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BRIEF COMMUNICATION

A Rapid and Simple HPLC Microassay for Biogenic Amines in Discrete Brain Regions

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DONZANTI, B. A. AND B. K. YAMAMOTO. *A rapid and simple HPLC microassay for biogenic amines in discrete brain regions.* PHARMACOL BIOCHEM BEHAV 30(3) 795–799, 1988.—A rapid microassay is described for the measurement of biogenic amines using an isocratic HPLC system with electrochemical detection. Catecholamines, indoleamines and their major metabolites were extracted with 150 μl of perchloric acid from brain tissue punches (<250 μg) using a simple one-step sample preparation method. These compounds were separated on a short (80 mm) column with 3 μm particle size packing, and electrochemically detected within a total run time of less than 6 minutes. Detection limit sensitivity was approximately 2–5 pg. This method, detailed in an easy-to-follow description, reduces assay time, minimizes the possibility for errors, maximizes efficiency, and requires only standard HPLC equipment and supplies.

HPLC Catecholamines Indoleamines Brain

A variety of assays utilizing radioenzymatic and fluorometric methods have been available for the determination of biogenic amines in brain tissue [5,10]. Due to rapid technological advances, these have now been replaced by more routine methods utilizing high-performance liquid chromatography (HPLC) coupled with electrochemical detection for the measurement of the monoamines dopamine, norepinephrine, serotonin, and their metabolites. Numerous studies have focused on either the chromatographic and electrochemical behavior of the species in question, the detection limit sensitivity, or the small sample size and minimal sample preparation needed. However, few studies have integrated these characteristics into a versatile and detailed, yet uncomplicated method and description for the non-chromatographer/neuroscientist. More recently, the advent of short (<250 mm) reversed-phase columns containing 3 μm packing materials have allowed for a substantial advancement in the resolution, sensitivity, and efficient quantitation of the biogenic amines within an abbreviated run time [1, 4, 14, 15]. In light of this, fewer studies have incorporated this recent technological advancement with the aforementioned desired assay characteristics into a routine and uncomplicated method [9,12].

We now report a method utilizing a rapid, simple, one-step sample preparation of small (<250 μg wet weight) brain tissue micropunches for the determination of biogenic amines and their major metabolites using HPLC with electrochemical detection. The following description includes an isocratic system using a short, reversed-phase column with 3 μm particle size that effectively resolves brain monoamines from their major metabolites in one short chromatographic step of less than 6 minutes. The ease of sample preparation and the sensitive quantitation of parent biogenic amines and their major metabolites in micropunched brain samples within a reduced assay time minimize the possibility for technical errors and maximize efficiency.

METHOD

Chemicals

Citric acid, sodium acetate, disodium ethylenediamine tetraacetate (Na_2EDTA), methanol (HPLC grade) and perchloric acid, 70% and phosphoric acid, 85% (HPLC grade) were purchased from Fisher Scientific Company (Pittsburg, PA). l-Octanesulfonic acid (OSA) sodium salt (HPLC grade) was purchased from Eastman Kodak Company (Rochester, NY). Diethylamine (free base), l-norepinephrine (NE) hydrochloride, dopamine (DA) hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptamine creatinine sulfate (5-HT), homovanillic acid (HVA) and 3,4-dihydroxybenzylamine (DHBA) hydrobromide were purchased from Sigma Chemical Company (St. Louis, MO).

