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SIMULTANEOUS DETERMINATION OF NOREPINEPHRINE, DOPAMINE, 5-HYDROXYTRYPTAMINE AND THEIR MAIN METABOLITES IN RAT BRAIN USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

ENZYMATIC HYDROLYSIS OF METABOLITES PRIOR TO CHROMATOGRAPHY

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SUMMARY

In order to measure turnover rates of the noradrenergic, dopaminergic, and serotonergic transmitter systems in rat brain, a method was developed by which norepinephrine, dopamine, and 5-hydroxytryptamine, and their main metabolites 3-methoxy-4-hydroxyphenylglycol, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, and 5-hydroxyindole-3-acetic acid, could be measured simultaneously. High-performance liquid chromatography in the reversed-phase mode, including ion pairing, separated the transmitters and their metabolites well. By means of enzymatic hydrolysis of the sample prior to chromatography, it was also possible to measure the conjugated forms of the metabolites. Since there was no prepurification step, the hydrolysed supernatants of tissue homogenates were injected directly into the chromatographic system; additional selectivity tests were necessary. Peak identification was confirmed by comparison of hydrodynamic voltagrams and capacity factors at different pH values of the mobile phase of the components in the sample and the standard solution. The method is demonstrated by analysing mediobasal hypothalamic tissues of probenecid-treated rats.

INTRODUCTION

The noradrenergic, dopaminergic and serotonergic transmitter systems play an important role in the control and regulation of many brain functions. In order to determine the activities of these transmitter systems, not only the tissue levels of the transmitters, but also the concentration of their main metab-

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olites should be measured. An estimation of the turnover in the transmitter systems can be obtained either from metabolite—transmitter relationships in steady-state conditions [1—3] or from the extent of metabolite accumulation after pharmacological blockage of their elimination from the brain by probenecid [4—6]. Before measurement, enzymatic hydrolysis of the sample is necessary, due to conjugation of the metabolites in the brain tissue [7—10].

The aim of this study was, therefore, to develop an analytical method in which norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT) and the free and conjugated forms of their main metabolites, 3-methoxy-4-hydroxyphenylglycol (MOPEG) [7, 8], 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA) [11, 12], and 5-hydroxyindole-3-acetic acid (5-HIAA) [13] can be measured simultaneously. The use of high-performance liquid chromatography (HPLC) with electrochemical detection (ED) in this context is described in many papers (for review see refs. 14 and 15). Most of them include prepurification steps for the sample, which makes simultaneous measurement of the substances mentioned above impossible. The use of an internal standard is also necessary. To overcome these disadvantages we used the method of direct injection of the brain homogenate supernatant into the HPLC system [16—19]. In spite of the high resolution of HPLC, additional selectivity tests became necessary.

The development of the method and the test of selectivity are demonstrated by means of the analysis of mediobasal hypothalamic tissues of probenecid-treated rats.

EXPERIMENTAL

Reagents and drugs

Chemicals were obtained from the following sources: 3,4-dihydroxyphenylglycol (DHPG), norepinephrine · hydrochloride (NE), L-epinephrine (E), 3-methoxy-4-hydroxyphenylglycol (MOPEG), 3,4-dihydroxybenzylamine · hydrobromide (DHBA), DL-normetanephrine · hydrochloride (NMN), 3,4-dihydroxyphenethylamine · hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid · dicyclohexylammonium salt (5-HIAA), 5-hydroxytryptamine · creatine-sulphate complex (5-HT), 3-methoxy-4-hydroxyphenylacetic acid (HVA), probenecid, sulphatase (from *Helix pomatia*, type H-5), and dibutylamine from Sigma (St. Louis, MO, U.S.A.); sodium octyl sulphate from Fluka (Buchs, Switzerland); and 3-methoxy-4-hydroxyphenylglycol-4-sulphate potassium salt from Hoffmann-La Roche (Basel, Switzerland). Glass-distilled methanol (Merck, Darmstadt, F.R.G.) was used; the deionised water was separated from organic components by vacuum filtration through a Norganic-patrone (Millipore, Bedford, MA, U.S.A.).

