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Note

Determination of rat brain tissue catecholamines using liquid chromatography with electrochemical detection

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The determination of catecholamine levels in the brain as well as in other biological tissues has been the focus of many investigators for a number of years. Liquid chromatography coupled with electrochemical detection (LC—EC) potentially has several advantages over fluorometric or radioenzymatic assays. LC—EC not only is more rapid and specific, but has the capacity to examine the metabolites as well as the parent catecholamines. Although previously published LC—EC procedures which use Waters μBondapak C_{18} columns [1—3] show that norepinephrine (NE) is separated from the solvent front, this separation is not consistent from column to column and deteriorates rapidly with column usage. Without an integrator, quantitation of NE is inconsistent because it is often not adequately resolved from the solvent front. When a peak is not adequately resolved, drawing a baseline is subjective and therefore not consistent from sample to sample. An electronic integrator determines a consistent baseline, even for unresolved peaks and therefore consistent values are obtained. This paper describes an improved LC—EC assay using a column with a straight-chain octadecyl (C_{18}) hydrocarbon bonded chemically to silica specifically designed to use with ion-pair agents.

MATERIALS AND METHODS

All the catecholamines and the metabolites were purchased from Sigma (St. Louis, MO, U.S.A.). Regis (Morton Grove, IL, U.S.A.) was the source of our sodium octyl sulfonate. Acid washed alumina was purchased from Bioanalytical

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Systems (West Lafayette, IN, U.S.A.) or alumina obtained from Calbiochem (Los Angeles, CA, U.S.A.) was washed by the procedure of Anton and Sayre [4]. Methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

The chromatography was performed with a Model 110A pump (Altex), Model 210 valve with a 20-μl loop (Altex), and a reversed-phase Altex Ultrasphere Ion Pair C_{18} column (25 × 0.46 cm I.D.). The column eluent was monitored with an electrochemical detector Model LC-4 and a glassy carbon electrode from Bioanalytical Systems. The detector potential was +0.72 V vs. an Ag/AgCl reference electrode. The flow-rate was maintained at 1.5 ml/min. The mobile phase was 1 volume of methanol and 9 volumes of 0.1 *M* potassium phosphate (pH 3.0), 0.2 m*M* sodium octyl sulfonate, and 0.1 m*M* EDTA. Water was deionized and the buffer was filtered through Millipore HAWP 0.45-μm filters. Millipore EHWP 0.5-μm filters were used to filter the methanol. The column was used at room temperature, but the buffer was maintained at 40—50°C to prevent bubble formation in the detector cell.

Male Sprague-Dawley rats (Holtzman) were killed by decapitation. Brains were rapidly removed, dissected on ice, frozen on dry ice, and stored at −80°C until assayed. The dissection was completed as follows: the cerebellum was peeled off the brain stem before the brain was removed from the skull. A cut was made through the corpus callosum and the cortex was peeled back, exposing the hippocampus and caudate which were carefully removed. Remaining white matter was removed from the cortex. To obtain the hypothalamus and thalamus, cuts were made anterior and posterior to the median eminence followed by sagital cuts on the two sides of the median eminence and a horizontal cut through the mamillothalamic tract. The dorsal tissue piece was used for thalamus and the ventral portion as the hypothalamus. Tissue was kept frozen at −80°C until assayed. No differences in NE or dopamine (DA) levels are observed upon storage for up to one month. However, tissues that were stored frozen for nine months at −80°C had catecholamine levels 30 to 40% of those assayed within one month of sacrifice.