Chromatography

The chromatographic system consisted of a Varian model 5000 liquid pump (Varian Associates Inc, Palo Alto, CA) equipped with a 10 μm inlet filter. A SSI model LP-21 pulse

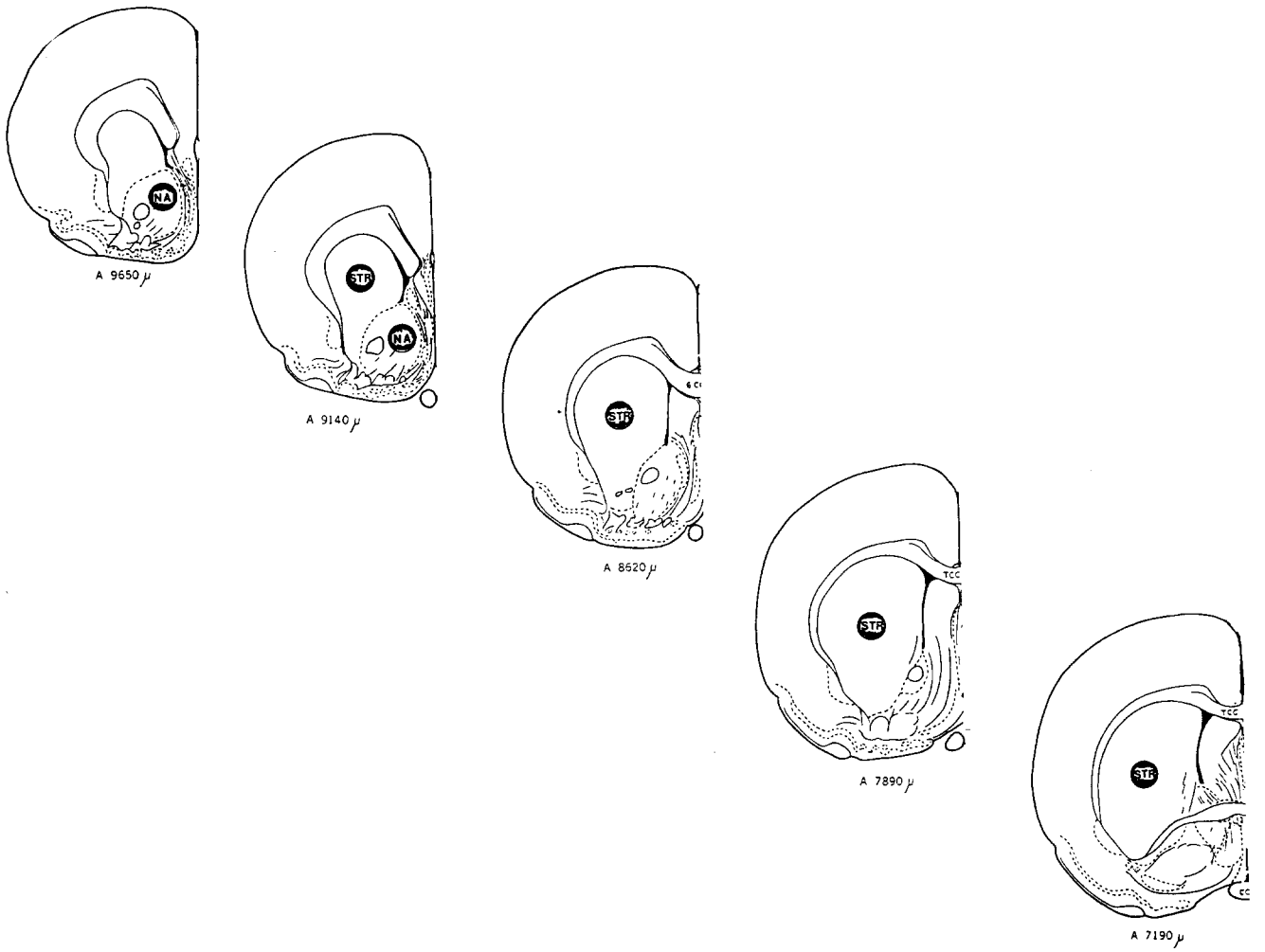

FIG. 1. Localization of micropunches in rat brain slices (520 μ thick). Regional punches were made in the 9650, 9140, 8620, 7890 and 7190 μ planes according to the atlas of König and Klippel [6]. STR, striatum; NA, nucleus accumbens.

