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INVESTIGATIONS OF THE PERFORMANCE OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM WITH AN ELECTROCHEMICAL DETECTOR

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SUMMARY

A procedure for the determination of norepinephrine and dopamine, based on high-performance liquid chromatography, is evaluated using an electrochemical detector system. The use of an inorganic mobile phase to provide resolution of low retention amines and extend column life is discussed. A high degree of correlation between estimations of endogenous catecholamine levels is reported using both electrochemical and fluorometric detector systems. Inter-assay reproducibility of the extraction method, and sensitivity and linearity of response of the electrochemical detector system are shown to be consistent across trials. The system described is determined to be accurate, sensitive, and reliable over time.

INTRODUCTION

The isolation and quantitation of catecholamines (CAs) in brain tissue are of major importance in neuroscience. Various methods have been developed for their detection, however some lack the necessary sensitivity, and others are too complicated for routine analysis. Several investigators have described procedures for CA determination based on high-performance liquid chromatography (HPLC) with either ultraviolet radiation detection [1], fluorescence detection after precolumn *o*-phthalaldehyde derivatization [2], or electrochemical detection (ED) [3—15]. Scratchley et al. [3] reported adequate separation and detection of 19 biologically active compounds using HPLC with various chromatographic conditions, including an ED system. A major difficulty mentioned by Scratchley et al. [3] was a shortened column life when using ion-pairing agents, e.g. sodium octyl sulfate (SOS). This problem is addressed later in the results section.

This report describes the performance of a HPLC system with an electrochemical detector. Determinations of sensitivity and linearity of response are

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made and compared across days to demonstrate the reliability of the detector system. A series of studies also examine (1) the use of various acids during the extraction procedure for eluting CAs from alumina, (2) the advantage of using an inorganic rather than organic mobile phase to improve resolution of low retention amines and extend column life, and (3) the correlation between electrochemical and fluorometric CA estimations of the same brain extract.

EXPERIMENTAL

Materials

A 15 cm × 3 mm I.D. HPLC stainless-steel column, packed with LiChrosorb RP-18, 10 μm (E. Merck, Elmsford, NY, U.S.A.) was equipped with an ED system consisting of a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model TL-7A detector cube and a Model LC-2A potentiastat with the electrochemical potential set at +0.72 V vs. Ag—AgCl reference electrode. The Model TL-7A uses a glassy carbon detector electrode, which improves the stability of the detector surface, and a facing auxilliary electrode, which extends the effective linear range of the detector system by two orders of magnitude. The mobile phase was 0.1 *N* nitric acid (pH 4.0 by sodium hydroxide), microfiltered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The ion pairing agent, SOS, was added (50 mg/l) and the solution was stirred, heated (40°C) and pumped by a Milton-Roy (Laboratory Data Control, Riviera Beach, FL, U.S.A.) mini-pump, Model 396-31 (1 ml/min), through a pressure gauge, pulse dampener, and a Rheodyne (Laboratory Data Control) loop injector, Model 7010. This instrument was assembled by the investigators and has been described previously by others [3, 4, 9]. The mobile phase consisted of triple-distilled water and analytical grade fuming nitric acid (Fisher Scientific, Cincinnati, OH, U.S.A.).

Sample preparation

The CA extraction method was from Felice et al. [16]. Rats were sacrificed by decapitation; the brains were excised and frozen immediately on dry ice. After sectioning and weighing, 100—500-mg samples were sonicated in 15-ml Pyrex centrifuge tubes which contained 1—2 ml of cold 0.05 *N* perchloric acid. Prior to sonication, some samples were spiked with the internal standard 3,4-dihydroxybenzylamine (DHBA). Certain samples were also equally split and spiked with norepinephrine (N) and dopamine (D) (50 ng/100 μl). The standards were prepared by serial dilution of fresh standards (Sigma, St. Louis, MO, U.S.A.) dissolved in 0.1 *N* hydrochloric acid at a concentration of 1 mg/ml.

The homogenate was centrifuged at 15,000 *g* for 15 min; the supernatant was then poured into a 2-ml conical vial containing 90 mg of alumina and 1.0 ml of 3.0 *M* Tris buffer (pH 8.6). After addition of the supernatant, the vials were vigorously shaken for 10 min. The alumina was allowed to settle and the supernatant removed by aspiration. The alumina was then washed once with Tris buffer (6 m*M*, pH 8.6) and twice with triple-distilled water. The last rinse was gently aspirated from the alumina. A 1-ml volume of acid (usually 0.05 *N* $HClO_4$) was added before shaking again for 10 min. After the alumina settled, 100 μl of the eluent were applied to the column via the loop injector system.

Investigations of the detector's response characteristics

One factor determining the magnitude of the response of the detector system to the CAs is the oxidation potential applied to the TL-7A detector cube by the potentiastat. The relationship between applied potential and the output current due to the oxidation of the CAs at the detector electrode surface was investigated. The output current, measure in nanoamperes, was determined following the injection of a constant volume (100 μl) and amount (100 ng) of N. The input oxidizing potential was varied from +400 to +800 mV vs. Ag—AgCl reference electrode, and the output was monitored with a dual-beam storage oscilloscope (Tektronix, Model 564). Applied potentials greater than +800 mV produce background currents too great to offset electronically; therefore potentials in this range were not tested.

