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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOGENIC AMINES AND METABOLITES IN BRAIN, CEREBROSPINAL FLUID, URINE AND PLASMA

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SUMMARY

A method for high-performance liquid chromatographic separation and electrochemical detection of biogenic amines and metabolites in a variety of biological matrices is described. The method employs either homogenization, precipitation or dilution followed by direct injection of the samples and permits the chromatographic resolution of dopamine, norepinephrine, epinephrine, serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) in brain; 3-methoxy-4-hydroxyphenylglycol, DOPAC, 5-HIAA and HVA in cerebrospinal fluid; 5-HIAA, HVA and 5-HT in plasma; and 5-HIAA and HVA in urine. Alterations in chromatographic conditions, voltammetry and in vivo pharmacological manipulations are employed to verify the identity of the putative neurotransmitter and metabolite peaks in the biological samples.

INTRODUCTION

In the last several years high-performance liquid chromatographic (HPLC) techniques coupled with electrochemical detection (ED) have largely supplanted fluorometric, radiometric and gas chromatographic—mass spectrometric methods for the routine determination of biogenic amines and their metabolites in a variety of biological matrices [1–8]. HPLC-ED techniques offer high sensitivity (in the nanomolar to picomolar range) and relative ease of assay. However, the majority of the HPLC—ED techniques have been optimized for analysis of biogenic amines in a single biological matrix [9—15]. If the investigator is interested in determining the relationship of neurotransmitter changes in the brain and cerebrospinal fluid (CSF) or relating

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changes in brain concentrations of neurotransmitters with changes in metabolite concentrations in plasma or urine in order to gain a readily available estimate of central nervous system function, different mobile phases and/or columns must be used. Typically, extensive sample preparations involving organic solvent extractions are required for analysis of the metabolites, particularly in plasma and urine [3, 16–20].

In this paper we describe a simple, rapid, sensitive HPLC–ED technique that involves minimal sample preparation. None of the sample preparative techniques require organic solvent or alumina extraction in order to provide baseline separation of compounds of interest. It is capable of simultaneously determining dopamine (DA) and its major metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (E), norepinephrine (NE) and serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) down to 50–150 pg in brain; similar quantities of 3-methoxy-4-hydroxyphenylglycol (MHPG), DOPAC, HVA and 5-HIAA in CSF; comparable amounts of HVA and 5-HIAA in urine; and comparable quantities of HVA, 5-HT and 5-HIAA in plasma.

Similarly, the actual HPLC analytical method requires only a single working electrode detection system and an isocratic mobile phase. These points, minimum sample preparation and the use of an isocratic mobile phase, are important when a large number of samples are to be analyzed or when samples will be analyzed with the use of an autosample injector where the use of a gradient mobile phase may lead to significant baseline drift. In instances where it is necessary or desirable to measure catecholamines (or metabolites possessing 3,4-hydroxyl groups, e.g. DOPAC) in plasma, where concentrations are below the detection limit or where they may not be resolved from interfering compounds, published techniques employing extraction and enrichment with activated alumina [21, 22] can be used with the chromatography described in this paper.

Because we have developed a chromatographic system based on the direct injection of homogenized or protein-precipitated samples rather than a system that employs selective clean-up or enrichment procedures, we must employ alternative methods to determine the identity of chromatographic peaks in the samples. These methods include systematic alterations in composition of the mobile phase (in which changes in retention times of peaks in the standard and sample are compared); comparisons of voltammograms of known peaks in the standard and putative peaks in the samples produced by systematically varying the voltage applied to the working electrode; and the selective use of pharmacologic agents that increase or decrease the endogenous concentrations of neurotransmitter metabolites. These methods will be presented in greater detail in Results and Discussion.

EXPERIMENTAL

Materials

All standards [vanillylmandelic acid (VMA), 3,4-dihydroxyphenylalanine (*l*-DOPA), MHPG, DOPAC, NE, 5-HIAA, E, HVA, DA, 5-HT and 3-methoxytyramine (3-MT)] were purchased as either the free acid or salt from Sigma

(St. Louis, MO, U.S.A.) as was the disodium salt of ethylenediaminetetraacetic acid (disodium EDTA), ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and 10% trichloroacetic acid (TCA). Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) and triethylamine (TEA) were purchased from MCB Manufacturing Chemists (Cincinnati, OH, U.S.A.), methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and octyl sodium sulfate (OSS) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Water was deionized, glass-distilled and stored in glass.