dampener (Rainin Instr. Co., Inc., Woburn, MA) followed in-line by an ESA Model 5020 guard cell (ESA Inc., Bedford, MA) were installed between the pump and a Rheodyne 7125 injection valve containing a 20 μl sample loop (Rainin Instr. Co., Inc.). A SSI 0.5 μm high-pressure filter (Alltech Assoc. Inc, Deerfield, IL) preceded the pulse dampener and a 0.2 μm high-pressure carbon filter element (ESA Inc.) preceded the guard cell. A 0.5 μm Rheodyne column inlet filter (Model 7335) preceded the 3 μm C18 HR-80 reversed-phase analytical column (8 cm × 4.6 mm i.d.; ESA Inc.) which was connected to a model 5011 high sensitivity analytical cell (ESA Inc.). A ESA 0.2 μm carbon filter element preceded the analytical cell.

Type 316 stainless steel tubing (0.01″ i.d.) was used to make connections throughout the system except between the inlet filter and pump, in which case, teflon tubing (0.10″ i.d.; Alltech Assoc. Inc.) was used. In addition, teflon tubing was also used to connect the outflow from the analytical cell to the mobile phase reservoir for recycling.

A model 5100A Coulometric electrochemical detector (ESA Inc.) containing a model 5011 dual electrode (coulometric-amperometric) high sensitivity analytical cell was used for sample analysis. The coulometric-amperometric cell arrangement allowed for the oxidation and reduction, in sequence, of the biogenic amines of interest. Based on the generation of hydrodynamic current-voltage curves (i.e., voltammograms) for each compound, the optimal working electrode potentials for our coulometric-amperometric analytical cell were +0.25V and −0.36V, respectively.

The mobile phase was prepared by dissolving citric acid (6.7 g), sodium acetate (7.4 g), Na_2EDTA (0.25 g) and OSA (0.05 g) in 930 ml of double distilled/deionized water. Diethylamine (1.2 ml) was then added to this solution prior to its filtration under vacuum through a nylon-66 0.2 μm membrane filter (Rainin Instr. Co. Inc.). To this mixture, vacuum-filtered methanol (70 ml) was added and thoroughly mixed. The pH of this final solution was adjusted to 4.50 with phosphoric acid (85%) and degassed under vacuum. The mobile phase was pumped through the system at a rate of 1.5 ml/min, producing a background pressure of approximately 2300 psi. This mobile phase was recycled for 2 weeks of continuous use before being replaced with a fresh solution.

The entire chromatographic system was run at ambient temperature. Catecholamine stock solutions were made up in 0.1N $HClO_4$. Indole stock solutions were made up in double distilled/deionized H_2O. These solutions remained stable for several weeks if kept refrigerated. Working standards were made up daily in 0.05 N $HClO_4$.

Tissue Preparation

Male Sprague-Dawley rats (180–200 g) were used throughout these experiments. Brains were removed following decapitation and frozen immediately on dry ice. Coronal sections (520 μm thick) were made using a Cryo-Cut microtome (American Optical Corp., Buffalo, NY). The frozen slices were then placed on a glass slide and kept on a thermoelectric cold plate for removal of discrete brain regions (FIG. 1) using the micropunch technique [9]. This was accomplished through the use of a 0.84 mm (i.d.) stainless steel tube with stylus to aid in the extrusion of the punched tissue disk. The same area on each side of the brain (i.e., two punches) was placed in a 1.5 ml polypropylene microcentrifuge tube (Bio-Rad, Richmond, CA or Fisher Scientific Co.) containing 400 pg of DHBA (as an internal standard) in 150 μl of filtered (0.2 μm nylon filter) 0.05 N $HClO_4$. The samples were then sonicated for 5 sec in a micro-ultrasonic cell disrupter equipped with a 3 mm o.d. probe (Kontes Scientific Glassware/Instrs., Vineland, NJ) followed by centrifugation at 13,000 × g for 5 minutes in a cold room. The supernatants were collected using pipettes and placed in a MF-1 ultrafiltration assembly containing 0.2 μm cellulose (RC58) membrane filters (BAS Inc., Lafayette, IN) for filtration by low speed centrifugation for 3 minutes. Twenty microliters of the filtered samples were then injected directly onto the HPLC system. Protein pellets were assayed for content using the Bradford method (Bio-Rad Protein Assay Kit).