Apparatus

The HPLC system consisted of a 6000A solvent delivery pump, a U6K injection system and a 5-μm C_{18} column RCM 100 (I.D. 8 mm) protected by a Guard-Pak precolumn insert, all obtained from Waters Assoc. (Milford, MA, U.S.A.). The electrochemical detector E656/641 from Metrohm (Herisau, Switzerland) with a glassy carbon electrode was used.

The mobile phase entering the detector cell was held at a constant temperature of 20° C by means of a water bath. The detector potential was normally maintained at 0.80 V versus an Ag/AgCl reference electrode. In order to obtain hydrodynamic voltamograms, step-by-step reductions were made from 0.90 to 0.35 V.

Chromatography

The mobile phase was a mixture of 0.1 *M* sodium acetate, 0.1 *M* citric acid, 0.5 m*M* sodium octyl sulphate, 0.15 m*M* EDTA, 1 m*M* dibutylamine, and 10% methanol (v/v), pH 3.70. For some experiments the pH value of the mobile phase was varied between 2.63 and 6.74 by changing the ratio of sodium acetate to citric acid, keeping the ionic strength of the solution constant. The mobile phase was filtered through a 0.22-μm filter (GVWP 04700, Millipore) and degassed under vacuum by means of ultrasonic agitation. All separations were performed isocratically at a flow-rate of 1.0 ml/min at room temperature.

Standards and calculations

The stock solutions of the standards were prepared in 0.1 *M* sodium acetate, 0.1 *M* citric acid pH 3.68 at a concentration of 6 m*M* and stored at −80° C. They were diluted 1:40,000 with the mobile phase to give the working solution; 25 μl of this were injected into the HPLC system (3.75 pmol). In order to test the linearity of the detector signal, a series of seven standard solutions were diluted from the stock solution, containing 0.5—9.4 pmol per injected volume.

The tissue levels of the substances were calculated by comparing the heights of the peaks in the sample with the heights of the peaks in the standard solution of known content. They were related to the protein content of the samples, expressed as pmol per mg protein. For comparison with the literature values results were expressed as pmol per mg wet tissue. Statistical comparison of results of different animal groups was carried out using the Mann-Whitney *U*-test.

Sample preparation

Male Wistar rats (190—230 g, from Mus Rattus, Brunnthal, F.R.G.) were treated with probenecid (400 mg/kg, intraperitoneally) as described by Gibson and Wurtman [20]: probenecid was dissolved in a minimum volume of 1 *M* sodium hydroxide, and the pH value was adjusted to 7.4 by adding 4 *M* hydrochloric acid. The final concentration of 100 mg/ml of this suspension was obtained with saline (0.9%, w/v) and injected intraperitoneally. In some experiments untreated animals were also used for reference.

Sixty minutes after the injection the animals were decapitated, and the brains rapidly removed and frozen on dry ice. The basal hypothalami were punched out with a needle (I.D. 4 mm) placed behind the optic chiasma. The heights of the tissue cylinders were 2 mm. Each of the tissue pieces was then placed in 600 μl of 0.12 *M* sodium acetate, pH 5.0 obtained by adding citric acid, and homogenized with an ultrasonic cell disrupter (Model B15, Branson, Danberg, CT, U.S.A.). Aliquots were taken from the homogenates for the analysis of protein according to the method of Lowry et al. [21]. The homog-

enates were then centrifuged for 15 min at 4°C at 25,000 *g*; 400 μl of each supernatant were separated for further processing.

In recovery experiments these samples were spiked with either 225 pmol of MOPEG sulphate or with 18.7 pmol, 37.4 pmol or 74.9 pmol of free NE, MOPEG, DA, DOPAC, HVA, 5-HT and 5-HIAA.

