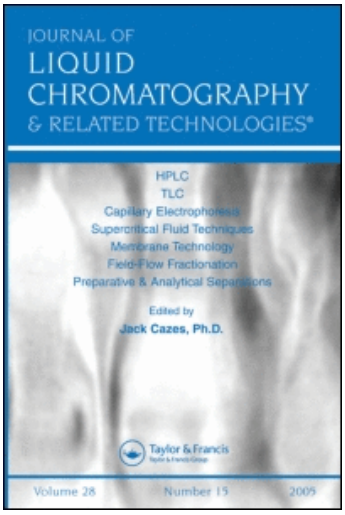

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SIMULTANEOUS HPLC ANALYSIS OF CATECHOLAMINES AND INDOLEAMINES IN MOUSE BRAIN TISSUE FOLLOWING ACETATE EXTRACTION AND TREATMENT WITH ASCORBATE OXIDASE

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ABSTRACT

A refined HPLC Method for the determination of monoamine levels in six brain regions is presented. Analyses are made for the olfactory tubercles, prefrontal cortex, septum, striatum, amygdala and hypothalamus of adult male ICR mice. This system permits the simultaneous analysis of norepinephrine, dopamine, serotonin and their major metabolites during a single run of approximately twenty-five minutes without prior clean-up of samples.

INTRODUCTION

Our laboratory has recently developed an HPLC technique that resolves more than thirteen catecholamines and indoleamines during a single run of less than twenty minutes (1). It allows for the simultaneous determination of most of the major monoamines of biological interest. As a result, the collection of a large amount of data is possible in a short period of time under identical experimental conditions. Specifically, norepinephrine (NE), epinephrine (EPI), dopamine (DA) and serotonin (5HT) are analyzed along with their precursors and metabolites. This includes the resolution of MHPG, the major metabolite of NE. In many systems, this neutral compound elutes so early that it is either totally or partially obscured by the solvent front.

Recently, we have applied this HPLC system to the analysis of brain tissue after extraction in perchloric acid (PCA) (2). We were able to detect nine monoamines, including both catechols and indoles, without prior clean-up of samples or separate extraction procedures. The compounds were NE and its metabolites, MHPG and normetanephrine (NM), DA and its metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3MT) and 5HT and its metabolite 5-hydroxyindole acetic acid (5HIAA). Results were reported for thalamic tissue obtained from the brains of adult male DBA/2 mice.

In the present study, we are reporting refinements of this technique and we have extended it to analyze monoamines in additional brain regions. The most important change is the adoption of an acetic acid/ascorbate oxidase extraction procedure (3,4). This materially decreases the front and allows for better preservation of the compounds.

MATERIALS AND METHODS

Instrumentation: The HPLC system was a Waters (Milford, Mass.) 590 programmable solvent delivery pump coupled to a refrigerated

Waters Intelligent Sample Processor (WISP) and Supelco (Bellefonte, PA) Supelcosil ODS 3 μ, C-18, 150 x 4.6 mm column. A BAS Biophase ODS 5 μ, C-18, 30 x 4.6 mm guard column and a BAS LC4B amperometric detector with glassy carbon electrode were used with this system. A Shimadzu C-R3A data processor equipped with a floppy disk drive and cathode ray tube (Shimadzu Scientific Instruments, Inc., Columbia, MD) recorded and integrated the signals received.

Chemicals: The following chemicals were purchased from Sigma (St. Louis, MO): 3-methoxy-4-hydroxyphenylglycol (hemipiperazine salt) (MHPG), arterenol bitartrate (norepinephrine bitartrate) crystalline (NE), epinephrine bitartrate (EPI), L-β-3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), DL-normetanephrine hydrochloride (NM), dopamine (3-4-dihydroxyphenylethylamine HCL) (DA), DL-metanephrine hydrochloride (MN), 5-hydroxyindole-3-acetic acid (free acid) (5HIAA), 3-methoxytyramine (3-methoxy-4-hydroxyphenethylamine) HCl (3MT), 5-hydroxytryptamine hydrochloride (serotonin) (5HT), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) (HVA), L-(-)-isoproterenol (IP), L-cysteine free base and L-ascorbic acid. We purchased 1-heptanesulfonic acid (HSA) from Fisher Scientific Co., Fairlawn, N.H., acetonitrile (CH_3CN) and sodium acetate from J.T. Baker Chemical Co., Philipsburg, New Jersey and ascorbate oxidase from Boehringer (Mannheim, W. Germany).