RESULTS

The response characteristics of the detector system described of applied potentials vs. nanoamperes of output current generated for the detection of N are shown in Fig. 1. The linearity of response and the consistency of the detector performance is shown in Fig. 2. Each data point in Fig. 2 represents ten determinations of serial dilutions of a standard on ten different occasions. The on-column detection limit was 500 pg per 100 μl injected volume with a signal-to-noise ratio of 2:1. Injected amounts greater than 1 μg saturate the detector and are not subject to quantitation.

Twenty-one estimations of percent recovery of CAs from spiked brain homogenate were made using the procedure out-lined above. Percent recovery was calculated according to the method by Felice et al. [16]. The values for

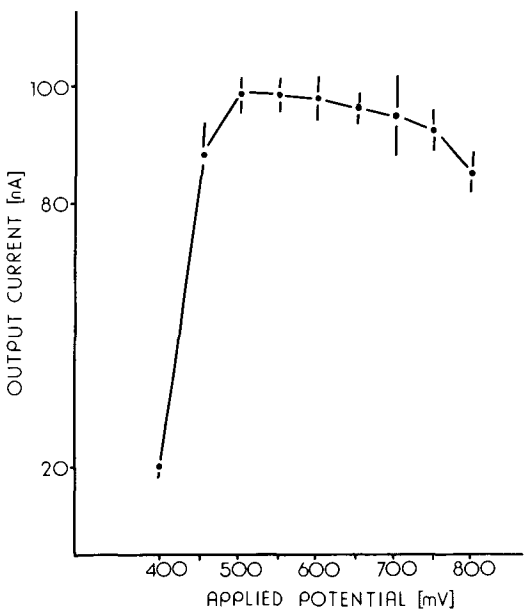

Fig. 1. Applied potential vs. output current. Each data point represents the $\bar{X}$ ± S.D. for five observations.

N, DHBA, and D were 84.3 ± 2.7, 65.4 ± 4.0 and 57.4 ± 5.3, respectively. These percent recovery values compare favorably with other reported values using more complicated extraction procedures [17, 18], and suggest a high degree of inter-assay consistency.

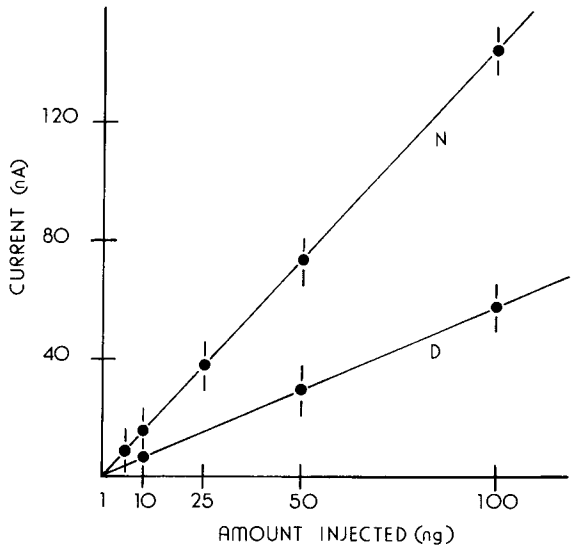

Fig. 2. Linearity of response to N and D. Each data point represents the $\bar{X}$ ± S.D. for ten observations. The various concentrations tested were made by serial dilution of a known standard. The figure also demonstrates a good inter-assay reproducibility.

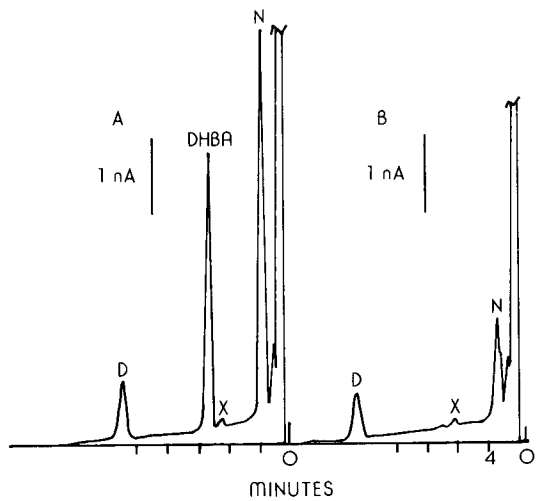

Fig. 3. Chromatograms of CAs from whole rat brains. (A) Spiked with 50 ng DHBA and 50 ng N: (B) endogenous levels. Chromatographic conditions: stationary phase, 30 cm × 3.9 mm I.D. LiChrosorb RP-18 (Merck); mobile phase, nitric acid—0.3 m*M* sodium octyl sulfate; flow-rate, 1 ml/min; temperature, ambient; electrode potential, +0.72 V vs. Ag—AgCl reference electrode; the CAs were eluted off the alumina in 0.05 *N* $HClO_4$; peak "x" is unknown.

