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SIMULTANEOUS DETERMINATION OF MONOAMINE TRANSMITTERS, PRECURSORS AND METABOLITES IN A SINGLE MOUSE BRAIN

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SUMMARY

A simple and sensitive procedure for simultaneous determination of monoamine transmitters and related substances including precursors and metabolites has been developed for a single mouse brain. The proposed procedure involves (1) primary butanol extraction, (2) separation of the substances into either acid or alkaline aqueous layers according to their physicochemical properties, and (3) determination by means of high-performance liquid chromatography with electrochemical detection. Three transmitters (noradrenaline, dopamine and 5-hydroxytryptamine) and their precursors (tyrosine, 3,4-dihydroxyphenylalanine and tryptophan) and major metabolites (normethanephrine, 3-methoxy-4-hydroxyphenylethylene glycol, 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid and 5-hydroxyindoleacetic acid) were selectively separated and sensitively detected in mouse whole brain sample. Although 3-methoxy-4-hydroxymandelic acid was also separated from other substances by authentic chromatography, the substance was not detected in mouse brain. Changes in levels of these substances were examined for drugs whose effects had been previously confirmed. These changes reflected putative effects of the drugs and confirmed that the procedure is effective for neurochemical research into the transmitter system.

INTRODUCTION

It is generally accepted that monoamines such as noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) exist in the central nervous system of mammals and function as neurotransmitters. There are many hypotheses concerning these monoamines as causative substances in a variety of neurological [1] and mental disorders [2, 3]. This has led to extensive research con-

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cerning the function and metabolism of these neurotransmitters. In this research, monoamines and related substances have been quantitatively determined in the brains of model animals and the physiological fluid of humans. Drugs used in the treatment of neurological and mental disorders affect the metabolism of catecholamines and indoleamines [4]. However, few studies have reported the simultaneous determination of wide-ranging substances relating to monoamine metabolism, including precursors and metabolites of catecholamines and indoleamines, in the same sample. This kind of assay would, if possible, provide highly important information on the physiological and pathological mechanisms of brain function, because it has been proposed that the central transmission system forms a composite multitransmitter system, which interacts to modulate brain function in a balanced manner [5]. During recent years our experimental focus has been on a simultaneous determination of monoamines and their precursors and metabolites in a single sample of mouse brain.

The most widely used methods of analysis for monoamines and related substances are variations of a fluorometric approach. The procedures involve the physical separation of each substance and conversion to the corresponding fluorescent derivative [6]. The procedures are, unfortunately, insufficiently sensitive for the concurrent determination of monoamine transmitters and metabolites in a single brain sample. Fluorometric detectors have been used in liquid chromatography [7]. However, detection is difficult in simultaneous quantitation because the substances have different fluorescence spectra [6]. Gas chromatography combined with electron-capture detection or mass fragmentography is also used for the determination of monoamines with varying degrees of success [8—10]. In these methods, the substance must first be converted to the volatile derivative. Biogenic substances, including monoamines and related substances, are generally water soluble and difficult to convert to volatile derivatives. It is, therefore, not reasonable to use gas chromatography for monoamine assay if other procedures are available. Recently, radioenzymatic techniques have been introduced for monoamine assay [11, 12]. Although it is extremely sensitive, the radioenzymatic assay possesses a fatal defect for simultaneous determination: it cannot be used to quantify the O-methylated metabolites of catecholamines, which are important in the metabolic pathway of the amines.