Pharmacologic agents included probenecid from Merck, Sharpe and Dohme (West Point, PA, U.S.A.), debrisoquin sulfate (Declinax®) from Hoffmann-La Roche (Nutley, NJ, U.S.A.), dextro-amphetamine (*d*-amphetamine) sulfate from Sigma and haloperidol (Haldol®) from McNeil Pharmaceutical (Spring Hill, PA, U.S.A.). Sodium heparin was also purchased from Sigma.

Mobile phase

The mobile phase was prepared by combining 840 parts of 100 m*M* $NaH_2PO_4 \cdot H_2O$ with 160 parts of methanol. The solution was then made $2.6 \cdot 10^{-3}$ *M* in OSS, $1.0 \cdot 10^{-4}$ *M* in disodium EDTA and $2.5 \cdot 10^{-4}$ *M* in TEA. After all reagents had dissolved, the apparent pH of the solution was measured with a glass electrode and adjusted to a pH between 3.85 and 4.25 (depending on the condition of the column and the biological matrix to be analyzed) with 3 *M* phosphoric acid. On a daily basis before use, the mobile phase was drawn under a vacuum through a 0.45-μm Type HA filter (Millipore, Bedford, MA, U.S.A.) for filtration and degassing.

Chromatography

A Model 6000A chromatography pump (Waters Assoc., Milford, MA, U.S.A.) was used to deliver the mobile phase at a flow-rate of 1.0 ml/min. Samples were injected with an automated sample injector (WISP Model 710B, Waters Assoc.) onto a single C_{18} 5-μm reversed-phase column (Supelcosil LC-18-DB, Supelco, Bellefonte, PA, U.S.A.) maintained at 35° C. No pre-column was used. A TL-5A glassy carbon working electrode, with a silver–silver chloride reference electrode (BioAnalytical Systems, West Lafayette, IN, U.S.A.) was used to oxidize the compounds of interest at 0.8 V versus the reference electrode. The resulting signal was amplified by a laboratory-built amperometric detector using a circuit designed by Kissinger et al. [23] and recorded on an HP3385A Data Automation System (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Preparation of standard solutions

All HPLC standards were initially dissolved in deionized, glass-distilled water at a concentration of 100 μg/ml. Aliquots (1 ml) were frozen at −80° C in 1.5-ml conical microcentrifuge tubes (Markson Scientific, Phoenix, AZ, U.S.A.). At the time of analysis, a frozen aliquot was thawed and diluted to 100 ng/ml with 0.1 *M* hydrochloric acid.

Experimental animals

Experimental subjects consisted of 250–300 g male Wistar-derived rats from the breeding facilities of the New York State Department of Health and male

Macaca nemestrina (pig-tailed macaque) weighing between 5 and 9 kg obtained from Charles River Primate Imports.

Collection and preparation of samples

Brain samples. Rats were killed by decapitation following i.p. pre-treatment with either saline, *d*-amphetamine sulfate (4 mg/kg, 35 min) or haloperidol (1 mg/kg, 120 min); the brains were rapidly removed and dissected on an ice-cooled aluminum block according to the method of Glowinski and Iversen [24]. The brain sections were weighed to the nearest milligram and immediately homogenized in a glass tube with a motorized PTFE pestle in 10–40 vols. of ice-cold 0.2 *M* perchloric acid containing $2.63 \cdot 10^{-4}$ *M* EGTA. The tubes were maintained on ice until the end of the session. At that time, 500 µl of brain suspension or the entire suspension, if the homogenization volume was less than 500 µl, were transferred to 1.5-ml conical microcentrifuge tubes and frozen at – 80° C until the time of analysis. Prior to analysis the samples were thawed and centrifuged at 13 000 *g* for 45 s. Depending on the brain region dissected and the homogenization volume used, 5–60 µl of supernatant were injected onto the column employing the above described mobile phase with pH adjusted to 4.25.

