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DETERMINATION OF PICOGRAM LEVELS OF BRAIN CATECHOLAMINES AND INDOLES BY A SIMPLIFIED LIQUID CHROMATOGRAPHIC ELECTROCHEMICAL DETECTION METHOD

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SUMMARY

A simple and rapid method for the simultaneous determination of norepinephrine, epinephrine, dopamine, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain regions by high-performance liquid chromatography (HPLC) with electrochemical detection has been developed. Perchloric acid extracts of the tissue were directly analyzed in the HPLC system. Each of these compounds gave a linear response over the range of 10-320 ng/ml cerebellar homogenate (0.2-6.4 ng on column). Analytical recoveries of these compounds added to the homogenates were complete when compared with standards dissolved in perchloric acid. The average between-run coefficients of variation for all these compounds were lower than 6.7%, over the range of 10-320 ng/ml, whereas the within-run coefficients of variation at 10 ng/ml were lower than 6.9%. Under the present instrumental and mobile phase conditions, all compounds were readily oxidized at 0.72 V *vs.* a Ag/AgCl reference electrode. The present method has been applied to a study determining the basal levels of these compounds in several rat brain regions as well as levels after medium raphe lesions.

INTRODUCTION

For the analysis of endogenous concentrations of biogenic amines in discrete regions of the brain tissues and biological fluids, high-performance liquid chromatography (HPLC), coupled with electrochemical detection, has proved to be a sensitive method that offers considerable selectivity. A great variety of HPLC systems with elaborate separation procedures are currently being utilized to measure catecholamines and indoles in brain tissue[1-20], plasma or serum[5,21-25], urine [2,5,26,27] and cerebrospinal fluid[5,20,28]. Deproteinization of samples is universally achieved by the addition of acid. However, currently employed HPLC procedures do not have sufficiently high resolution to permit the measurement of catecholamines and indoles in the same sample unless these are first isolated and purified by ion-exchange chromatography, alumina adsorption or organic extraction before injection into the HPLC system. A few methods have been described which permit the simultaneous determination of both classes of compounds[3,6,8,9,18].

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Recently, methods with only minimal sample pre-treatment, to precipitate proteins, have been introduced for the determination of tryptophan and its metabolites[13] and for group separation of catecholamines and indoles in brain tissues[16,19,20]. These methods greatly reduce the time required in sample preparation while still permitting a high sensitivity. We report here the sensitive and rapid simultaneous determination of norepinephrine (NE), epinephrine (E), dopamine (DA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) by high-resolution reversed-phase HPLC, combined with detection by electrochemical oxidation. The method employs a very simple and rapid deproteinization in perchloric acid as the sole extraction/purification step before application of the sample to the HPLC system. Ascorbic acid is added to prevent oxidation of the compounds during chromatography, and an ion-pairing reagent is used to improve resolution of all the compounds.

As a biological validation of the present method, it was applied to a study of the effect of median raphe lesions on the concentrations of biogenic amines in rat brain. Catecholamine and indole levels were measured in samples of various regions of the brains of individual rats.

MATERIALS AND METHODS

Materials

Materials and their sources were as follows: NE bitartrate (No. A-9512), E (No. E-4375), DA hydrochloride (No. H-8502), 5-HT creatinine sulfate complex (No. H-7752), 5-HIAA (No. H-8876) and 3,4-dihydroxybenzylamine hydrobromide (DHBA, D-7012) from Sigma, St. Louis, MO, U.S.A.; citric acid (B-10081), disodium hydrogen orthophosphate (No. 10249), sodium pentobarbital (No. 88512) and L-ascorbic acid (B-44006) from BDH, Toronto, Canada; disodium ethylenediaminetetraacetic acid (EDTA, S-311) and 70% perchloric acid (ACS Reagent, A-229) from Fisher, Fair Lawn, NJ, U.S.A.; octyl sodium sulfate (No. 10577) from Eastman Kodak, Rochester, NY, U.S.A.; methanol (HPLC Grade) from Caledon Laboratories, Georgetown, Canada. Water was deionized and glass-distilled.

