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Determination of Dopamine, Norepinephrine, Serotonin and Their Major Metabolic Products in Rat Brain by Reverse-Phase Ion-Pair High Performance Liquid Chromatography With Electrochemical Detection

CONNIE KOTAKE, THOMAS HEFFNER, GEORGETTA VOSMER AND LEWIS SEIDEN[1]

The University of Chicago, Department of Pharmacological and Physiological Sciences
947 East 58th Street, Chicago, IL 60637

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KOTAKE, C , T HEFFNER, G. VOSMER AND L SEIDEN *Determination of dopamine, norepinephrine, serotonin and their major metabolic products in rat brain by reverse-phase ion-pair high performance liquid chromatography with electrochemical detection* PHARMACOL BIOCHEM BEHAV 22(1) 85–89, 1985 —A method is described for the separation and quantitation of catecholamines, serotonin, and their major metabolites with use of reverse-phase, ion-pair liquid chromatography with electrochemical detection This method employs columns packed with a microparticulate C-18 resin, octyl sodium sulfate as the ion-pairing agent, and isocratic elution with a citrate-phosphate buffer containing methanol Conditions are described for the separation of norepinephrine, normetanephrine, 3-methoxy-4-hydroxyphenylglycol, epinephrine, metanephrine, dopamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, serotonin, and 5-hydroxyindole-3-acetic acid and for their quantitation in extracts of rat brain tissue

High performance liquid chromatography Catecholamine analysis Serotonin Amine metabolites

THE use of high performance liquid chromatography (HPLC) for the quantitation of biogenic amines has been described in a number of reports (e g., [5,8]). With use of reverse-phase ion-pair HPLC coupled with electrochemical detection, norepinephrine (NE), dopamine (DA), serotonin (5-HT) and metabolites of these compounds can be determined in biological tissues [2,9]. However, most available methods for the determination of these compounds require preparative chromatographic procedures prior to application of tissue extracts onto a HPLC column Such methods require that several fractions from the original tissue extract be analyzed by separate HPLC procedures

The purpose of the present study was to determine if an HPLC method could be devised which would permit quantitation of endogenous amines and metabolites from homogenates of rat brain parts without the use of extensive preliminary fractionation procedures The method described employs HPLC columns packed with a microparticulate C-18 resin, octyl sodium sulfate as the ion-pairing agent, and isocratic elution with a citrate-phosphate buffer. This method proved suitable for the determination of NE, DA, 5-HT, epinephrine (E), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole-3-acetic acid (5-HIAA) in deproteinized homogenates of rat hypothalamus or caudate putamen. The method may also be suitable for the determination of normetanephrine (NMN) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in rat brain parts Although development of such chromatograms required 90 minutes, more rapid determinations could be achieved by separating the homogenate into two portions, the catechols were determined in one portion after alumina absorption while 5HT, 5HIAA and HVA were determined in the remaining portion with use of a mobile phase containing methanol.

METHOD

Chemicals

The following standards were purchased from the Sigma Chemical Co (St Louis, MO) d,l-norepinephrine (NE) hydrochloride, 3-methoxy-4-hydroxyphenylglycol (MHPG) piperazine, d,l-normetanephrine (NMN) hydrochloride, L3,4-dihydroxyphenylalanine (DOPA), l-epinephrine (E)

[1]Requests for reprints should be addressed to L Seiden

bitartrate, 3,4-dihydroxybenzylamine (DHBA) hydrobromide, dopamine (DA) hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3MT) hydrochloride, serotonin (5-HT) creatinine sulfate, 5-hydroxyindole-3-acetic acid (5-HIAA), and d,l-metanephrine (MN) hydrochloride. Octyl sodium sulfate (OSS) was purchased from Eastman Kodak Co (Rochester, NY) Aluminum oxide (Woelm, activity grade 1) was purchased from ICN Nutritional Biochemicals (Cleveland, OH) N-tris-hydroxymethyl-methylglycine (Tricine) was purchased from Sigma Chemical Co (St Louis, MO) All other chemicals were reagent grade and were purchased from Fisher Scientific Co (Pittsburgh, PA)

Chromatography

The chromatography systems consisted of Milton Roy minipumps (Milton Roy Co., Riviera Beach, FL) equipped with pulse dampeners (Bioanalytical Systems Inc , Lafayette, IN), Model 7010 stainless steel sample injectors (Rheodyne Co., Cotati, CA) equipped with 100 μl sample loops, and stainless steel columns (2 50×4.6 mm) packed with RSIL, C18HL reverse phase resin, 5 micron particle size (Alltech Associates Inc , Deerfield, IL) The electrochemical detectors were purchased from Bioanalytical Systems Inc (Lafayette, IN) and consisted of Model LC-3 amperometric detectors, TL5 glassy carbon working electrodes, and RE-1 silver silver chloride reference electrodes Peaks due to the oxidation of compounds in the column eluates were recorded on strip chart recorders (Model 355, Linear Instrument Corp , Irvine, CA) The working electrode was maintained at a potential of +0.9 V relative to the reference electrode