In order to hydrolyse the conjugated metabolites 0.7 mg (19.7 units) of sulphatase (7 mg/ml in 0.12 *M* sodium acetate, pH 5.0) were added to each sample and all were incubated for 3 h at 37°C. The samples were subsequently cooled in an ice bath and adjusted to give them the same composition as the mobile phase by adding 40 μl of 1.3 *M* citric acid, 7.5 m*M* sodium octyl sulphate, 2.25 m*M* EDTA, 13.5 m*M* dibutylamine and 60 μl of methanol (final volume of the sample 600 μl). For purposes of comparison some samples were not hydrolysed; 0.12 *M* sodium acetate, pH 5.0, without sulphatase was added to them at the same volume (100 μl) as to the others and the incubation was omitted. The further processing is described above.

The samples were then centrifuged for 15 min at 4°C at 25,000 *g* to pellet precipitated protein; the supernatants were diluted 1:1.5 and filtered through a 0.22-μm filter (GVWP 01300, Millipore); 25 μl of filtrate were then injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatographic conditions

The aim of this study — to detect NE, DA, 5-HT and their main metabolites simultaneously — makes considerable demands on the chromatographic system, since it entails separating compounds of different polarity, such as basic (NE, DA, 5-HT), neutral (MOPEG), and acidic substances (DOPAC, HVA, 5-HIAA). HPLC in the reversed-phase mode, including ion-pairing, offers the wides range of possibilities for method development. The composition and conditions of the mobile phase are the main determinants of solute separation [15]. By varying the pH, the concentration of the ion-pairing reagent and the content of the methanol, we found an optimal separation of NE, DA, 5-HT and many of their metabolites under conditions shown in Fig. 1a. At pH 3.70 the amino groups of the substances are fully protonated, and the dissociation of carboxyl groups is suppressed [22]. The uncharged acidic metabolites are retained long enough at the hydrocarbonaceous stationary phase. The charged functional groups of the amines interact with the ion-pairing reagent in the mobile phase and on the stationary phase prolonging the retention times of the amines [14]. With the methanol as an organic modifier the retention times of all substances were shortened to avoid a lengthy period of separation.

A flow-rate of 1.0 ml/min was found to give the best resolution.

After 80 injections the back pressure of the column had risen to 1.5 times the initial value. Exchanging the guard column diminished this increase. Up to about 400 injections the resolution of the column was sufficient. Then the retention times of the analytes had been shortened to such a degree that NE began to interfere with the front peak. Therefore the column cartridge had to be changed.