HPLC apparatus

HPLC determinations were performed with a Beckman Model 330 Isocratic Liquid Chromatograph and a Model 110A pump, a Bioanalytical Systems Model LC-4B amperometric detector, and a Hewlett-Packard 3390A recording integrator. A glassy carbon working electrode was set at 0.72 V *vs.* a Ag/AgCl reference electrode. Sensitivity of the detector was maintained from 0.2 to 1.0 nA full scale, depending on the concentration. Separation 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 by isocratic elution.

Mobile phase

The mobile phase (pH 4.2) contained 35 m*M* citric acid, 12.5 m*M* disodium hydrogen orthophosphate, 0.25 m*M* octyl sodium sulfate and 0.05 m*M* EDTA, and 20% methanol (v/v) was added. In some runs, the effect of 0.5 m*M* octyl sodium sulfate was also tested. The mobile phase was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and then de-gassed under vacuum before use. A flow-

rate of 1.0 ml/min (2000 p.s.i.) at ambient temperature was employed in the present study.

Preparation of standard solutions

Standard solutions of NE, E, DA, 5-HT, 5-HIAA and DHBA (each 0.1 mg/ml, expressed as the free compounds rather than the salts) were prepared in 0.1 *M* perchloric acid containing 0.05 m*M* ascorbic acid (this is referred to below as 0.1 *M* PCA), which was previously filtered and de-gassed. These solutions were stored at −70°C and freshly prepared every 2 weeks.

Animal procedures

Experiment I. Adult, male Sprague Dawley rats (CBL, Montreal, Canada) weighing 190–210 g were housed in an environmentally controlled room at 21–23°C and 40% relative humidity, with lighting on from 07.00–19.00 hr, for 1 week before use. They received food and water *ad libitum,* except that food was removed 24 h prior to sacrifice. The rats were decapitated and their brains were removed rapidly. The brains was dissected on an ice-chilled glass plate, and the striatum, hypothalamus and hippocampus were separated as described by Glowinski and Iversen[29], frozen on dry ice, and stored at −70°C until assayed. The cerebellum was saved, to make a pooled cerebellum homogenate for use in the preparation of standard curves, as described below.

Experiment II. Adult, male Wistar rats (CBL) were housed individually and maintained at a body weight of 280–320 g by appropriate restriction of the daily ration of chow. Water was available *ad libitum.* These animals had been used previously in a study of pentobarbital tolerance, but they were not employed for the present purpose until at least three weeks after the last exposure to pentobarbital.

For the production of brain lesions, rats were anesthetized with a 50 mg/kg intraperitoneal (i.p.) injection of sodium pentobarbital. Lesions were performed on a David Kopf stereotaxic apparatus with nickel chromium electrodes, insulated except for 0.5 mm at the tip. Lesions of the median raphe nuclei were produced by passing a 2-mA current for 15 sec at the coordinates AP 8.2, LM 00, and ventral 6.8, according to König and Klippel[30]. Sham-lesioned rats were exposed to the same procedure with the exception that no current was delivered. Lesion sites were verified by histology in a separate group of rats. Sixty-five days after production of the lesions, the rats for neurochemical study were decapitated, and samples of brain regions were prepared as in Experiment I.

Preparation of tissue samples

Individual brain regions (64–120 mg sample weight) were placed in polypropylene tubes set in an ice-water bath, and were homogenized in 1.0 ml of 0.1 *M* PCA, containing 40 ng of DHBA as an internal standard, for 45 sec with the use of a Polytron (Brinkman Instrument, NY, U.S.A.). Homogenates were centrifuged at 4°C for 20 min at 35,550 *g*. The supernatants were separated and either immediately analyzed or stored at −70°C for assay later, usually within 2 weeks. Supernatants were injected into the HPLC system in a volume of 20 μl, by means of a 50-μl Hamilton syringe.

Standard curve

Known amounts of NE, E, DA, 5-HT and 5-HIAA (10–320 ng) in 1.0 ml aliquots of the pooled cerebellar homogenates (90 mg of tissue per ml of 0.1 *M* PCA) were taken through the entire procedure, DHBA (40 ng) being added to each of these samples as an internal standard. An identical set of samples of these compounds was made up in 1.0 ml of 0.1 *M* PCA. The measured levels of endogenous NE, E, DA, 5-HT and 5-HIAA contained in aliquots of the same homogenate without these added exogenous compounds were subtracted from those of the corresponding standard samples. To construct the standard curve, the NE/DHBA, E/DHBA, DA/DHBA, 5-HT/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 into the HPLC system were 0.2–6.4 ng, while the amount of DHBA was 0.8 ng.