Cerebrospinal fluid samples. Lumbar CSF was collected from the non-human primate, *Macaca nemestrina*, under baseline conditions (no drug) or following treatment with probenecid (100 mg/kg, i.v.). 5 h after drug administration, the monkeys were sedated with a quickly acting, non-barbiturate anesthetic, ketamine · HCl, 10 mg/kg, i.m. (Ketaset®, Bristol Labs., Syracuse, NY, U.S.A.) and two 1-ml samples of CSF were collected by lumbar puncture within 15 min of their receiving the anesthetic. The second CSF aliquot was analyzed for biogenic amine metabolite concentrations following a 2:1 dilution with 0.4 *M* perchloric acid and centrifugation. Under baseline conditions 50 µl of the CSF– perchloric acid mixture were injected onto the column; when probenecid was used, the volume necessary for quantification was reduced to 9 µl. The mobile phase was adjusted to pH 4.25.

Urine samples. Urine collections (24 h) were made from laboratory rats maintained for the duration of the experiments in stainless-steel metabolism cages (Allentown Caging Equipment, Allentown, NJ, U.S.A.). Both before and after i.p. treatment with debrisoquin sulfate (20 mg/kg), urine was collected in 50-ml beakers containing 1 ml of 3 *M* hydrochloric acid (to prevent degradation of catecholamines and their metabolites) and 5 ml of paraffin oil (Fisher Scientific, Rochester, NY, U.S.A.) (to prevent evaporation of the urine). The urine was centrifuged (2500 *g*, 10 min), the volume was recorded and 1-ml aliquots were frozen at –80° C in 1.5-ml conical microcentrifuge tubes until the time of analysis. At the time of analysis the thawed urine was again centrifuged (13 000 *g*, 2 min) and diluted with 19 parts of distilled water prior to injection of 5–10 µl of the diluted urine onto the column. Baseline resolution of HVA and 5-HIAA was achieved employing the mobile phase at pH 4.00.

Plasma assay for the acidic metabolites of dopamine (HVA) and serotonin (5-HIAA). Fresh whole human blood treated with sodium heparin was spun to separate the plasma fraction from the cellular components. To 100 µl of plasma

was added an equal volume of 10% TCA for protein precipitation. After spinning in a microcentrifuge (13 000 *g*, 2 min), 100 µl of the supernatant were directly injected onto the column employing the above described mobile phase with the pH adjusted to 3.85.

Determination of hydrodynamic voltammograms

Starting with the applied potential difference of 1.00 V versus the reference electrode a series of injections was made (standard, brain, CSF, urine and plasma) at decreasing applied potential differences in 0.05-V steps. Voltammograms were then plotted by expressing the observed response as a percentage of the maximum achieved response.

RESULTS

Standard

A chromatogram of a standard consisting of 2 ng each of VMA, *l*-DOPA, MHPG, DOPAC, NE, 5-HIAA, E, HVA, DA, 5-HT and 3-MT is shown in Fig. 1. All compounds are baseline-resolved.

Determination of biogenic amines and metabolites in regionally dissected rat brain