Since Takata and Muto [13] introduced the principle and Refshauge et al. [14] demonstrated its application in monoamine assays, high-performance liquid chromatography (HPLC) with electrochemical detection has been extensively used in the quantitation of monoamines and related substances in the brain [15—19]. The comprehensive principle and basic methodology of this new method has recently been reviewed [20]. It has been suggested that electrochemical detection is highly sensitive for substances having phenolic hydroxy groups [7, 13, 20]. All substances involved in monoamine metabolism possess the OH group(s) on their molecules [6] except tryptophan, a precursor amino acid for 5-HT. This amino acid has, fortunately, been reported to be detected electrochemically despite its molecular characteristic of having no OH group [17]. In the present study, therefore, HPLC with electrochemical detection was chosen as the experimental tool for the simultaneous determina-

tion of monoamine transmitters and their metabolites and precursors in a single mouse brain sample.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was set up with commercially available components including a thin-layer voltammetric detector with a glassy carbon electrode (Yanagimoto VMD-101, Kyoto, Japan). The chromatographic column was LiChrosorb RP-18 reversed-phase resin (average particle size, 10 μm) prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column (Altex, Berkely, CA, U.S.A.). The detector potential was set at +700 mV or 600 mV vs. Ag/AgCl reference electrode for amine and metabolite assays, respectively.

Reagents

All chemicals for extraction and chromatography were obtained from a single source (Mallinkrodt, Paris, KT, U.S.A.) and were analytical reagent grade. The reagents were used as obtained commercially without further purification. Each authentic standard was prepared at a concentration of 1 mg/ml in 0.025 *N* hydrochloric acid for amines and precursors, and in 0.05 *M* phosphate buffer (pH 8.5) for metabolites. These standard stock solutions were stable for one month when stored in a refrigerator at 4°C. A working standard was made from the stock solution on the day of assay. The standard solution which contained substances in concentrations similar to those in the samples to be assayed, was taken through the entire procedure for extraction. Isoproterenol (ISO) and 3,4-dihydroxyphenylpropionic acid (DOPPA) were used as internal standards for the amine and metabolite assays, respectively.

Two different types of mobile phases were used according to the purpose of the assay. For the assay of monoamines (including amine metabolites, 3-methoxytyramine (3-MT) and normethanephrine (NMN) and precursor amino acids, a 0.025 *M* citrate buffer (pH 5.0) containing 1% tetrahydrofuran was used. On the other hand, a 0.05 *M* phosphate buffer (pH 6.9) containing 10% methanol was used for the metabolite assay. The mobile phase was pumped at a flow-rate of 1.0 ml/min or 0.7 ml/min for the amine and metabolite assays, respectively.

Animals

The animals were male Swiss—Webster mice each weighing about 35 g at the time of sacrifice. They were maintained on a 12-h light/12-h dark cycle and allowed access to food and water ad libitum. They were sacrificed at a fixed time between 10:00 a.m. and 12:00 noon to exclude daily fluctuation in biogenic amine metabolism. The brain was removed as quickly as possible after decapitation and stored on dry ice until the assay could be performed. In general, the assay was completed within one week after sacrifice.

Drug injections

The following drugs were used; 300 mg/kg of *p*-chlorophenylalanine (PCPA), 1 mg/kg of reserpine (RES), 1 mg/kg of haloperidol (HAL) and α-methyl-*p*-

tyrosine (α-MPT). All drugs were administered intraperitoneally. Time intervals between injection and decapitation were selected to produce the maximum drug effect: 48 h for PCPA; 24 h for RES; 1 h for HAL; and 2 h for α-MPT. Control animals received no injection.

Extraction procedure

Each brain was weighed and transferred to a glass tube containing 200 μl of 0.1 *N* hydrochloric acid, 20 μl of 0.1 *M* EDTA, 500 ng of ISO and 250 ng of DOPPA. Immediately after the addition of 10 ml of butanol, the brain was homogenized by means of a homogenizer (Tissuemizer, Janke and Kunkel, G.F.R.). The homogenate was transferred to a screw-capped glass tube containing 2 g of solid sodium chloride and was shaken in a reciprocal shaker for 60 min. After centrifugation at 4000 rpm for 10 min, 5 ml of the butanol layer was pipetted into another screw-capped tube containing 200 μl of 0.1 *N* hydrochloric acid and 10 ml of *n*-heptane. The tube was shaken again for 10 min and centrifuged at 4000 rpm for 5 min. The organic layer was transferred to another tube and stored overnight in a refrigerator at 4°C. The aqueous layer in this step was used for the assay of monoamines and precursor amino acids. A 10-μl aliquot of the aqueous layer was injected onto the column through a six-port valve equipped with 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). On the next day, 200 μl of 0.1 *M* phosphate buffer (pH 7.5) was added to the organic layer stored in a refrigerator and the tube was shaken for 60 sec on a Vortex mixer. The tube was then centrifuged at 3000 rpm for 60 sec. A 50-μl aliquot of the buffer aqueous layer was taken from the bottom of the tube and injected onto the column for the metabolite assay.