Statistical analysis

Group means were compared by using Student's *t* test.

RESULTS AND DISCUSSION

Representative chromatograms obtained for NE, E, DA, 5-HT and 5-HIAA standards in 0.1 *M* PCA, and for the same compounds occurring endogenously in rat hypothalamus homogenate, are shown in Fig. 1. No interfering endogenous compounds were apparent.

The linearity of the concentration–response relation for each of these compounds was established over the range of 10–320 ng in 1.0 ml of cerebellar homogenate. The correlation coefficients for all these compounds were higher than 0.9976 (Table I). Analytical recoveries of each of these compounds (10–320 ng), added to the homogenates, were calculated by comparison of peak areas with results obtained with the corresponding samples in PCA, as well as with that of internal standard DHBA. The recoveries were essentially complete (Table II). The average between-run and within-run coefficients of variation for all these compounds were lower than 6.7 and 6.9%, respectively (Table II).

Table III presents the levels of NE, DA, 5-HT and 5-HIAA in various brain regions. The present data are in reasonable agreement with those obtained previously with alternative HPLC methods[4,11,13,15,17,19]. The wide range of levels reported in the literature (Table IV, inter-assay variation as high as 80% for different amines) may indicate methodological problems, such as endogenous interference or lack of sensitivity, difficulties associated with the isolation and detection scheme, and variability in tissue sampling techniques.

Table V shows the NE, DA, 5-HT and 5-HIAA levels in the hypothalamus, hippocampus and striatum, following sham and median raphe lesions. Since no statistical differences were found in the levels of these compounds between rats previously treated either with pentobarbital or with water, the data of the two groups were combined. The 5-HT and 5-HIAA levels were significantly decreased in hypothalamus and hippocampus after lesion treatment. No significant difference was found in the levels of 5-HT and 5-HIAA in striatum and no differences in NE and DA in any of the regions. Most of the forebrain 5-HT has been shown to originate

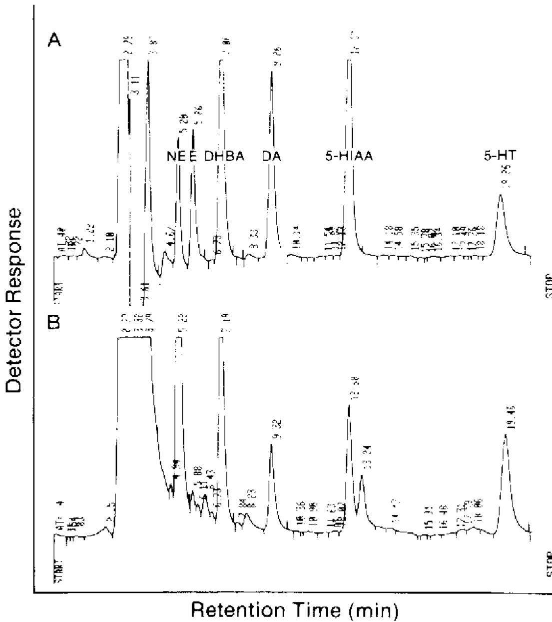

Fig. 1. A, Reversed-phase HPLC separation of NE, E, DA, 5-HT and 5-HIAA standards (each 0.4 ng), and the internal standard DHBA (0.8 ng) in PCA. B, Representative chromatogram, demonstrating the HPLC separation and detection of NE, DA, 5-HT and 5-HIAA from a portion of a single rat hypothalamus (20 μl PCA extract equivalent to 1.3 mg of tissue). DHBA was used as an internal standard. Chromatographic conditions: column, Ultrasphere ODS C_{18}; mobile phase (pH 4.2), 80:20 (v/v) mixture of the citrate (35 m*M*) phosphate (12.5 m*M*) and methanol with octyl sodium sulfate (0.25 m*M*) and EDTA (0.05 m*M*); sensitivity, 0.2 nA; sample size, 20 μl; flow-rate, 1.0 ml/min; electrode potential, +0.72 *vs.* Ag/AgCl reference electrode; temperature, ambient.