Data Analysis

Data were collected on a model D5000 strip chart recorder (Houston Instr., Austin, TX). Sample peaks were quantitatively analyzed by the peak height ratio method using DHBA as an internal standard to correct for sample loss. Biogenic amine tissue contents were expressed as ng/mg protein (Mean±S.E.M.). Statistical comparisons were made using an ANOVA or two-tailed *t*-test.

RESULTS AND DISCUSSION

Methodological Considerations

During the course of these experiments, there were several technical observations worth noting. With regard to chromatographic design, the use of 3 μm particle size columns appear to have major advantages (e.g., speed, sensitivity and resolution) over the more traditional 5 and 10 μm columns as reported by others [1,4]. However, it was also observed that these columns are much more sensitive to minute changes in mobile phase content. For example, changes in pH drastically altered retention times of the acid metabolites resulting in coeluting peaks, while changes in the methanol concentration (<0.5% v/v) resulted in peaks eluting in the solvent front. Secondly, the use of a guard column to prolong the life of the analytical column does not seem advisable. We found that it substantially increased retention times and induced severe tailing in peaks eluting as early as 10 minutes. An alternative approach would be to use a series of in-line filters as described above. The major advantages of filters over guard columns are low backpressure, economy, and minimal effects on analytical column efficiency and peak resolution. Finally, the use of a guard cell for precolumn oxidation (or reduction) of chemical impurities in the mobile phase in order to decrease baseline interference does not appear necessary if an adequate water filtration system is used.

The dual cell detection system used in the present system has some distinct advantages over a single cell detection system. The most obvious was the substantial amount of solvent front clean-up, particularly when used in the redox mode. This allowed for the use of faster flow rates without the loss of early eluting peaks in the solvent front.

With regard to sample preparation, we have found perchloric acid (0.05 N) to be a good protein-precipitating homogenizing solution since: (1) it does not produce a large solvent front in the redox mode and (2) it does not decrease column efficiency following 20 μl injections as does homogenizing solutions containing organic modifiers [10]. It was also found that sample filtration prior to injection is a simple and economical way to prevent increasing backpressure (due to clogging of column frits), prolong column life and decrease detector cell contamination. However, with our small sample volumes (150 μl), we did not find the MF-1 filtration system to be sufficiently reliable since we frequently observed 100% sample loss. An excellent alternative seems to be the 0.2 μm nylon disposable syringe filters manufactured by Alltech Associates Inc. These filters, although relatively more expensive than the MF-1 assembly, appear to provide greater (95–99%) and more consistent sample recovery for analysis, thus making them more cost effective. Finally, it was observed that perchloric acid leached unidentified contaminants from some Fisherbrand 1.5 ml polypropylene microcentrifuge tubes (Lot No. 123186A) which coeluted with the catecholamines. This problem did not occur when using Bio-Rad microcentrifuge tubes.

Chromatography

Figure 2 shows typical 5.8 minute chromatographic runs obtained with standards and brain tissue. This analysis time, along with minimal sample preparation, allowed for the analysis of approximately 60 samples per day. Increasing the flow rate to 2 ml/min decreased analysis time to 4.4 minutes; however, the increase in backpressure and pump demands necessary to maintain this flow rate does not appear to warrant its use for long-term routine analysis. A major factor in reducing the analysis time is the incorporation of diethylamine in the mobile phase which reduces the elution time of later peaks (5HT and 5HIAA) relative to earlier peaks (NE), resulting in an abbreviated chromatogram.