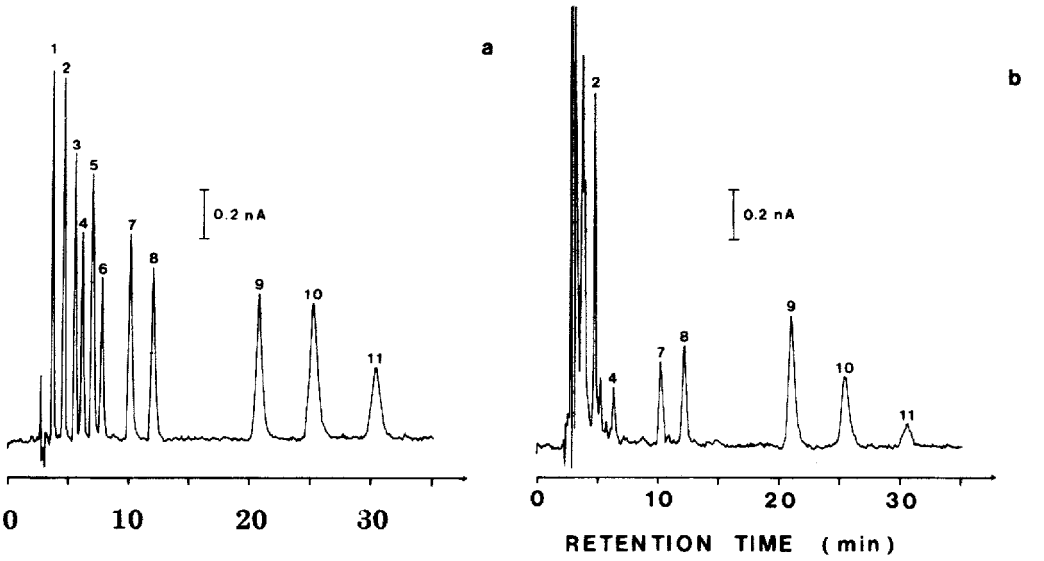

Fig. 1. (a) Chromatogram of a standard solution; 3.75 pmol of each standard dissolved in 25 μl of the mobile phase were injected. (b) Chromatogram of mediobasal hypothalamic tissue. The animals were treated with probenecid and the samples hydrolysed with sulphatase. Injection volume was 25 μl. Stationary phase, 5-μm C_{18} RCM 100; mobile phase, 0.1 *M* sodium acetate, 0.1 *M* citric acid, 0.5 m*M* sodium octyl sulphate, 0.15 m*M* EDTA, 1 m*M* dibutylamine, 10% methanol (v/v), pH 3.70; detector potential, 800 mV versus Ag/AgCl reference electrode. Peaks: 1 = DHPG, 2 = NE, 3 = A, 4 = MOPEG, 5 = DHBA, 6 = NMN, 7 = DA, 8 = DOPAC, 9 = 5-HIAA, 10 = 5-HI, 11 = HVA.

Linearity and sensitivity

The relation between the injected amount of standards and the detector response was linear for all analytes in the tested range (0.5—9.4 pmol). The sensitivity of the measurement varied mainly according to the condition of the detector. The noise of the detector cell was a limiting factor, which was primarily dependent on the condition of the reference electrode.

The detection limits, calculated by doubling the noise ratios, and expressed in terms of pmoles of the components injected were: 0.15 ± 0.02 (NE), 0.22 ± 0.02 (MOPEG), 0.28 ± 0.04 (DA), 0.33 ± 0.04 (DOPAC), 0.75 ± 0.10 (HVA), 0.43 ± 0.05 (5-HT), 0.38 ± 0.05 (5-HIAA) (n = 7).

Selectivity

Fig. 1b shows a chromatogram of mediobasal hypothalamic tissue of probenecid-treated rats. The samples had been hydrolysed with sulphatase. The long analysis time of 35 min was not reduced by increasing the methanol content of the mobile phase, since reduction of the retention times would lead to overlapping of the peaks in the first part of the chromatogram. The peaks were first identified by comparison of their retention times with those of the standards. Spiking the samples with standards provided additional reference points. Omitting prepurification steps in processing the samples renders this identification inadequate. Two additional tests were therefore carried out to ensure that correct peaks were identified and that there were no impurities coeluting with the compounds under investigation.

Variation of the potential of the detector cell (hydrodynamic voltamograms). By increasing the potential step by step, the catecholamines and indoleamines and their metabolites are oxidised at distinghishable potentials [23, 24]. Fig. 2 shows the comparison of the "relative current ratios" at different potentials between the provisionally identified components in the sample and the standard. The substances can be separated by their half-wave potentials ($E_{1/2}$) into three classes: dihydroxyphenols, indoles, vanillic compounds (in following the order of the increasing $E_{1/2}$). It is shown that the half-wave potentials of the provisionally identified components and their standards are almost identical.

