compounds were lower than 6.9 and 9.7%, respectively (Table I).

Use of the paired-ion 0.5 mM octyl sodium sulfate in the mobile phase resulted in a high resolution of all compounds. Increasing the paired-ion concentration (up to 1.0 mM) was found to increase the retention times, and this resulted in overlapping of the NE and DOPAC peaks. A lower concentration of paired-ion, *i.e.*, 0.45 mM, gave poor resolution of NE and MHPG. When no paired ion was used, NE and MHPG emerged unresolved with the solvent front. Use of higher methanol concentration (20% or more) also resulted in overlapping of NE, MHPG, and DOPAC. Concentrations of methanol lower than 12% resulted in the overlap of HVA and DA. Increasing the ionic strength two-fold was found to increase the retention time of all compounds in the column, with no changes in sensitivity or resolution. Changes in acidity in the range of pH 4.0 to 4.5 also did not affect the resolution of all compounds.

TABLE II

EFFECTS OF PROBENECID, HALOPERIDOL AND YOHIMBINE ON LEVELS OF BIOGENIC AMINES AND METABOLITES IN THE RAT STRIATUM

A single intraperitoneal dose of either probenecid (200 mg/kg), haloperidol (2 mg/kg), or yohimbine (10 mg/kg) was given 90 min prior to sacrifice. Each value represents mean ± S.E.M. for 7–8 rats (conc., ng/g wet weight). Significant difference from control groups is indicated by p values (in parentheses) of less than 0.05, 0.005, or 0.001 (Student's t test for unpaired data).

Compounds	*Control*	*Probenecid*	*% Change (p) from control*	*Haloperidol*	*% Change (p) from control*	*Yohimbine*	*% Change (p) from control*
NE	169 ± 17	180 ± 27	+7	123 ± 25	−27	163 ± 8	−4
MHPG	48 ± 7	110 ± 11	+129 (0.005)	56 ± 10	+17	71 ± 16	+48
DA	10772 ± 665	11816 ± 1257	+10	9565 ± 781	−11	9676 ± 465	−10
DOPAC	1193 ± 90	1397 ± 166	+17	4157 ± 369	+248 (0.001)	2952 ± 144	+146 (0.001)
HVA	676 ± 58	1173 ± 109	+74 (0.001)	3009 ± 240	+345 (0.001)	2086 ± 110	+209 (0.001)
5-HT	406 ± 59	607 ± 60	+50 (0.05)	347 ± 31	−15	437 ± 47	+8
5-HIAA	175 ± 29	251 ± 24	+43 (0.05)	171 ± 16	−2	163 ± 19	−7

TABLE III

EFFECTS OF PROBENECID, HALOPERIDOL AND YOHIMBINE ON LEVELS OF BIOGENIC AMINES AND THEIR METABOLITES IN THE RAT HYPOTHALAMUS

A single intraperitoneal dose of either probenecid (200 mg/kg), haloperidol (2 mg/kg) or yohimbine (10 mg/kg) was given 90 min prior to sacrifice. Each value represents mean ± S.E.M. for 7–8 rats (conc., ng/g wet weight). Significant difference from control groups is indicated by p values (in parentheses) of less than 0.05, 0.025, 0.01, 0.005, or 0.001 (Student's t test for unpaired data).

Compound	*Control*	*Probenecid*	*% Change (p) from control*	*Haloperidol*	*% Change (p) from control*	*Yohimbine*	*% Change (p) from control*
NE	2825 ± 188	2417 ± 219	−14	2627 ± 189	−7	2025 ± 132	−28 (0.005)
MHPG	59 ± 7	122 ± 13	+107 (0.005)	75 ± 11	+21	134 ± 13	+127 (0.005)
DA	421 ± 22	538 ± 40	+28 (0.01)	402 ± 17	−5	501 ± 40	+19 (0.05)
DOPAC	100 ± 7	165 ± 13	+65 (0.001)	172 ± 14	+72 (0.001)	193 ± 17	+93 (0.001)
HVA	43 ± 6	68 ± 6	+58 (0.01)	104 ± 12	+141 (0.001)	94 ± 9	+119 (0.001)
5-HT	1112 ± 98	1003 ± 100	−10	815 ± 53	−27 (0.025)	1207 ± 160	+9
5-HIAA	337 ± 28	339 ± 51	+0.6	261 ± 34	−23	259 ± 20	−23 (0.05)