Standard curves (1–1000 pg) generated linear correlation coefficients between 0.9997–0.9999 for each compound. Detection limits, based on twice the baseline height, were approximately 2–5 pg for the catechols and 5–10 pg for the indoles. Initial studies showed the reversibility of NE and DA in the redox mode to be essentially 100% while that of DOPAC, 5-HT, and 5HIAA to be 35%, 10% and 5%, respectively.

The redox mode appears to provide an excellent approach for rapid and sensitive analysis of biogenic amines and their major metabolites. A disadvantage of this technique, at present, is that HVA can not be detected unless the oxidation potential of the first electrode was increased to +0.33V. Al-

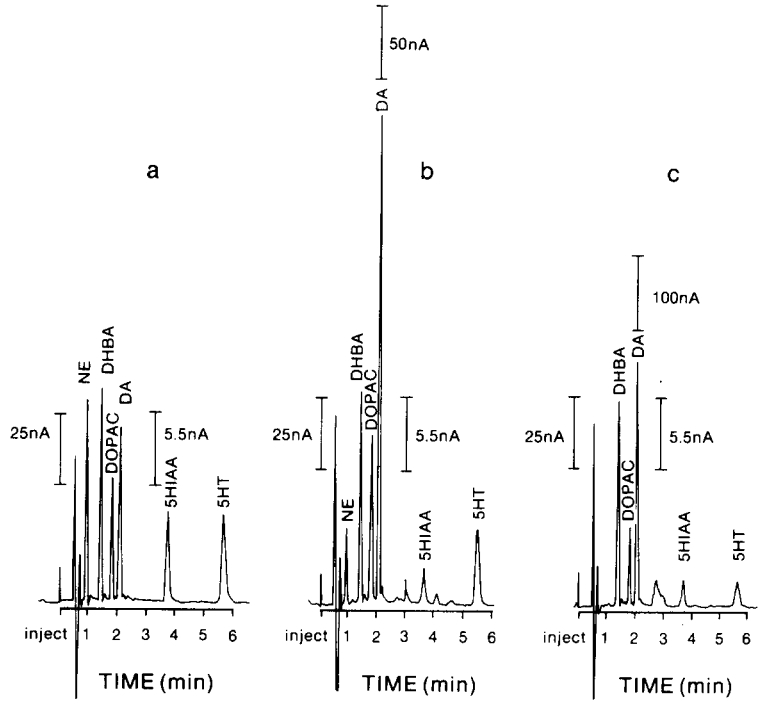

FIG. 2. Chromatograms obtained from: (a) an external mixture of standards containing 250 pg of each compound except DHBA (400 pg), (b) nucleus accumbens and (c) striatum. Each brain sample contained DHBA (400 pg) as an internal standard. Detector response time was set at 4 sec. Chart speed was 1 cm/min.

though this could be easily accomplished, an unknown compound coelutes with 5HT. Thus, HVA and 5HT cannot be detected simultaneously using this assay in the redox mode. This problem could possibly be solved by using the dual cell detection system in the screen mode. In this mode, a relatively small oxidation potential is applied to the first electrode to serve as a partial clean-up of the sample (and mobile phase) prior to its exposure to the second electrode for biogenic amine oxidation. Although we have found (unpublished observations) this method to detect all of the biogenic amines and metabolites mentioned above, including HVA, the solvent front is still sufficiently large enough to interfere with the detection of NE. One may avoid this problem by decreasing the organic modifier (e.g., methanol) content to increase the retention time of NE. The disadvantage of this is the substantially longer retention times (>15 min) for 5HT even on a 3 μm column. In fact, we see no advantages of this design over a much less expensive thin-layer single cell design (e.g., Bioanalytical Systems Inc.) in which we have eluted (unpublished observations) all of the previously mentioned compounds including HVA on a 3 μm column in less than a 12 minute run time with a detection limit of approximately 10 pg.