Calculations and statistics

All quantitations were based on peak heights of the resulting chromatogram. Ratios of the peak heights for the substances and the corresponding internal standard were compared for samples and standards taken through the entire extraction procedure, as described previously [14, 15]. Statistical significance was examined by use of the Student's *t*-test.

RESULTS AND DISCUSSION

The present study has demonstrated a simple and sensitive method for the simultaneous determination of wide-ranging substances related to monoamine metabolism in the same sample of mouse brain. The procedure consists of two parts; extraction of the substances by acidic butanol and electrochemical detection following separation by reversed-phase HPLC.

For catecholamine assay, the alumina absorption technique is useful for purification of the sample and has frequently been applied for the brain [14—16] and physiological fluid [21]. However, the procedure is ineffective for indole compounds as well as for 3-methoxylated metabolites of catecholamines. Organic solvent extraction procedures are also used for the pretreatment of monoamine quantitations. Although the solvent extraction is not specific for monoamine transmitters or metabolites, this procedure can be extremely useful if an adequate chromatographic separation is available. Sasa

and Blank [19] have reported successful application of the extraction procedure in liquid chromatography for determination of DA and 5-HT, but not of NA, in mouse brain. In the present study, therefore, the acidic butanol method with minor modifications was used for extraction.

Monoamines and related substances, which had initially been extracted in the butanol layer, were re-extracted in 0.1 *N* hydrochloric acid, and then in alkaline buffer according to their physicochemical properties. It is therefore essential to know the distribution coefficients of the substances for both aqueous layers. Recovery of alkaline compounds (amines) was not influenced by changing the concentration of hydrochloric acid. However, the recovery of 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG), a neutral metabolite of NA, in the hydrochloric acid layer was decreased in proportion to an increase in the concentration. This resulted in a slight increase in recovery in alkaline buffer (Fig. 1). On the other hand, no acid metabolites appeared in the hydrochloric acid layer. While increasing the hydrochloric acid concentration seemed to be convenient for MOPEG determination, the HCl molecule interfered with the proper determination of NA because of its own peak. The concentration of hydrochloric acid for re-extraction was finally set at 0.1 *N* which was the same as previously reported [19].

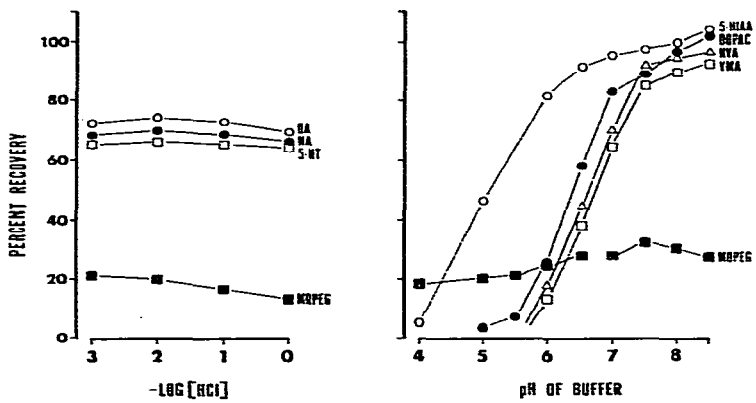

Fig. 1. Changes in recovery rates of monoamine transmitters and metabolites. Left panel shows effects of concentrations of hydrochloric acid on the recoveries of monoamines and a neutral transmitter, MOPEG. Right panel shows effects of pH values of buffer on the recoveries of metabolites.