TABLE IV

EFFECTS OF PROBENECID, HALOPERIDOL AND YOHIMBINE ON LEVELS OF BIOGENIC AMINES AND THEIR METABOLITES IN THE RAT HIPPOCAMPUS

A single intraperitoneal dose of either probenecid (200 mg/kg), haloperidol (2 mg/kg) or yohimbine (10 mg/kg) was given 90 min prior to sacrifice. Each value represents mean ± S.E.M. for 7–8 rats (conc., ng/g wet weight). Significant difference from control groups is indicated by p values (in parentheses) of less than 0.05, 0.025, 0.01, or 0.005 (Student's t test for unpaired data). ND, not detected.

Compounds	*Control*	*Probenecid*	*% Change (p) from control*	*Haloperidol*	*% Change (p) from control*	*Yohimbine*	*% Change (p) from control*
NE	257 ± 24	201 ± 18	−22 (0.05)	246 ± 18	−4	207 ± 21	−19
MHPG	33 ± 6	43 ± 8	+30	38 ± 9	+15	69 ± 9	+109 (0.005)
DA	55 ± 7	60 ± 10	+9	35 ± 4	−35 (0.05)	40 ± 5	−27
DOPAC	ND	ND		ND		ND	
HVA	11 ± 2	27 ± 6	+145 (0.025)	21 ± 3	+91 (0.025)	19 ± 3	+73 (0.025)
5-HT	603 ± 59	341 ± 51	−43 (0.01)	508 ± 76	−16	543 ± 31	−10
5-HIAA	193 ± 23	168 ± 15	−13	146 ± 16	−24	104 ± 16	−46 (0.005)

Hydrolysis of MHPG-sulfate

Much attention has been paid to the changes of MHPG, the major metabolite of NE in the rat brain. In the present study, hydrolysis of MHPG-sulfate was generally carried out for 12 min at 130°C, conditions which provide the best compromise between effectiveness of the MHPG-sulfate hydrolysis (84%) and stability of free MHPG (29% destruction). Estimation of MHPG-sulfate content in biological samples could be made independently of this limited MHPG destruction and incomplete MHPG-sulfate hydrolysis, since a linear relationship in the concentration–response relationship for freed MHPG was obtained under the experimental conditions used in the present study.

Effects of drugs on the levels of biogenic amines and their metabolites in different brain regions

The present analytical method was applied to the study of the effects of probenecid, haloperidol and yohimbine, drugs with fairly well understood mechanisms of action, on the concentration of NE, DA, and 5-HT and their metabolites.

Administration of probenecid was expected to result in increased brain concentrations of acid metabolites such as HVA, DOPAC, and 5-HIAA and neutral metabolite MHPG. In agreement with this prediction and with other reports, elevated levels of MHPG and HVA were found in all brain regions of probenecid-treated rats (Tables II–IV). DOPAC level was also found to be significantly increased by probenecid in the hypothalamus. However, the concentration of this DA metabolite in the striatum (Table II) remained unaffected by this treatment. In agreement with the findings by Nielsen and Johnston[15], probenecid administration also produced a significant increase in the 5-HIAA concentration in the striatum (Table II), but not in the hypothalamus or hippocampus. In addition, in this latter region (Table IV), probenecid produced a marked decrease in 5-HT content and to a lesser extent in the levels of NE.

In agreement with many reports in the literature[15,16,24], it was revealed that there were large increases (2–4-fold) in the striatal content of DOPAC and HVA after haloperidol (Table II). Similarly, increased DA turnover in the hypothalamus and hippocampus of haloperidol-treated rats was indicated by the significantly higher levels of DOPAC and HVA, relative to those of the controls (Tables III and IV). In addition, DA levels in the hippocampus were found to be significantly decreased in haloperidol-treated rats.

In agreement with the known effects of yohimbine, administration of this drug resulted here in marked increases in the levels of MHPG in all three brain regions studied (Tables II–IV), and in the levels of DOPAC and HVA in the striatum and hypothalamus. In addition, the levels of 5-HIAA were found to be markedly decreased by yohimbine.

In conclusion, a simple method was described for the simultaneous measurement of catecholamines and indoles and their respective metabolites in small biological samples. This method was capable of demonstrating the known pharmacological effects of probenecid, haloperidol, and yohimbine on catecholaminergic and indoleaminergic neurotransmission.

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