Chromatograms of supernatants from regionally dissected and perchloric

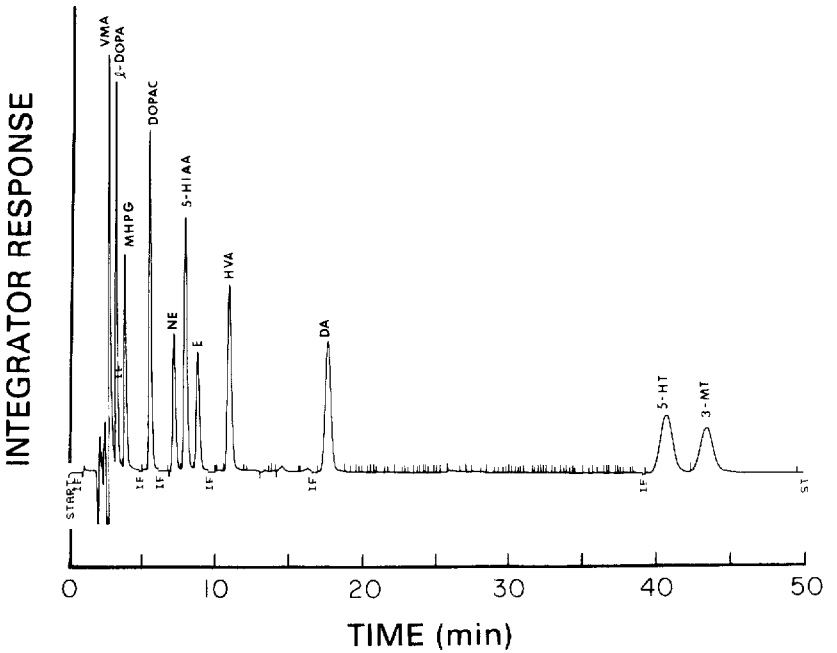


Fig. 1. Chromatogram of a standard consisting of 2 ng each of the following compounds with the number in parentheses referring to the retention time (min) of the compound: vanillylmandelic acid, VMA (2.73); 3,4-dihydroxyphenylalanine, *l*-DOPA (3.19); 3-methoxy-4-hydroxyphenylglycol, MHPG (3.74); 3,4-dihydroxyphenylacetic acid, DOPAC (5.33); norepinephrine, NE (6.93); 5-hydroxyindoleacetic acid, 5-HIAA (7.61); epinephrine, E (8.51); homovanillic acid, HVA (10.40); dopamine, DA (17.05); serotonin, 5-HT (39.36); 3-methoxytyramine, 3-MT (42.06).