Chromatographic Conditions: The mobile phase was a mixture of 0.10 M citric acid, 0.06% diethylamine, 0.05mM Na_2EDTA, 200 mg/L HSA and 4.5% CH_3CN at pH 2.5. These components were dissolved in deionized water (<1 megohm resistance), then filtered through a 90 mm, 0.2 μ Whatman filter (Maidstone, England). The solvent was sparged with helium gas to deaerate it. All separations were performed isocratically at a flow rate of 1.3 ml/min at room temperature, a detector setting of 0.85 V and a sensitivity setting of 2 na.

Standards: Fresh standard compounds were ordered in brown bottles and were kept in a dessicator in the freezing compartment. Stock solutions were prepared in 0.5% PCA at a concentration of 1 mg/ml then diluted serially to give the working solution of 20 ng/ml. 50 µl aliquots were injected into the HPLC system from the refrigerated sample compartment.

Tissue Preparation: Brain tissue was obtained from 5-7 week old ICR male mice. They were killed by cervical dislocation and the brains were rapidly removed and frozen in liquid nitrogen. Dissections were performed over liquid nitrogen on a frosted plate. Olfactory tubercles (OT), prefrontal cortex (PC), septum (SP), striatum (ST), amygdala (AMY), and hypothalamus (HT) were obtained from appropriate coronal sections. The tissues were then weighed, extracted in 200 µl of a sodium acetate buffer pH 5.0 (3.0 g sodium acetate and 4.3 ml glacial acetic acid per liter) containing the internal standard (20 ng/ml IP), and homogenized (Tekmar Tissumizer, Cinninnati, OH). Filtration of the homogenate through Isolab (Akron, OH) QS-GS filter columns was performed in a refrigerated superspeed centrifuge at 2,300 G and -4°C for 5 minutes. The supernatant was transferred to new vials containing 5 µl of a 0.1% ascorbate oxidase solution (Frankfurt et al., 1985). 50 µl of the tissue samples were then injected into the HPLC system. The brains from eight animals were analyzed.

RESULTS AND DISCUSSION

The resolution of monoamines in brain tissue by this refined chromatographic method is shown in Fig. 1 for a representative region, the HT. Nine monoamines, including the internal standard, IP, were identified. The peaks seen just before NE represent unidentified components of the tissue front. This method features MHPG eluting after NE and well separated from the front. Many HPLC studies have omitted analysis of this neutral noradrenergic metabolite since it tends to interfere with the solvent front