The pH value of the alkaline buffer affected the recovery of acid metabolites (Fig. 1). While the metabolites recovered well in a high pH buffer, the maximum recovery of MOPEG was at pH 7.5. Since MOPEG is a major metabolite of NA in the brain [22, 23], determination of this substance is essential for understanding the activity of noradrenergic neurons in the central nervous system. The pH value for re-extraction of metabolites was, therefore, decided at 7.5 because of the MOPEG quantitation. Although the recovery rates of other metabolites were lower at this pH value than their maximum, relative deviations for the rates were within 4% and were enough for simultaneous determination of the metabolites.

Under these re-extraction conditions, recovery rates for the substances

TABLE I

RECOVERY RATES OF SUBSTANCES IN THE PRESENCE OF TISSUE SAMPLE

A brain was homogenized in 1 ml of 0.025 *N* hydrochloric acid containing 20 μl of 0.1 *M* EDTA. The homogenate was divided into two equal portions and transferred to two screw-capped glass tubes. Known quantities of authentic standard substances and internal standards were added to one tube, and only internal standards to the other. After additions of sodium chloride and butanol, each tube was subjected to the extraction procedure described in the text. The recovery rates shown in this table were calculated by comparing the peak height of each substances in the sample. The values represent means ± S.D. from six determinations. Abbreviations: DOPA = 3,4-dihydroxyphenylalanine; VMA = 3-methoxy-4-hydroxymandelic acid; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = 3-methoxy-4-hydroxyphenylacetic acid; 5-HIAA = 5-hydroxyindoleacetic acid.

Precursors		Amines		Metabolites	
Tyrosine	56.7 ± 2.8	NA	60.0 ± 3.7	VMA	68.8 ± 4.3
DOPA	61.9 ± 3.1	DA	64.7 ± 3.4	MOPEG	31.1 ± 2.3
Tryptophan	39.2 ± 4.6	5-HT	54.6 ± 2.9	DOPAC	84.6 ± 2.5
		NMN	61.1 ± 4.2	HVA	76.9 ± 3.2
		3-MT	51.8 ± 3.6	5-HIAA	79.0 ± 4.6

quantified in the present study were calculated in the presence of tissue sample (Table I). The values were higher than those of a previous report [19], while the recovery of catecholamines was less than those obtained by the alumina absorption technique [21]. The recovery rate of each substance was included in the estimation of tissue concentration.

Electrode responses depend upon the applied voltage [8, 13, 20]. Although increases in applied voltages result in more responses, the increases are accompanied by increases in background and noise currents which interfere with suitable determination of the substances. Fig. 2 shows the current—voltage curves for listed substances.

The substances tested in the present study can be classified according to their electrochemical behavior (Fig. 2). The most sensitive substances for electrochemical detection were catechol compounds which have two OH radicals on the benzene nucleus. In this group, the current was initiated at an applied voltage of about +200 mV. 3-Methoxy-4-hydroxy derivatives of catecholamine (3-methoxylated metabolites) required higher voltage for producing the response currents than did catecholamines. The findings suggested that the responses to lower applied voltages were due to the dual oxidation of OH groups on their molecules. Both catecholamines and 3-methoxyiated metabolites represented a plateau in the current—voltage curves and further increases of the applied voltages resulted in lowered responses. Such effects might be due to interfering electrochemical reactions among adjacent radicals in the molecule.