Since it is more appropriate to use an indoleamine as an internal standard for the determination of 5HT and 5HIAA, we attempted to incorporate 5-hydroxyindole in the samples similar to DHBA. Unfortunately, this resulted in peak coelution which could not be resolved unless the run time was significantly increased. Although not tested in the present system, N-methyl-5HT may be an alternative choice for an indole internal standard. This compound has been successfully used, along with DHBA, for catechol/indole analysis on a 3 μm column [9]. However, for many investigators, the relatively high cost of this compound may not justify its use for routine analysis. Regardless of the internal standard used, it is essential that the standards be treated the same as the samples to insure accurate quantitation.

The speed, sensitivity and resolution of 3 μm columns would no longer appear to warrant the use of 5 and 10 μm analytical columns for brain biogenic amine analysis. Although the 3 μm column does not demonstrate the same sensitivity range as microbore columns [3], the faster analysis time and lower cost of the 3 μm column would strongly advocate its use for routine neurochemical analysis.

Neurochemical Findings

Table 1 summarizes the regional distribution of biogenic amines and their major metabolites in the rat striatum and nucleus accumbens. In the striatum, there was as significant rostrocaudal gradient for DA, $F(3,12)=7.70$; 5-HT, $F(3,12)=11.02$; and 5-HIAA, $F(3,12)=3.82$. DA content was highest in the rostral striatum while 5-HT and 5-HIAA levels were highest in the caudal sections. There was no observable rostrocaudal gradient for DOPAC, $F(3,12)=2.57$. NE was not detected in sufficient quantities for accurate analysis. These results are similar to other studies using discrete micropunches in the same coronal planes [2,16]. In addition, we have found a significant rostrocaudal gradient for catecholamines within the nucleus accumbens. NE content was highest in the most caudal region while DA and DOPAC levels were highest in the rostral region. In contrast to the striatum, there was no rostrocaudal gradient for accumbal 5-HT or 5-HIAA. It was also observed that within the same coronal section (9140 μ), DA levels were significantly higher in the striatum while 5-HT and 5-HIAA levels were significantly higher within the nucleus accumbens. It is interesting to note

TABLE 1

REGIONAL DISTRIBUTION OF BIOGENIC AMINES AND MAJOR METABOLITES IN THE STRIATUM AND NUCLEUS ACCUMBENS

Plane (μ)	NE	DA	DOPAC	5-HT	5-HIAA
			Striatum		
9140	n.d.	187.2 ± 9.1*†	33.2 ± 2.7†	1.3 ± 0.1*†	1.8 ± 0.2*†
8620	n.d.	160.5 ± 11.7	34.0 ± 2.3	1.7 ± 0.1	2.1 ± 0.1
7890	n.d.	130.3 ± 11.7	43.3 ± 4.0	1.7 ± 0.1	2.2 ± 0.1
7190	n.d.	121.4 ± 10.6	31.7 ± 2.1	2.3 ± 0.2	2.5 ± 0.2
			Nucleus Accumbens		
9650	5.8 ± 0.5*	124.6 ± 2.9*	49.6 ± 5.0*	3.4 ± 0.2	2.5 ± 0.3
9140	16.4 ± 1.3	95.3 ± 8.9	26.3 ± 2.6	3.6 ± 0.2	2.7 ± 0.2

n.d. = not detectable.

Values represent the mean ± S.E.M., expressed in ng/mg protein from 4 rats. Significant difference across planes: (*$p<0.05$, ANOVA for striatum and t-test for nucleus accumbens). Significant difference vs. accumbal content at 9140 μ plane (†$p<0.05$, t-test).

that striatal and accumbal DA and DOPAC levels are substantially lower and 5HT and 5HIAA levels substantially higher when whole brain regions are analyzed [6,8] as opposed to discrete areas within the nuclei. Thus, these data confirm and extend the existence of biogenic amine heterogeneity within the striatum and nucleus accumbens.

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