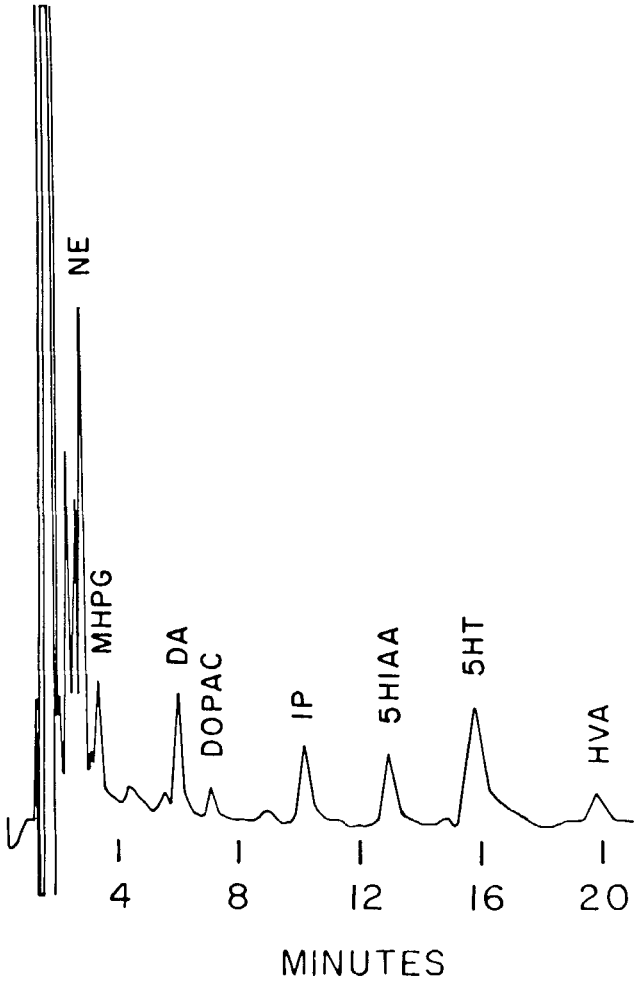

Figure 1

(4-8). The present mobile phase and extraction procedures were designed to overcome this deficiency, to identify both catechols and indoles and to resolve a large number of them during a single run. The front can be reduced still further by using a higher concentration of ascorbate oxidase than was employed in the present study. Quality control was carried out by mass spectrometry and by other HPLC methods to determine purity of those peaks which may conflict with other compounds. The contribution of each solute and the rationale for its choice have been discussed previously (1).

In table 1, the values for the compounds measured are given in picograms/mg original tissue ± S.E. for six brain regions. Note the recovery in the low picogram range for several compounds depending upon the tissue. In most instances, these values are similar to these reported by others (4-8). NM and EPI were also detected in the PC (41 ± 6 ng/g and 5.5 ± 1.3 ng/g) respectively.

Dopaminergic compounds were richest in the ST and OT. NE was highest in the HT and least in the OT and ST. The serotonergic compounds were highest in the HT. The low levels of 5HT in the ST is due to sampling in the anterior portion of this nucleus.

TABLE 1

HPLC VALUES (PG/MG±S.E.) FOR MONOAMINES IN SIX MOUSE BRAIN REGIONS (N=8)

	OT	PC	SP	ST	AMY	HT
NE	34±4.4	85±9.4	143±8.3	13±2.8	116±8.1	384±22
MHPG	32±1.2	22±2.6	43±3.3	48±6.3	33±2.1	19±2.3
DA	1776±106	20±2.8	130±11	3118±406	129±25	84±4
DOPAC	257±21	9±2	30±3.6	235±28	25±4.1	28±2.2
HVA	88±7.3	18±4	N.D.	362±22	45±9.9	23±7
3MT	145±14.6	N.D.	N.D.	241±33	N.D.	N.D.
5HT	231±7.1	165±15	221±26	131±14	238±14	431±17
5HIAA	50±2.7	46±4.1	110±7.1	72±12	82±5.8	142±11

See Methods for abbreviations; ND = not detected.

In the present method, we are using acetate buffer as an extraction medium for brain tissue to reduce the tissue front. To further enhance this effect, ascorbate oxidase is added to the tissue filtrates. Endogenous ascorbic acid contributes significantly to the size of the front. In addition, exogenous ascorbate is added to tissue extracts in some methods to prevent oxidation of monoamines (6). This can be avoided by substituting 1% cysteine as an antoxidant since it produces a sharp peak in the solvent front instead of the broad shoulder that is typically produced by ascorbic acid (Personal communication, BAS, W. Lafayette, IN).

Though the present method is suitable for analysis of monoamines in mouse brain, additional modifications are necessary for identification of NE and MHPG in the rat brain, including a higher concentration of ascorbate oxidase and the liberation of congegated MHPG with sulfatase (4).

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