5-Hydroxyindole compounds differed from both catecholamines and 3-methoxylated metabolites in their electrochemical responses. In 5-HT and 5-HIAA, the current was initiated at a relatively low applied voltage, and responses gradually increased in proportion to the applied voltage without a plateau (Fig. 2). This response might be due to one molar oxidation of the

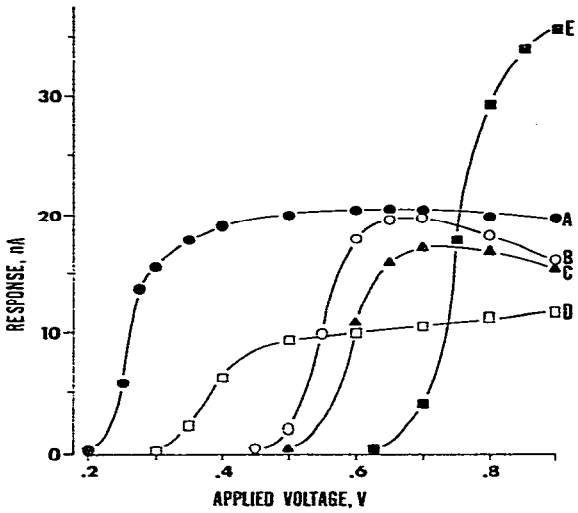

Fig. 2. Current—voltages curves of standard substances. A, for catechol compounds (NA, DA, DOPA and DOPAC) typified with 10 ng of NA; B, for 3-methoxylated amines (3-MT and NMN) typified with 10 ng of 3-MT; C, for 3-methoxylated metabolites (VMA, MOPEG and HVA) typified with 10 ng HVA; D, for indole compounds (5-HT and 5-HIAA) typified with 10 ng of 5-HT; and E, for amino acids (tyrosine and tryptophan) typified with 30 ng of tyrosine.

hydroxy group on the indole nucleus and to the absence of radicals in adjacent positions.

The precursor amino acids, tyrosine and tryptophan, required higher applied voltage to initiate the electrode response than other substances examined. Although tyrosine possesses one phenolic OH, but not tryptophan, in the molecule, both amino acids represented similar responses in the current—voltage curves. This means that the current is generated by the oxidation of not only radicals on nucleus rings but also carboxyl and/or amino groups in these molecules.

The detector response was measured for injections from 0.2 ng to 50 ng and provided a linear calibration for each substance in this range. The overall precision of the procedure was found to be less than ± 4.6% standard deviation for each substance.

Typical chromatograms for monoamine and metabolite assays are shown in Fig. 3. In the chromatographic study mentioned above, eight peaks were detected for the monoamine-related substances on the chromatogram for the monoamine assay in which 20 μl of the hydrochloric acid layer had been injected (Fig. 3A). The identification of each peak was accomplished by the procedure previously reported [17], in which the peak was compared for both chromatographic and electrochemical behavior with the standard substance taken through the entire extraction procedure. In a previous experiment using the butanol extraction method [19], NA could not be detected because of an interfering front peak. This peak can be attributed to the ascorbic acid added to prevent the oxidation of substances during extraction, because, in the present chromatography, this substance had a retention time similar to that of the front peak. With the antioxidant removed, the front peak on the present