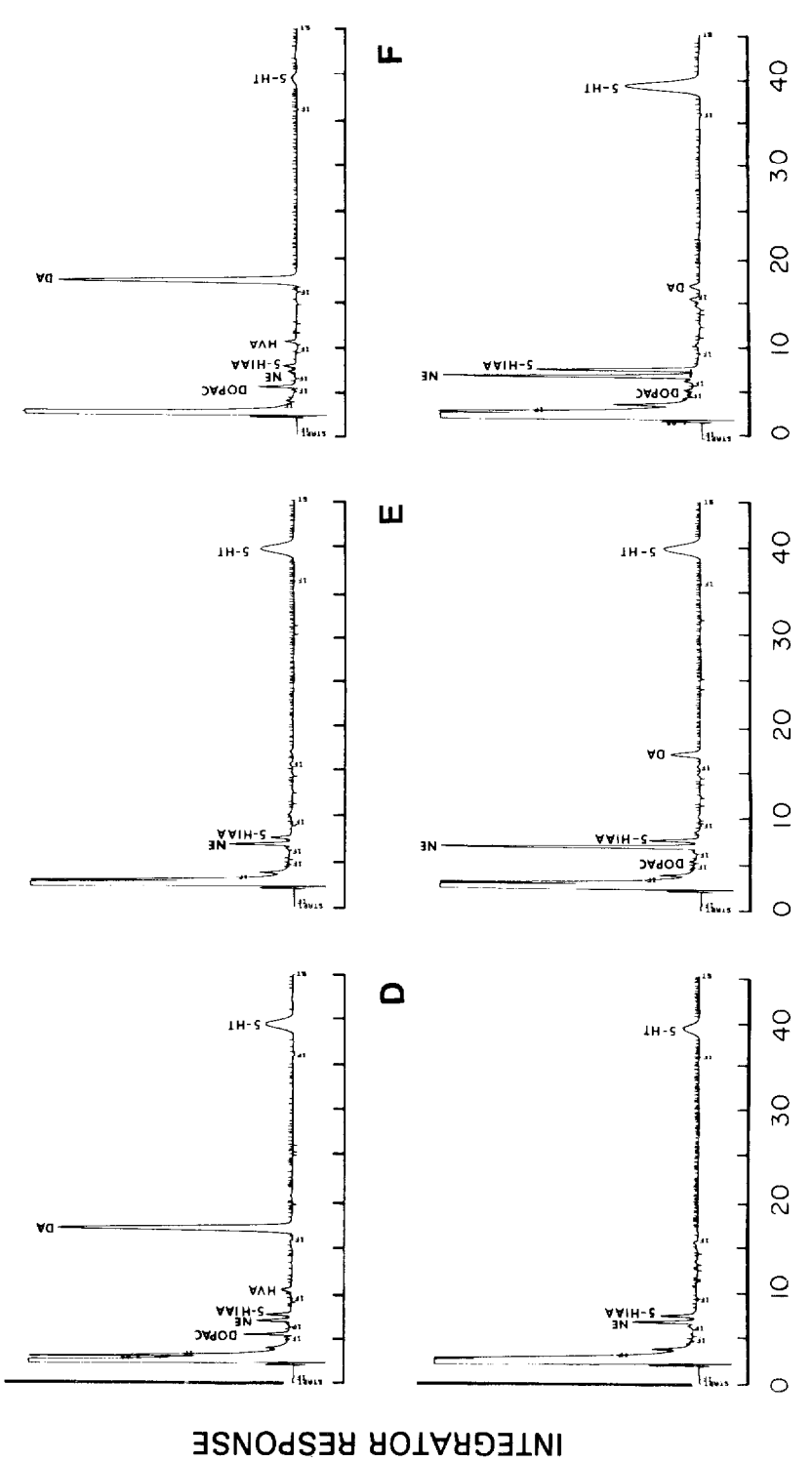


Fig. 2. Chromatograms of regionally dissected rat brain: (A) a 40-μl injection of supernatant from a 1:40 perchloric acid diluted lateral olfactory tract; (B) a 40-μl injection of supernatant of a 1:20 perchloric acid diluted frontal cortex; (C) a 5-μl injection of supernatant from a 1:20 perchloric acid diluted caudate; (D) a 60-μl injection of supernatant from a 1:20 perchloric acid diluted hippocampus; (E) a 40-μl injection of supernatant from a 1:20 perchloric acid diluted hypothalamus; (F) an 80-μl injection of supernatant from a 1:10 perchloric acid diluted brainstem.

TABLE I

REGIONAL RAT BRAIN CONCENTRATIONS OF BIOGENIC AMINE NEUROTRANSMITTERS AND METABOLITES UNDER CONTROL CONDITIONS

DA = Dopamine; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; NE = norepinephrine; 5-HT = serotonin; 5-HIAA = 5-hydroxyindoleacetic acid. N.D. = Not detected at the injection volumes routinely analyzed.

	n	Concentration (mean ± standard error of the mean) (ng/mg wet weight)					
		DA	DOPAC	HVA	NE	5-HT	5-HIAA
Frontal cortex	14	N.D.	N.D.	N.D.	0.81 ± 0.02	0.89 ± 0.04	0.15 ± 0.01
Lateral olfactory tract	12	5.83 ± 0.34	0.49 ± 0.04	0.25 ± 0.02	0.57 ± 0.03	1.04 ± 0.06	0.28 ± 0.02
Caudate nucleus	14	15.20 ± 0.31	1.08 ± 0.04	0.73 ± 0.03	N.D.	0.50 ± 0.02	0.37 ± 0.03
Hippocampus	20	N.D.	N.D.	N.D.	0.87 ± 0.03	0.45 ± 0.01	0.27 ± 0.01
Hypothalamus	14	0.35 ± 0.02	N.D.	N.D.	3.44 ± 0.13	1.09 ± 0.04	0.42 ± 0.01
Brainstem	14	0.050 ± 0.002	N.D.	N.D.	1.16 ± 0.02	0.66 ± 0.02	0.34 ± 0.01

TABLE II

CONCENTRATIONS OF DOPAMINE AND DOPAMINE METABOLITES IN RAT CAUDATE NUCLEUS INDUCED BY VARIOUS PHARMACOLOGIC MANIPULATIONS

Per cell n = 5.