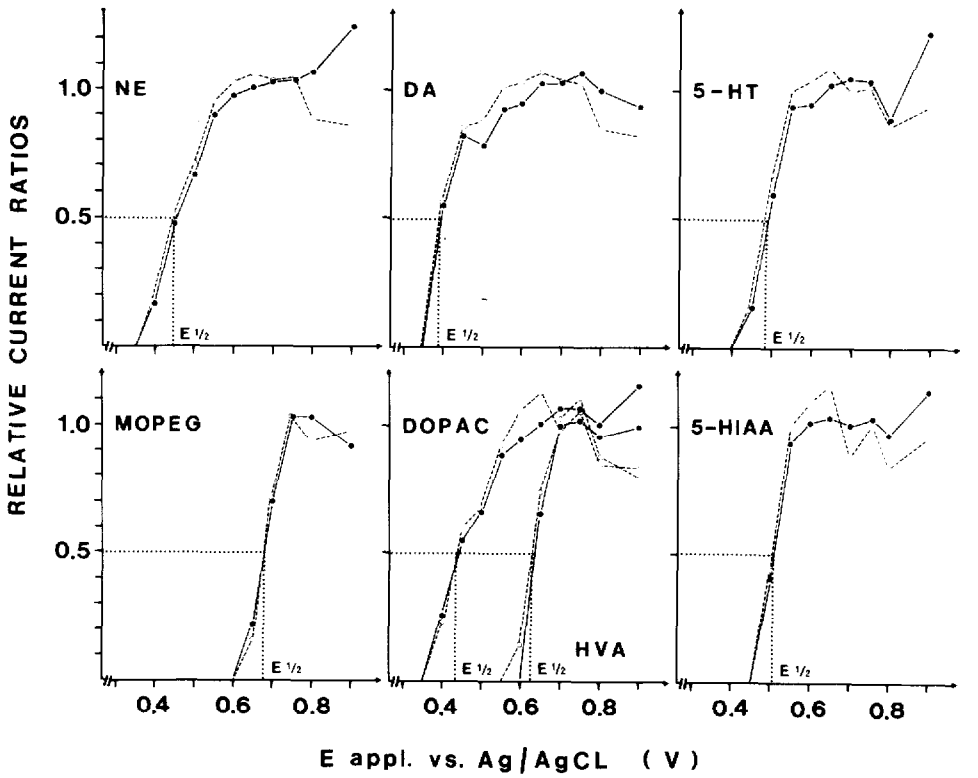

Fig. 2. Hydrodynamic voltammograms of the sample and the standard solution. The mediobasal hypothalami of probenecid-treated animals were pooled and hydrolysed with sulphatase; 25 µl of the processed samples were injected. The response (current) at several potentials was recorded, and the ratios of the current at any given potential to that of the average response at the plateau level were calculated and compared to the relative current ratios of the standards. Each point represents the mean of two determinations. (•——•), Sample; (○- - - -○), standards. The average deviation of the duplicates from their mean values was 2.0 ± 2.2%.

Variation of the pH value of the mobile phase. Depending on the pK_a values of the functional groups of the measured compounds, they are protonated to different degrees at certain pH values of the mobile phase. Fig. 3 shows the comparison of the capacity factors of the provisionally identified components of the sample with those of the standards dependent on the eluent pH. The