Recovery from tissue: the effect of eluting acid

Felice et al. [16] reported that using perchloric acid to elute the CAs from alumina provides adequate recovery while not producing a large void volume response. However, the investigators were using a citrate—phosphate mobile phase. In this study the mobile phase was nitric acid. When phosphoric acid is used to elute the CAs off alumina, a smaller void volume response is produced than with perchloric acid (Figs. 3 and 4). The reduced response provides better resolution of low retention amines, such as N. Table I indicates that recovery is actually improved by using phosphoric acid.

At low levels N is usually overlapped by the void volume response. Generally, changing chromatographic conditions to increase N retention also greatly increases both D retention and analysis time. The system described thus provides increased sensitivity of N without the expense of time.

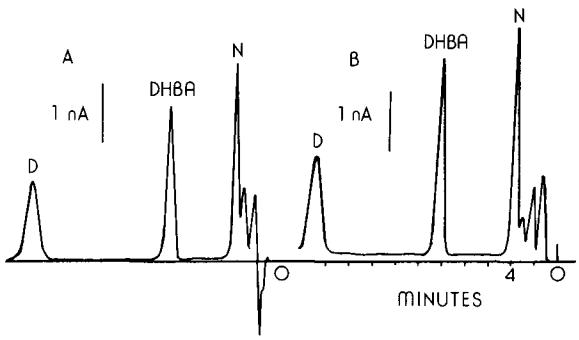

Fig. 4. Chromatograms of CAs from whole brain using perchlorid acid (A) and phosphoric acid (B) as the eluting vehicle. The chart speed was increased to demonstrate the components of the void volume response. Conditions as in Fig. 3. The homogenates were spiked with 50 ng of DHBA.

TABLE I

PERCENT RECOVERY FROM TISSUE: THE EFFECTS OF VARIOUS ELUTING ACIDS

Acid	Normality	Recovery (%)*		
		N	D	D**
Perchloric	0.05	84	75	75
Hydrochloric	0.01	9	5	—
Hydrochloric	0.10	0	55	—
Sulfuric	0.05	98	86	88
Phosphoric	0.05	98	92	97
Acetic	0.50	96	77	92

* % Recovery = $\frac{\text{ng (spike + endogenous)} - \text{ng (endogenous)}}{\text{ng (spiked amount)}}$ using 75—100 mg of tissue.

**Dopamine recovery values form Felice et al. [16]. Norepinephrine values were not reported.

Electrochemistry vs. fluorimetry: a comparison

Fifteen whole rat brains were homogenized and the CAs extracted and eluted in 0.05 *N* hydrochloric acid according to the procedures of Anton and Sayre [17, 18]. The amounts of N and D in the same extract were determined by HPLC—ED and by fluorimetry (Table II).

A paired *t*-test of dependent samples showed that the electrochemical and fluorimetric data do not differ significantly. A linear regression analysis was performed on the data and a correlation coefficient determined ($r = 0.81$).

TABLE II

ELECTROCHEMISTRY AND FLUORIMETRY: A COMPARISON

$\bar{X}$ ± S.D. of ng amounts of CAs/g wet weight tissue. All reported levels were for endogenous CAs.

Animal No.	Concentrations of CAs (ng/g tissue*)			
	Norepinephrine		Dopamine	
	Electrochemical	Fluorimetry	Electrochemical	Fluorimetry
1	403	418	964	878
2	447	395	1094	934
3	452	398	1243	1120
4	435	418	1245	1123
5	426	405	1613	—**
6	394	420	854	879
7	397	405	850	885
8	367	383	916	1012
9	419	452	955	977
10	451	425	867	929
11	408	408	925	880
12	410	410	930	884
13	431	436	960	943
14	447	459	1033	976
15	380	372	1029	915
$\bar{X}$ ± S.D.	417 ± 26	413 ± 23	1031 ± 202	925 ± 83
		$r = 0.81$		

*Wet weight.
**Lost sample.

DISCUSSION

In the present work, the HPLC—ED system is shown to be simple, sensitive, accurate, and reproducible; therefore suitable for the determination of small changes in CA concentrations in localized areas of the rat brain.

The HPLC system with an ED has been utilized by several investigators to detect CAs in various biological matrices. With appropriate modifications of the extraction procedure, and under varying chromatographic conditions, picogram levels of CAs have been analyzed in brain tissue [7], urine [5], and in small cell chambers containing brain slices following pharmacological manipulations [4]. The versatility of the method is due in part to its simplicity and primarily to its remarkable sensitivity.

The simplicity of the procedure encourages multiple sample analysis. Overnight storage may therefore become necessary if automatic sampling systems are not available. Storage of the eluant on alumina overnight at 4°C greatly reduces recovery. However, overnight storage of the homogenate at −20°C had no adverse effects on overall recovery.

Nitric acid is reported to improve column performance and extend column life. The LiChrosorb RP-18 (10-μm bead) used in this study performed adequately for 10 weeks and over 250 injections. The column was stored in the buffer and only rinsed with methanol—water (50:50, v/v) on weekends.

The method described is shown to be reliable, and to perform consistently with a minimum of maintenance. The detector is accurate when compared to existing methods, and is a versatile tool when used with the highly efficient microparticulate columns.

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