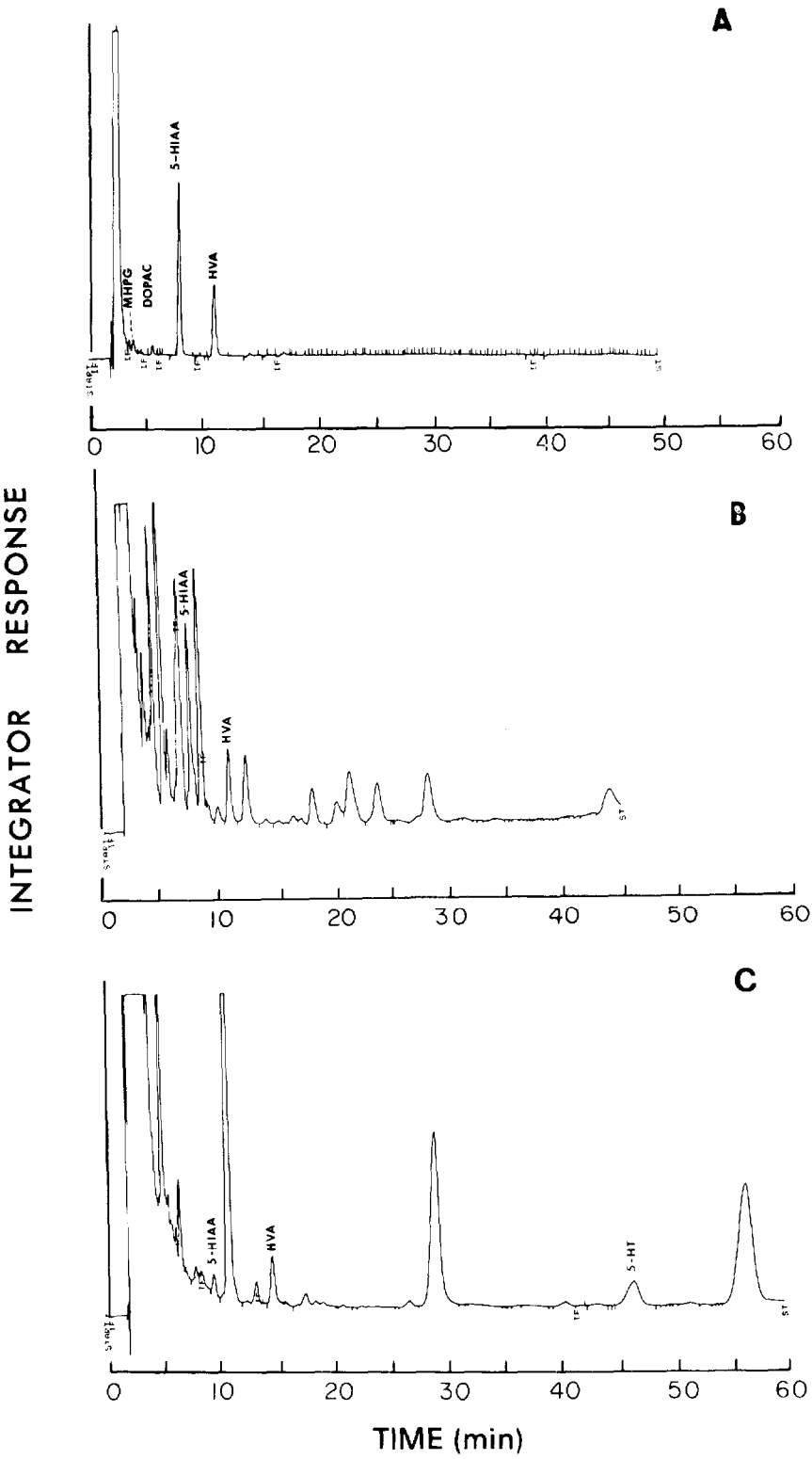
Treatment	Concentration (mean ± standard error of the mean (ng/mg wet weight)			DOPAC/DA × 100	HVA/DA × 100
	DA	DOPAC	HVA		
Vehicle	15.4 ± 0.8	0.98 ± 0.06	0.90 ± 0.07	6.46 ± 0.55	5.92 ± 0.68
d-Amphetamine sulfate (2 mg/kg)	17.2 ± 0.8	0.64 ± 0.03	0.78 ± 0.06	3.66 ± 0.17	4.44 ± 0.19
Haloperidol (1 mg/kg)	15.6 ± 1.5	4.84 ± 0.38	4.18 ± 0.18	31.3 ± 1.6	27.3 ± 1.7

acid-homogenized rat brains are shown in Fig. 2; lateral olfactory tract (Fig. 2A), frontal cortex (Fig. 2B), caudate nucleus (Fig. 2C), hippocampus (Fig. 2D), hypothalamus (Fig. 2E) and brainstem (Fig. 2F). Concentrations of the neurotransmitters and their respective metabolites under control conditions in the different brain sections are shown in Table I.