The mobile phase contained 125 mM citric acid, 125 mM sodium phosphate, 0.01% (w/v) ethylenediamine tetracetic acid (EDTA) and OSS (10, 20 or 30 mg per liter). The pH of the mobile phase was 3 7, in some cases, the pH was adjusted to 2 5 with phosphoric acid (85%) The mobile phase was passed through a filter with 0 45 μm pores (Type HATF, Millipore Corp , Bedford, MA) and was degassed under vacuum In some cases, absolute methanol (degassed by sonication) was added to the filtered, degassed mobile phase in place of equal volumes of water to provide concentrations of 5, 7 5, 10 or 15% (v/v) methanol The mobile phase was pumped through the column at a rate of 1 ml per min

The HPLC columns were washed with 100 ml of absolute methanol and then with 100 ml of distilled, deionized water Adaptation of the columns to mobile phases containing OSS was accomplished by passing 1.5 liters of mobile phase through the columns For increasing the OSS concentration in the mobile phase, 1 liter of new mobile phase was passed over the columns, for decreasing the OSS concentration, the columns were washed in succession with 50 ml of water, 50 ml of methanol, and 50 ml of water prior to adapting the columns to 1 5 liters of the new mobile phase Adaptation of the columns to a mobile phase containing methanol or to a change in the pH of the mobile phase was accomplished by passing 200 ml of the new mobile phase through the columns

Tissue Preparation

Rats obtained from the Holtzman Co (Madison, WI) were sacrificed by decapitation and the brains were removed and rapidly dissected [4] to provide hypothalamus and caudate putamen Brain regions were stored at −80°C until analyzed Brain tissue (30–50 mg, wet weight) was homogenized with use of a sonic tissue disruptor (Heat Systems Inc , Melville, NY) in 1 ml of 0 4 N perchloric acid containing 0 05% (w/v) EDTA, 0 1% (w/v) sodium metabisulfite, and DHBA as an internal standard After centrifugation (20,000×g, 30 min, 4°C), 100 μl of the supernatants were in some cases applied directly to the HPLC columns In other instances, catechols present in the supernatant were first isolated with alumina [3] Aliquots of the supernatants (0 5–1 ml) were combined with 20 mg of activated alumina in 15 ml conical plastic tubes and the pH was adjusted to 8 6 with 0 1 M tricine containing 2 5% (w/v) EDTA and 2 1% (w/v) sodium hydroxide After mechanically shaking the tubes for 5 min, the alumina was sedimented by centrifugation (1000×g, 2 min, 4°C) and the supernatant was aspirated and discarded The alumina was then washed three times with 5 ml of water by vortex mixing for 5 sec and aspirating and discarding the supernatant The catechols were eluted from the alumina in 150–500 μl of 0 1 M phosphoric acid by shaking the tubes mechanically for 5 min After centrifugation (1000×g, 2 min, 4°C), the eluates were aspirated and stored at −80°C until analyzed The tissue pellets obtained after centrifugation of the homogenates were suspended in 0 1 M sodium hydroxide and protein was determined by the Biuret procedure [6]

Calculations

The amounts of amines eluted from the HPLC columns were calculated from comparisons between the heights of the recorded peaks from standards and tissue extracts Recovery of compounds was estimated from the recovery of known amounts of DHBA added during tissue homogenization. Preliminary studies established that the recovery of each of the catechols from alumina was within 5% of the recovery of DHBA and that the recorded peak heights were proportional to the amount of sample applied to the columns The amounts of amines in tissue were corrected for recoveries and were expressed as ng/mg of protein The retention of the compounds by the HPLC columns was expressed in minutes

RESULTS

For each of three concentrations of OSS in the mobile phase (10, 20, or 30 mg per liter), retention was determined under five concentrations of methanol (0, 5, 7 5, 10, and 15%, v/v) at each of two pHs (3 7 and 2 5) The presence of OSS in the mobile phase improved the resolution of the compounds examined Increasing the concentration of OSS from 10 to 30 mg per liter increased the retention times of most compounds by 38–69%, except for DOPAC and 5HIAA, the retention times of which were relatively unaffected by this change, and MHPG and HVA, the retention times of which were slightly reduced (14–24%) by increasing amounts of OSS As shown in Fig 1, decreasing the pH from 3 7 to 2 5 decreased the retention times of most compounds from 20–60%, with the exception that the retention time of DOPA was increased by 20% The addition of methanol to the mobile phase decreased the retention times of all compounds examined, increasing the methanol concentration from 0 to 15% (v/v), reduced retention times by 32–89% with the compounds that normally displayed the strongest retention (MN, 5HIAA, 5HT, 3MT and HVA), and showed the greatest reduction in retention time in the presence of methanol Increasing the methanol concentration to 15%