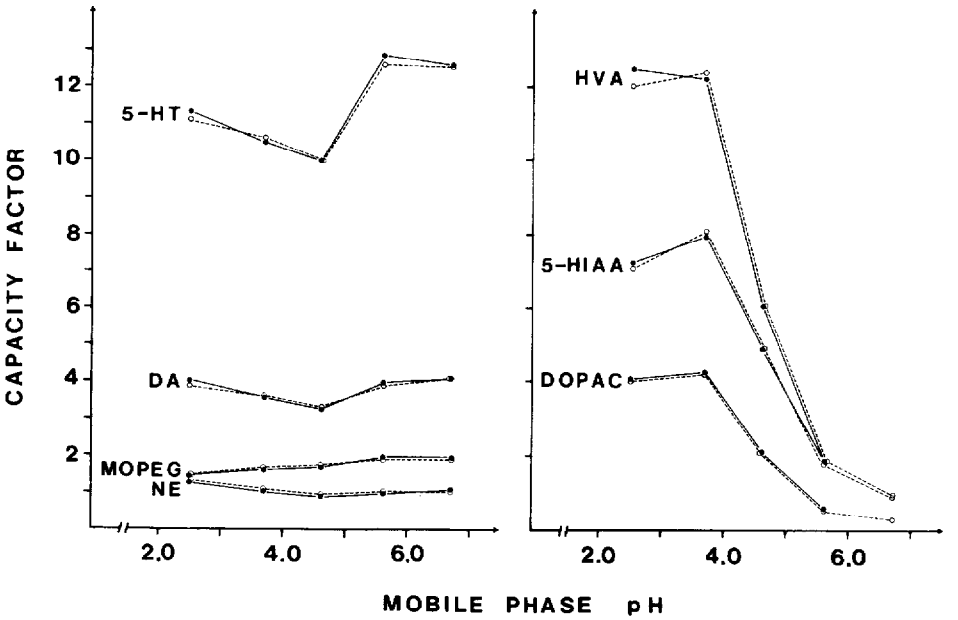

Fig. 3. Effect of mobile-phase pH on capacity factors of components in samples and standard solution. The mediobasal hypothalami of probenecid-treated rats were pooled and hydrolysed with sulphatase; 25 μl of the processed samples were injected. The pH of the mobile phase was varied by changing the ratio of sodium acetate—citric acid, maintaining a constant ionic strength. Each point represents the mean of two determinations. (•——•), Sample; (○- - - -○), standards. The average deviation of the duplicates from their mean values was 0.84 ± 0.63%.

capacity factors of the catecholamines and indoleamines do not change: their amine groups remain protonated over the pH range tested. The glycol MOPEG is not affected either. The carboxyl groups of the acidic metabolites dissociate at higher pH values and their capacity factors decrease. At pH values above 5.60 the capacity factors of the acidic metabolites in the sample could not be shown, since at these pH values their peaks interfered with the front peaks. Accurate identification became impossible. Comparison of the curves of the substances in the sample and of the curves of the standards showed almost no difference between the two.

In summary, the initial identification of the sample peaks was confirmed. Time-consuming prepurification of the sample to increase the selectivity of the method can therefore be omitted.

Precision and recoveries

The analysis of samples spiked with 18.7 pmol, 37.4 pmol or 74.9 pmol of free NE, MOPEG, DA, DOPAC, HVA, 5-HT and 5-HIAA gave recovery values independent from the added amount of analyte. Table I shows, therefore, the mean recoveries ± S.D. of all experiments. They ranged from 91% to 101%. This indicates that the substances are not affected by the sample processing, even during 3 h of incubation at 37°C. The good within-run precision of the estimations, given in the last column of Table I, together with the high recovery rates do not necessitate the use of an internal standard.

TABLE I

ESTIMATION OF THE PRECISION AND THE RECOVERIES OF SAMPLES SPIKED WITH KNOWN AMOUNTS OF STANDARDS

The mediobasal hypothalami of probenecid-treated animals were pooled and each of six samples was spiked with 18.7 pmol, 37.4 pmol or 74.9 pmol of standards. The samples were hydrolysed with sulphatase and 25 μl of each processed sample were injected.

	Endogenous amount (pmol per sample)	Mean recovery (%) ± S.D. (n = 18)	Precision, s_{rel} (%) (n = 24)
NE	167.4	99 ± 17	4.3
MOPEG	48.6	91 ± 9	5.3
DA	71.3	93 ± 7	3.3
DOPAC	85.3	100 ± 10	3.9
HVA	56.7	97 ± 10	5.4
5-HT	115.0	101 ± 10	3.1
5-HIAA	159.3	95 ± 16	3.4