from the dorsal and median raphe nuclei located in the midbrain[31,32]. Lesions of these two nuclei markedly depleted forebrain 5-HT[33–36]. Studies have been performed to determine the specific brain projections of these two nuclei. Lesions of the median raphe nuclei have been found to produce a fall in 5-HT in the hippocampus and hypothalamus, whereas lesions of the dorsal nuclei affect only the hypothalamus. In contrast, median raphe lesions have no effect on striatum, but dorsal raphe lesions do decrease striatal 5-HT[35,36]. Therefore, it has been suggested that the hippocampus

TABLE I

LINEAR REGRESSION ANALYSIS ON SLOPES AND INTERCEPTS DERIVED FROM STANDARD CURVES IN CEREBELLAR HOMOGENATES

	Slope	*Y-Intercept*	r^2
NE	0.7638	0.0665	0.9993
E	0.7988	0.0008	0.9984
DA	1.6978	0.0932	0.9976
5-HT	1.0637	0.0117	0.9991
5-HIAA	2.4564	0.0643	0.9998

TABLE II

RECOVERY AND PRECISION OF THE HPLC DETERMINATION OF NE, E, DA, 5-HT AND 5-HIAA IN CEREBELLAR HOMOGENATES

	*Recovery (%)**,**	*Between-run C.V. (%)****	*Within-run C.V. (%)*§
NE	98 ± 4	4.1	6.3
E	98 ± 5	5.1	6.0
DA	107 ± 3	2.8	5.3
5-HT	104 ± 7	6.7	6.9
5-HIAA	105 ± 4	3.8	6.4

* Relative to value for PCA solution.
** Mean ± SD over the range of 10 320 ng/ml homogenates ($n = 8$).
*** Determinations over the range of 10 320 ng/ml homogenates (at each concentration, $n = 8$).
§ Determinations at 10 ng/ml homogenate ($n = 8$).

receives most if not all of its serotonergic input from the median raphe nucleus, whereas the dorsal nucleus innervates the striatum, and the hypothalamus receives innervation from both median and dorsal raphe nuclei[36]. The present data are entirely consistent with this.

The large improvement in resolution of the amines and metabolites reported here is due in part to the use of an ion-pairing reagent and a mobile phase, citrate–disodium phosphate, having a higher pH than is generally used. In previous studies of HPLC methods for morphine, we found improved resolution in the pH range of 4.2–4.5. Since morphine has a phenolic ring, it seemed possible that the same conditions would improve resolution of the amines and metabolites, and this appears to be the case. Under the instrumental and mobile phase conditions described here, all catecholamines and indoles were readily oxidized at +0.72 V. A working electrode potential of +0.8 V and higher can be employed, but it increases the baseline noise and makes the detection limits worse. Catecholamines, especially NE and E, have short retention times and therefore tend to be superimposed on the frontal elution of unretained compounds on the C_{18} column. However, they can be resolved by use of an ion-pairing reagent, octyl sodium sulfate. A recent report describes the principles and methodology of liquid chromatography with electrochemical detection and ion-pairing reagents for the separation of phenolic compounds[37]. Subsequently,

TABLE III

CONTROL VALUES OF NE, DA, 5-HT AND 5-HIAA (ng/g WET WEIGHT) IN VARIOUS REGIONS OF RAT BRAIN

The PCA extracts equivalent to 2.4, 1.3 and 2.0 mg for striatum, hypothalamus and hippocampus, respectively, containing 0.8 ng DHBA as an internal standard, were injected into the HPLC system. Results are means ± S.E.M. of 10 preparations.

Brain region	*NE*	*DA*	*5-HT*	*5-HIAA*
Striatum	183 ± 24	7478 ± 404	807 ± 28	330 ± 21
Hypothalamus	2603 ± 96	356 ± 24	1249 ± 51	576 ± 35
Hippocampus	354 ± 43	48 ± 2	546 ± 20	339 ± 7

TABLE IV

REGIONAL CATECHOLAMINE AND INDOLE LEVELS REPORTED IN THE LITERATURE

References cited in parentheses for lowest and highest values found.