TABLE 1

CONCENTRATION OF CATECHOLS IN RAT BRAIN REGIONS DETERMINED WITH USE OF ALUMINA

Tissue	NE	E	DA	DOPAC
Hypothalamus	20 65 ± 0 84	0 92 ± 0 10	2 33 ± 0 10	0.82 ± 0 06
Caudate putamen	2.97 ± 0 15	NM*	106 9 ± 7 5	15 29 ± 1 13

*Not measurable

The mobile phase contained 30 mg OSS per liter and no methanol, the pH was 2 5 An alumina eluate, in 0.1 M H_3PO_4, was injected on the column

Values represent the mean and S.E M , expressed in ng per mg of protein, from 8 rats

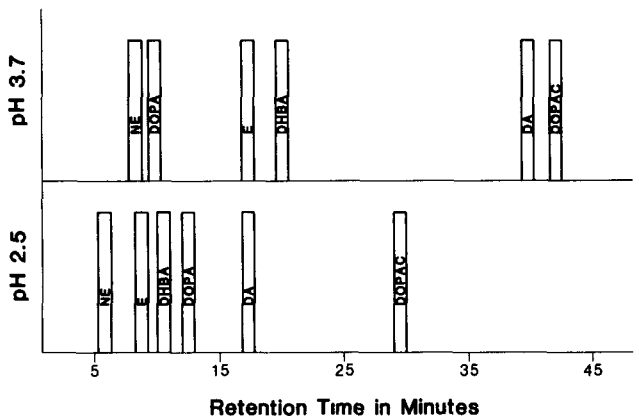

FIG 1 The effect of increased pH on retention time is shown here for NE, E, DHBA, DA and DOPAC The mobile phase contained 20 mg OSS per liter and no methanol Increasing the pH of the mobile phase from 2 5 to pH 3 7 results in increased retention times for all compounds but DOPA Although not illustrated, an increased pH similarly affected the mobile phase systems containing 10 or 30 mg OSS/l and various concentrations of methanol.

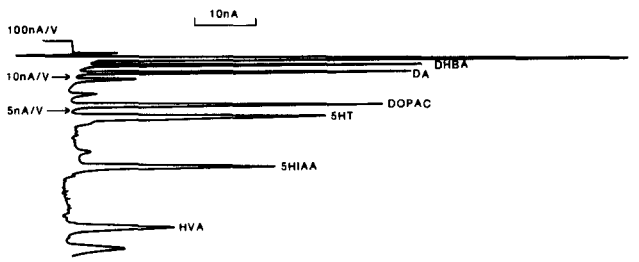

FIG 2 This chromatogram was obtained following direct injection of caudate tissue homogenate Thirty two (32) mg of tissue was homogenized in 1 ml 0 4 N PCA containing 200 ng DHBA The mobile phase contained 20 mg OSS/liter and 10% (v/v) methanol at pH 2 5 The flow rate was 1 ml/min and the potential of the working electrode was maintained at +0 9 V relative to the reference electrode

(v/v) or greater resulted in loss of resolution for many compounds.

The mobile phase best suited for the determination of the catechols (NE, E, DHBA, DA, and DOPAC) in alumina eluates was a citrate-phosphate buffer (pH 2.5) containing 30 mg OSS per liter, pH 2.5, without methanol. Lower amounts of OSS resulted in poor resolution of NE from the large solvent front obtained with use of tissue extracts as well as broadening of later peaks due to DA and DOPAC. The mean (±S E.M.) recovery of DHBA with use of the alumina procedure was 64.2±4 2% for the caudate putamen (n=6) Typical concentrations of catechols in brain tissue determined with use of the alumina procedure are shown in Table 1. Those values are in close agreement with previously reported values [5, 7, 9] including those determined in our laboratories with use of a radioenzymatic assay procedure [4]