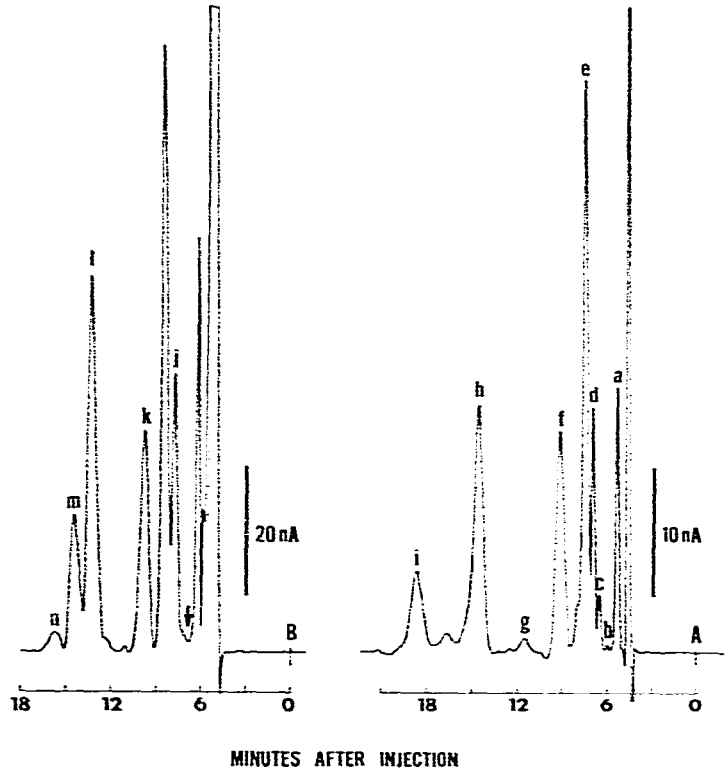

Fig. 3. Typical chromatograms for monoamine (A) and metabolite (B) assay of mouse whole brain. For extraction procedure, see text. Chromatographic conditions: stationary phase, LiChrosorb RP-18 (average particle size, 10 μm) prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column; mobile phase, 0.025 *M* citrate buffer (pH 5.0) containing 1% tetrahydrofuran and 0.05 *M* phosphate buffer (pH 6.9) containing 10% methanol for monoamine and metabolite assays, respectively; detector applied voltage, +700 mV and 600 mV vs. Ag/AgCl reference electrode for monoamine and metabolite assays, respectively. Peaks: a = NA; b = DOPA; c = NMN; d = tyrosine; e = DA; f = isoproterenol (internal standard); g = 3-MT; h = 5-HT; i = tryptophan; j = DOPAC; k = 3,4-dihydroxyphenylpropionic acid (internal standard); l = 5-HIAA; m = HVA; n = MOPEG. An arrow shows a retention time for VMA which was not detected in the present assay. Two recorders with different sensitivities were used for routine determinations.

chromatogram was so small that NA was detected without disturbance.

In previous reports [14—16, 19], 3,4-dihydroxybenzylamine (DHBA) was used as the internal standard substance. Since it has a retention time identical to that of DOPA, the substance is not a suitable standard in the precursor amino acid quantification. Other candidates for the internal standard are α-methyl dopamine [21] and ISO. Because the former has a retention time similar to that of 3-MT, ISO was finally selected as the internal standard in the present study.

In the metabolite assay injected with 50 μl of buffer aqueous sample, five major metabolites were completely separated (Fig. 3B). However, VMA was not detected and MOPEG concentration was low in the present sample of mouse whole brain. These findings suggest that the main metabolic pathway for NA is the formation of MOPEG followed by a rapid conjugation process

with sulfuric acid [6, 22, 23] and that conjugated MOPEG is not extracted in the acidic butanol layer. The minimum detectable amount of each substance by the present procedure was as follows: 200 pg for catechol compounds; 500 pg for 3-methoxylated metabolites and indoles; and 2 ng for precursor amino acids.

In order to ascertain the feasibility of the present procedure, the effects of several drugs were investigated (Table II). These effects have previously been confirmed by a large number of studies.

RES acts primarily by depleting catecholamines and indoleamines from the nervous system as well as the adrenal medulla [4, 24, 25]. In the present study, the drug significantly decreased the concentration of three monoamine transmitters 24 h after a 1 mg/kg intraperitoneal injection. The reducing effect was stronger on NA than on 5-HT. Catecholamine metabolites were also reduced by the RES administration. However, the drug did not affect the intracerebral 5-HIAA level in spite of a significant reduction of the parent transmitter. Although the effect on the 5-HIAA level requires further experiments for confirmation, it is likely that the present effect indicates that the indoleamines are restored more rapidly than catecholamines after RES-induced depletion. These findings also suggest agreement with the possibility that catecholamines play a more important role than 5-HT in the effects of the drug [25].

HAL is a typical neuroleptic drug which is used in the treatment of psychiatric disorders, especially schizophrenia [5]. DA was decreased, while the metabolites were increased, in mouse brain by an injection of the drug. The present results were expected in view of the drug's effect on the dopaminergic system. Vogt [26] has demonstrated similar effects on the dopaminergic system in which the drug decreases the transmitter and increases a metabolite, HVA. These effects have been interpreted as indicating that the drug blocks dopamine receptors [3, 25, 27, 28]. The blockade effect on the receptor results in more DA being released which causes a compensatory increase in the turnover rate of the transmitter. The drug had other effects in the present study, i.e., increasing the levels of MOPEG and 5-HIAA. These findings suggest the possibility that HAL has effects not only on the dopaminergic but also on the noradrenergic and serotonergic systems.