Brain region	*Compounds*	*Range of concentrations (ng/g wet weight)*
Striatum	NE	140 ± 19 (17)
	E	–*
	DA	6640 ± 329 (17) to 11888 ± 1840 (4)
	5-HT	1745 ± 422 (19)
	5-HIAA	281 ± 23 (17) to 555 ± 19 (19)
Hypothalamus	NE	1232 ± 63 (15) to 5481 ± 288 (19)
	E	36 ± 2 (15)
	DA	293 ± 12 (15) to 597 ± 15 (19)
	5-HT	661 ± 44 (11) to 2643 ± 141 (19)
	5-HIAA	400 ± 28 (11) to 650 ± 38 (19)
Hippocampus	NE	321 ± 22 (15) to 795 ± 51 (19)
	E	–*
	DA	23 ± 5 (17) to 39 ± 13 (15)
	5-HT	435 ± 30 (11) to 1322 ± 18 (19)
	5-HIAA	200 ± 12 (11) to 421 + 38 (19)

* Not reported.

other investigators found that ion-pairing reagents increase the capacity factors for the catecholamines and improve their resolution. In the present study, 0.25 m*M* octyl sodium sulfate was effective in separating NE and E, but 0.5 m*M* doubled the reten-

TABLE V

REGIONAL NE, DA, 5-HT AND 5-HIAA LEVELS (ng/g WET WEIGHT) IN RAT BRAIN AFTER MEDIAN RAPHE AND SHAM LESIONS

Results are means ± S.E.M. (n = 11–12).

Brain region	*Compounds*	*Lesion group*		*% Change after lesion (p)*
		Sham	*Median raphe*	
Hypothalamus	NE	5438 + 507	5993 ± 352	+ 10 (n.s.)
	DA	687 ± 110	579 ± 112	−16 (n.s.)
	5-HT	1441 ± 201	735 ± 141	−49 ($p < 0.005$)
	5-HIAA	445 ± 64	278 ± 33	−38 ($p < 0.025$)
Hippocampus	NE	1313 ± 65	1318 ± 195	0 (n.s.)
	DA	73 ± 30	49 ± 28	−33 (n.s.)
	5-HT	538 ± 49	216 ± 44	−60 ($p < 0.001$)
	5-HIAA	317 ± 20	148 ± 21	−53 ($p < 0.001$)
Striatum	NE	377 ± 78	380 ± 76	+ 1 (n.s.)
	DA	6783 ± 601	7126 ± 507	+ 5 (n.s.)
	5-HT	1172 ± 81	1064 ± 84	− 9 (n.s.)
	5-HIAA	521 ± 34	489 ± 24	− 6 (n.s.)

tion times of all compounds. For example, the retention time for 5-HT was increased from 20 min to 40 min. Under the present chromatographic conditions, the retention and resolution of the compounds were constant over 1500 analyses on the same column. Column rejuvenation to the same efficiency was accomplished by washing the column with 800–1000 ml of methanol–water (40:60) after 100 analyses and replacing the guard column after every 250 analyses.

The techniques of ion-exchange chromatography, alumina adsorption or organic extraction eliminate many endogenous compounds and ions that react with the electrochemical detector, and are reputed to prolong the life of the column. In general, the previously reported methods require elaborate and extensive isolation and purification procedures to permit effective determination of catecholamines and indoles, and yields vary from 20–90%. A significant advantage of the direct injection technique is the ability to measure the levels of these compounds in the same chromatographic analysis without loss in recovery. Tryptophan and its metabolites have been separated by direct injection[13]. In the procedure described by Nielsen and Johnston[20], the eluates were filtered through pre-washed glass wool, and a large volume (500 μl) was injected for the determination of NE, DA and 5-HT. The resolution of NE was poor and E was not separated. A rapid one-step analysis for the determination of DA metabolites and 5-HIAA in rat cerebrospinal fluid has also been described[28]. Most recently, Zaczek and Coyle[19] reported the direct injection of the supernatant and quantitation of NE, E, DA, 5-HT and 5-HIAA with an elution time of 40 min. No statistical data were given for this method, or for other reports on this technique[13,16,20], and thus its precision can not be compared with that of the present method.

In the present study, the supernatant of the homogenate was injected directly onto the column, eliminating the commonly adopted steps of ion-exchange chromatography, adsorption on alumina or organic extraction. This eliminated a possible source of error, a loss in sensitivity and an unsatisfactory recovery. No glassware used in the procedure was silanized. The major advantages of the present method are simplicity of sample processing (elution time of 20 min for all these compounds), and its high sensitivity and reproducibility. During a normal working day, 24 samples can easily be analyzed.

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