For the determination of DA metabolites not isolated with use of alumina as well as 5-HT and 5-HIAA, the supernatants from tissue homogenates were applied directly to HPLC columns adapted to a mobile phase containing 20 mg OSS per liter (pH 2 5) and 10% (v/v) methanol As shown in Fig. 2, peaks corresponding to DHBA, DA, DOPAC, 5-HT, 5-HIAA and HVA are resolved from other identified and unidentified peaks with use of this mobile phase. Although 5-HT and 3-MT are not resolved with use of this mobile phase, the low levels of 3-MT present in rat brain do not normally present a problem for the determination of 5-HT. The mean (±S.E M.) recovery of DHBA from the hypothalamic and caudate tissue homogenates (n=6) was 87.3±3.9% and 86.0±4.7%, respectively. Typical concentrations of the five compounds determined in rat caudate putamen and

TABLE 2

CONCENTRATION OF DA, 5-HT AND THEIR MAJOR METABOLITES IN RAT CAUDATE PUTAMEN

	DA	DOPAC	HVA	5-HT	5-HIAA
Mean	112 0	17 28	9 37	3 77	4 92
S E M	8 5	1 23	0 79	0 25	0 36

The mobile phase contained 20 mg OSS per liter and 10% (v/v) methanol, the pH was 2 5 A supernatant from tissue homogenate, in 0 4 N $HC10_4$, was injected on the column

Values represent the mean and S E M , expressed in ng per mg of protein, from 8 rats

TABLE 3

CONCENTRATION OF NE, DA, 5-HT AND THEIR METABOLITES IN RAT HYPOTHALAMUS

	NE	NMN	MHPG	E	DA	DOPAC	5-HT	5-HIAA
Mean	25 62	0 46	0 08	1 08	2 40	0 96	7 33	6 12
S E M	1 63	0 06	0 01	0 10	0 16	0 84	0 47	0 37

The mobile phase contained 30 mg OSS per liter (pH 2 5) without methanol A supernatant from tissue homogenate, in 0 4 N $HCLO_4$ was injected on the column

Values represent the mean and S E M , expressed in ng per mg of protein from 8 rats

hypothalamus are shown in Table 2 These values are in agreement with previous reports [2, 4, 9, 12]

Although the concentrations of DA, DOPAC, 5-HT, 5-HIAA and HVA in the hypothalamus could be determined with use of a mobile phase containing 20 mg OSS per liter (pH 2 5) and 20% methanol, compounds with retention times shorter than DA were either not resolved from the solvent front (NE, E) or were not resolved from unidentified peaks found in tissue (NMN, MHPG) In order to determine NE, E, DHBA, DA, DOPAC, 5-HT, 5-HIAA, and HVA in tissue extracts, the supernatant from the tissue homogenate can be divided into two aliquots One aliquot can be used for the determination of catechols with use of alumina, as described above, while the other aliquot can be injected directly onto a column adapted to a mobile phase containing 10% methanol for the determination of 5-HT, 5-HIAA and HVA This method, however, does not provide for the determination of NMN, MHPG and MN In an attempt to determine these compounds in hypothalamic extracts, the supernatant from a hypothalamic homogenate was injected directly onto a column adapted to a mobile phase containing 30 mg OSS per liter (pH 2 5) without methanol The strong retention of HVA by the resin adapted to this mobile phase resulted in a late, very broad peak which was not suitable for quantitation of this compound Table 3 shows the concentrations of hypothalamic NE, E, NMN, MHPG, DA, DOPAC, 5-HT and 5-HIAA determined in this manner These values are in general agreement with previous reports [2, 4, 5, 7, 9, 11] The concentration of NMN within the hypothalamus was less than the sensitivity of this assay procedure

DISCUSSION

The present results demonstrate the suitability of RSIL reverse-phase columns for the analysis of biogenic amines in brain tissue The sensitivity of this method was estimated by determining the amounts of standards necessary to provide peaks which were twice the height of the maximal variation in baseline recordings during analysis of tissue samples The estimated sensitivities range from 40–120 pg The amine and metabolite values determined in rat brain tissues are in close agreement with previously published values

A problem with the present method is that 3MT can not be resolved from 5-HT, or in some cases, 5-HIAA While the low levels of 3MT present in brain permit the accurate assessment of tissue levels of 5-HT and 5-HIAA, 3MT itself can not be determined with use of any of the mobile phases tested A possible method for determination of 3MT could involve an initial chromatography procedure to separate 5-HT and 5-HIAA from 3MT [9]

The present method appears suitable for the determination of a number of compounds in rat brain tissue During routine use for amine analyses in our laboratories, we have found that the columns are useful for 4–6 months, with occasional replacement of the contaminated resin on top of the columns The retention times of the compounds described on new columns (n=20) varied on the average of 6% These methods appear to be applicable to the determination of amines and their metabolites in the caudate putamen and hypothalamus, as well as in other rat brain regions

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