PCPA and α-MPT are biosynthetic inhibitors of indoleamines and catecholamines, respectively. The former blocks tryptophan hydroxylase, the rate limiting enzyme [29]; and the latter, tyrosine hydroxylase [30, 31]. In the present study, PCPA affected the serotonergic, but not the catecholaminergic system, decreasing both levels of 5-HT and 5-HIAA. It has been reported that a 316 mg/kg injection of the drug significantly decreases the 5-HT level without affecting NA [29]. On the other hand, α-MPT affected only the catecholaminergic system, decreasing the concentrations of transmitter substances and metabolites. These results are reasonable for its effects. α-MPT, but not PCPA, influences the levels of precursor amino acids. These results may indicate the difference in substrate availabilities of the enzymes.

The steady-state concentrations of monoamines, precursors and metabolites were simultaneously determined in the same sample of mouse brain by means of HPLC with electrochemical detection. It is impracticable to compare whole values with those of previous reports. The concentrations of three monoamine

TABLE II

EFFECTS OF DRUGS ON MONOAMINE TRANSMITTERS, PRECURSORS AND METABOLITES IN MOUSE WHOLE BRAIN

Values are expressed in ng/g wet tissue and means ± S.D. from six animals.

	Control	RES	HAL	PCPA	α-MPT
Tyrosine	6837 ± 563	6794 ± 289	6918 ± 218	6775 ± 391	7728 ± 431*
DOPA	4.1 ± 0.5	3.3 ± 0.9	6.8 ± 2.4	3.4 ± 0.5	not detectable
Tryptophan	3459 ± 415	3295 ± 247	3623 ± 213	3232 ± 119	3478 ± 372
NA	418 ± 26	194 ± 16*	408 ± 32	458 ± 22	317 ± 17*
NMN	10.9 ± 1.2	11.1 ± 1.4	10.6 ± 1.1	10.5 ± 0.9	6.8 ± 1.0
MOPEG	32.0 ± 5.4	25.8 ± 3.9*	48.3 ± 5.5*	36.2 ± 5.0	13.8 ± 2.5*
DA	887 ± 39	657 ± 38*	714 ± 51*	941 ± 19	526 ± 39*
3-MT	25.2 ± 2.1	14.5 ± 1.6*	47.7 ± 6.6*	21.2 ± 2.9	17.3 ± 1.6*
DOPAC	72.5 ± 5.1	44.8 ± 6.7*	31.2 ± 35*	67.8 ± 5.3	29.5 ± 7.5*
HVA	152 ± 13	103 ± 18*	351 ± 32*	132 ± 17	70 ± 10*
5-HT	429 ± 36	256 ± 16*	425 ± 41	347 ± 20*	433 ± 17
5-HIAA	223 ± 19	239 ± 10	340 ± 27	140 ± 13*	224 ± 16

*Significantly different from control group ($P < 0.01$).

transmitters (NA, DA and 5-HT) were, at least, well within the range of determination reported [19, 32, 33].

The present procedure has merit in the possible determination of a series of substances related to monoamine metabolism from precursor amino acids through end-metabolites in a single brain sample of the mouse, although one problem remains to be resolved. MOPEG, a major metabolite of NA, has the properties of an alcohol and remains, in large part, in the organic layer in the re-extraction process. This resulted in a lower recovery rate in comparison with other substances. Furthermore, a major portion of the substance is converted to a sulfate conjugate [6, 22] which was ineffective for butanol extraction. For these two reasons, the determination of MOPEG was restricted in the present study. However, the effects of drugs were readily seen in the changes in the concentrations of the substances studied, including MOPEG.

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