Frozen tissues were weighed and homogenized on ice in 3 ml of 0.05 *M* perchloric acid containing varying amounts of dihydroxybenzylamine (DBA) with a Polytron distributed by Brinkmann. Whole brains were homogenized in 10 ml of 0.05 *M* perchloric acid containing 100 ng DBA per ml for NE and DA determinations. For determining NE and DA in cerebral cortex only one side was homogenized with 90 ng DBA. One of the caudate nuclei was homogenized with 450 ng of DBA to determine DA and the other with 45 ng of DBA to determine NE. Both hippocampi were homogenized with 30 ng DBA. The hypothalamus was homogenized with 60 ng DBA, the thalamus with 45 ng DBA, the cerebellum with 90 ng DBA, and the midbrain with 90 ng DBA. From each homogenate two 1-ml aliquots were placed in 12 × 75 mm polypropylene tubes (Sarstedt 526). One of the two aliquots was supplemented with 1 μg/ml NE and/or DA in 0.05 *M* perchloric acid to be used as working standards. The following provided reasonable supplements: whole brain, 50 ng NE and 25 ng DA; cortex, 10 ng NE and 10 ng DA; caudate for DA, 50 ng DA; caudate for NE, 5 ng NE; cerebellum, 10 ng NE; hippocampus, 5 ng NE and 5 ng DA; hypothalamus, 10 ng NE and 5 ng DA; thalamus 5 ng NE and 2.5 ng DA; and midbrain, 10 ng NE and 5 ng DA.

All the samples were centrifuged at 9000 *g* for 15 min. Supernatants from the samples were transferred to 12 × 75 mm conical polystyrene tubes (Sarstedt 477) containing 10 mg acid washed alumina and 1 ml of 0.5 *M* Tris—HCl, pH 8.6. The tubes were capped and shaken for 15—30 min at top speed on a New Brunswick Scientific Shaker. After allowing the alumina to settle the supernatants were aspirated. The alumina was washed three times with 1 ml 5 m*M* Tris—HCl, pH 8.6. Elution of the catecholamines was with 100 μl of 0.2 *M* acetic acid. The 100-μl aliquots of 0.2 *M* acetic acid containing the eluted catecholamines were filtered with a Flath-Lundin filter syringe purchased from Hamilton (Reno, NV, U.S.A.). Millipore HAWP 0.45-μm filters were used. A 20-μl aliquot of the filtered samples was injected. Catecholamine levels were calculated as described by Felice et al. [1].

RESULTS AND DISCUSSION

Fig. 1A shows an isocratic separation of pure standards of NE and DA as well as a number of their major metabolites using the methanol—phosphate mobile phase described in detail in the Methods section. From this chromatogram it is clear that NE is markedly separated from the solvent front. Further-

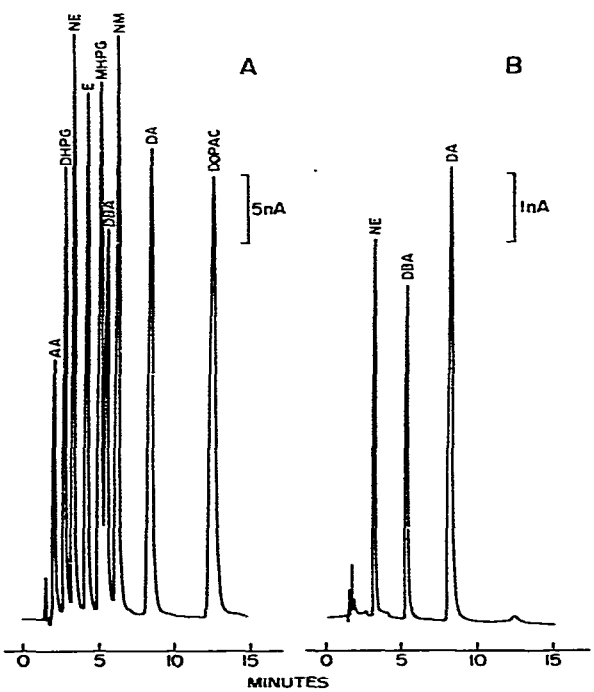

Fig. 1. Chromatograms of standards (A) and of catecholamines from adult rat whole brain with DBA added as an internal standard (B). Details for the chromatographic conditions and the sample preparations are in the Methods section. For (A) abbreviations and amounts injected in nanograms: AA = ascorbic acid, 15; DHPG = dihydroxyphenylglycol, 8; NE = norepinephrine, 15; E = epinephrine, 30; MHPG = hydroxymethoxyphenylglycol, 25; DBA = dihydroxybenzylamine, 10; NM = normetanephrine, 400; DA = dopamine, 15; and DOPAC = dihydroxyphenylacetic acid, 25.

more, NE is not only resolved from ascorbic acid but also from dihydroxyphenylglycol (DHPG), a major NE metabolite, which is not the case with another widely used LC column [1]. Under these conditions DA elutes from the column within 10 min. That is a remarkably rapid elution with baseline separations for NE, DBA and DA.