Sample preparation

Enzymatic hydrolysis. The conjugated forms of the metabolites cannot be measured electrochemically. Hydrolysis is therefore necessary. The use of sulphatase preparation from *Helix pomatia* (type H-5, from Sigma) did not interfere with the chromatography of the sample. To test whether the hydrolysis of the conjugated compounds was complete, we spiked six samples

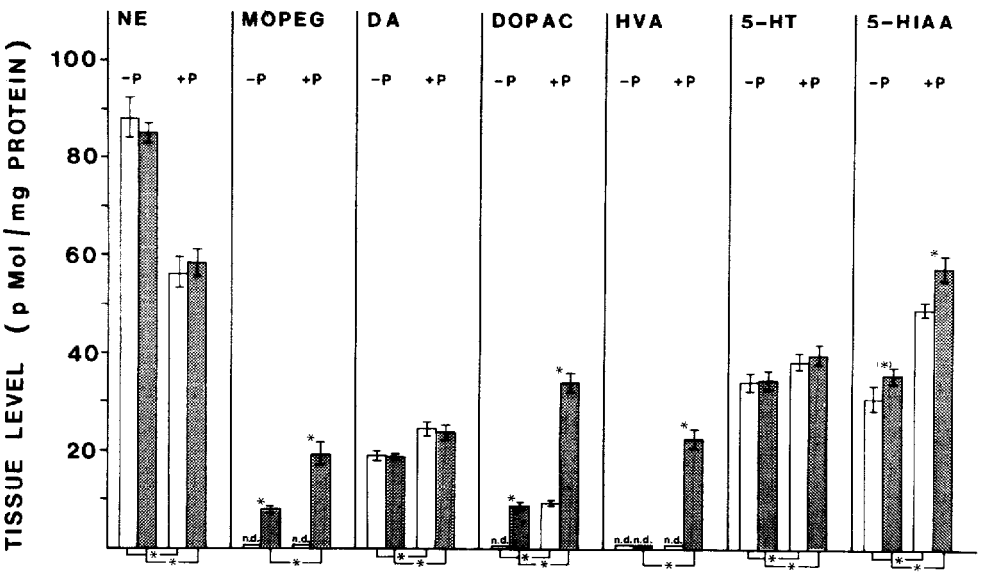

Fig. 4. Effect of probenecid treatment and enzymatic hydrolysis. The mediobasal hypothalami of probenecid-treated or untreated rats were pooled. The samples were then divided and independently processed by either incubating them with sulphatase or by omitting hydrolysis; 25 μl of each processed sample were injected. The heights of the columns represent the mean values ± S.D. of six determinations (n.d. = not detectable). —P = Untreated rats; +P = probenecid-treated rats; (□), unhydrolysed sample; (▨), hydrolysed sample. *: $p < 0.01$; (*): $p < 0.1$.

from probenecid-treated rats, as well as six samples from untreated animals, with quantities of MOPEG sulphate five to ten times higher than that found in mediobasal hypothalamic tissue. We estimated a recovery of 98 ± 5% and 101 ± 3%, respectively. We may assume, therefore, that under the conditions chosen all conjugated components will be hydrolysed completely. Fig. 4 compares data of samples with and without hydrolysis. It can be seen that the metabolites are partially or totally conjugated. Their levels were increased after hydrolysis. This is in agreement with the literature [7—10]. The catecholamines or indoleamines were not affected by this incubation procedure and their levels remained constant. After incubation the composition of the sample was adjusted to that of the mobile phase. This procedure resulted in very small front peaks in the chromatograms. The methanol content thus obtained was sufficient to precipitate protein, as also shown by others [17, 19].