The mobile phase we used with the Altex Ultrasphere C_{18} Ion Pair column contained a relatively large percentage of methanol and a relatively low concentration of ion-pairing agent. Therefore, there are a number of things one can do to the mobile phase to further separate these and other catecholamines and their metabolites. Decreasing the methanol concentration and/or increasing the ion-pair concentration will increase retention times. With use, LC columns generally lose some of their resolving power(s); therefore the ability to dramatically increase the polarity of the solvent should lengthen the expected useful lifetime of the column. On the other hand, even when we used the more polar mobile phases containing no methanol, as described by Felice et al. [1] and Asmus and Freed [5], NE did not completely separate from the solvent front on a Waters μBondapak C_{18} column. The addition of more ion-pairing agent markedly increased the retention time of DA but had little or no effect on the retention time for NE. EDTA is an essential component of the mobile phase, since it dramatically decreases the spread of the solvent front. Without EDTA; ascorbic acid, DHPG and NE are not resolved from the solvent front. A possible explanation for this effect is that the samples are in acid which may cause metal ions to be injected from the syringe, the injector, or the pumping system which are oxidized and consequently observed with the electrochemical detector.

Fig. 1B is a chromatogram of an alumina extraction from a male Sprague-Dawley rat whole brain that included DBA as an internal standard. The amount of NE, 345±10 ng/g wet weight and DA, 818±21 ng/g wet weight in whole brain that we calculated using this LC—EC method is comparable to that obtained by other investigators [1, 6—8]. Calculations were performed using DBA as an internal standard as described in detail by Felice et al. [1]. The amounts of NE and DA found in the various regions of male Sprague-Dawley rat brains are presented in Table I. These values compare favorably with those reported by other investigators [1, 6, 7].

TABLE I

NOREPINEPHRINE AND DOPAMINE CONTENT OF SEVERAL RAT BRAIN REGIONS

Each region was assayed in duplicate from 3 to 14 animals. Results are expressed as a mean ± S.E.M.

Region	Norepinephrine (ng/g of tissue)	Dopamine (ng/g of tissue)
Whole brain	345 ± 10	818 ± 21
Cerebral cortex	205 ± 10	51 ± 4
Cerebellum	124 ± 15	13 ± 1
Caudate nucleus	277 ± 37	9253 ± 213
Hippocampus	201 ± 21	11 ± 0.5
Hypothalamus	1446 ± 87	298 ± 33
Thalamus	554 ± 41	191 ± 31
Midbrain	392 ± 7	129 ± 17

This method gave linear responses both with the detector and with the tissue sample in the tested range of 1—100 ng NE, DA, and DBA. The extraction method has not been optimized for maximum recovery. Using 0.2 *M* acetic acid to extract catecholamines from alumina gave recoveries of approximately 40%. Acetic acid was chosen because it probably is least detrimental to the column packing material and the sample injection valve. In addition, it is volatile which means concentrating samples does not increase the acid concentration.

Using this method we could assay five samples per hour. The system stabilizes very quickly (15—20 min) if one leaves the power on overnight for the electrochemical detector and keeps the column equilibrated with the methanol—phosphate mobile phase by pumping at 0.1 ml/min. We found it takes 3—4 h to obtain a stable baseline if the power is turned off overnight. A similar amount of time is needed to re-equilibrate the column if one stores the column in 70 to 100% methanol.

With a buffered mobile phase the column must have a continual flow-rate to avoid salts crystallizing on the column. We have found our baseline to be stable enough that we anticipate one could successfully analyze catecholamines with a fully automated system, i.e. an autosampler and an integrator.

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