Probenecid treatment of the animals. Fig. 4 demonstrates that probenecid causes an accumulation of all metabolites measured. It blocks the active transport system which eliminates them from the brain [5]. The dependence of the elimination on this transport system was demonstrated for MOPEG sulphate [4, 6], DOPAC sulphate [10], HVA and 5-HIAA [5, 6, 25]. The increase of unconjugated DOPAC after probenecid treatment shown here may not be due to inhibition of transport but is secondary to the end-product inhibition of the DOPAC-conjugating enzyme by the DOPAC conjugate [10]. The high dose of probenecid that we used (400 mg/kg) to guarantee complete blockage of the elimination system for MOPEG sulphate [4] may have been responsible for the significant changes of catecholamine and indoleamine levels after treatment. Other studies have shown that even at lower doses probenecid can change the metabolic rates of these transmitter systems [4, 26, 27]. An estimation of turnover rates by the probenecid method alone may therefore be problematic.

Accuracy

Evidence of the accuracy of this method has already been given by the performed selectivity tests and the high degree of recovery. Certainty in this connection could only be gained by a comparison of this method with a reference method, at present not available for this purpose. Therefore, a comparison of the values of the presented method with literature values of other studies has been made (Table II). The values obtained in this study are in general agreement with the others. None of the reported investigations determined all components simultaneously. Taylor et al. [28] used two different mobile phases to measure either the amines or their related metabolites.

Any comparison of metabolite determinations must take into consideration the fact that the results of the MOPEG, DOPAC and HVA determinations, presented in Table II in this investigation, include the free as well as the conjugated forms of the metabolites. The results of the HVA determination was additionally obtained from probenecid-treated animals. The results are therefore higher than the reported values. Only Kohno et al. [31] determined the free and the conjugated forms of MOPEG, and Smythe and co-workers [1, 2] included the sulphated form of metabolites in their determinations by using trifluoroacetic anhydride for derivatization of the samples. The apparent

TABLE II

RESULTS OF AMINE AND METABOLITE DETERMINATIONS BY THE PRESENT METHOD COMPARED TO LITERATURE VALUES FOR CERTAIN COMPOUNDS IN THE MALE RAT HYPOTHALAMUS

The values of the present method were obtained by six independent determinations of a pooled mediobasal hypothalamic homogenate. Mean values are given.

Investigation method	Concentration (pmol per mg wet tissue)						
	NE	MOPEG	DA	DOPAC	HVA	5-HT	5-HIAA
This investigation	9.71	0.87*	2.11	0.97*	2.53*,**	3.78	3.62
HPLC—ED [28]	14.91	0.43	2.65	0.59	0.14	4.68	1.10
HPLC—ED [29]	8.66	—	1.44	0.30	0.16	—	2.41
HPLC—ED [30]	8.93	—	2.72	—	—	2.91	—
Fluorometry [31]	8.25	1.12	—	—	—	—	—
Radioenzymatic assay							
[32]	9.54	—	3.06	—	—	—	—
[33]	—	—	—	—	—	2.53	—
Gas chromatography—mass spectrometry							
[2]	14.31	—	3.72	—	0.52	5.43	2.96
	13.40	—	2.66	—	0.47	7.56	2.48
	10.30	0.50	3.19	0.95	—	6.19	2.93
	10.10	—	2.35	—	0.64	4.21	2.17
	13.77	—	3.04	—	0.61	5.29	2.53
[1]	10.7	—	3.14	—	0.70	4.65	2.41
	—	—	4.09	—	0.84	6.2	3.5

*Determination of samples hydrolysed with sulphatase.
**Determinations of a pooled homogenate of probenecid-treated rats.

differences between the other values are probably a reflection of the differences in the methods as well as differences in the dissection technique of the hypothalamus. Furthermore, rats of different breed, age and sex were used.

CONCLUSION

This study offers a simple method for measuring NE, DA, and 5-HT and their main metabolites simultaneously, omitting time-consuming sample pre-purification. By means of enzymatic hydrolysis of the sample, which can be easily carried out prior to chromatography, it is also possible to measure conjugated metabolites. The additional tests of selectivity performed ensure adequate peak identification in the samples. This method is thus useful for the estimation of turnover rates in the selected transmitter systems.

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