Data from experiments employing *d*-amphetamine sulfate and haloperidol are presented in Table II to illustrate the value of both simultaneously determining concentrations of DA and its metabolites in a single analytical run and employing pharmacological tools to alter endogenous concentrations of the above compounds to aid in identifying the putative peaks in biological samples.

Determination of biogenic amine metabolites in non-human primate cerebrospinal fluid

HVA, DOPAC and 5-HIAA concentrations in CSF of non-human primates were determined under baseline (no drug) and probenecid (100 mg/kg, i.v.) conditions. A chromatogram of CSF showing baseline levels of HVA, DOPAC and 5-HIAA is presented in Fig. 3A. Probenecid inhibits the transport of acid metabolites from the central nervous system resulting in significant increases in concentrations of HVA, DOPAC and 5-HIAA [25]. For example, analysis of


A
MHPG
DOPAC
5-HIAA
HVA
0
10
20
30
40
50
60
B
5-HIAA
HVA
0
10
20
30
40
50
60
C
5-HIAA
HVA
5-HT
0
10
20
30
40
50
60
INTEGRATOR RESPONSE
TIME (min)

CSF collected by lumbar puncture 5 h following the i.v. administration of 100 mg/kg probenecid resulted in HVA concentrations increasing from a mean (± S.E.M.) of 19.3 ± 3.3 ng/ml of CSF to 198 ± 59 ng/ml of CSF (n = 4). Thus, concentrations of acidic metabolites of biogenic amines in CSF are higher following the administration of probenecid and provide a further means of identifying putative HVA, DOPAC and 5-HIAA peaks in CSF.

Determination of biogenic amine metabolite concentrations in urine

A chromatogram of a 5-μl injection of a diluted 24-h urine sample from a laboratory rat is presented in Fig. 3B. The analysis requires no pre-column clean-up or organic extraction. However, because urine contains so many electroactive compounds, the pH of the mobile phase must be lowered from 4.25 to 4.00 to chromatographically separate HVA and 5-HIAA from neighboring interfering compounds and to maintain baseline resolution. In order to verify that the column maintains adequate chromatographic resolution and that the reporting integrator provides reliable estimates of the peak area of the desired compounds, we routinely determined HVA and 5-HIAA concentrations in a pooled rat control urine. We have found that maintaining the pH of the mobile phase between 3.85 and 4.0 provides values for HVA and 5-HIAA concentrations in the control urine that vary by no more than ± 3%.

We have employed debrisoquin sulfate, a monoamine oxidase inhibitor that does not cross the blood-brain barrier and thus inhibits peripheral production of HVA and 5-HIAA to aid in determining the identity of the putative HVA and 5-HIAA peaks in urine. After two 20 mg/kg i.p. injections of debrisoquin sulfate, HVA and 5-HIAA concentrations, as expected, were reduced by 42 and 19%, respectively.

Determination of HVA, 5-HIAA and 5-HT concentrations in plasma

Values for concentrations of HVA, 5-HIAA and 5-HT obtained from the analysis of pooled human plasma were 11.9, 12.4 and 15.9 ng/ml, respectively. These values agree with reported values in the literature [26, 27]. A representative chromatogram is shown in Fig. 3C.

Determination of peak purity by hydrodynamic voltammetry

In order to determine the purity of the putative peaks in the biological samples, we have generated voltammograms for known peaks in the standard and compared them with voltammograms from peaks thought to represent the same compounds in the biological samples. Results for HVA in the four biological matrices are presented in Fig. 4 as a percentage of the maximum obtained response as the voltage across the working electrode was lowered from 1.00 V in 0.05-V steps. Although graphic results are presented only for HVA (there were no discernible differences in voltammetric responses of the synthetic and endogenous HVA), similar results were also found for the other compounds of interest.

Fig. 3. Chromatograms of (A) a 50-μl injection of a 2:1 perchloric acid diluted lumbar cerebrospinal fluid sample from *Macaca nemestrina*, (B) a 5-μl injection of a 1:19 distilled water diluted rat urine and (C) a 100-μl injection of supernatant of a 1:1 trichloroacetic acid precipitated human plasma.

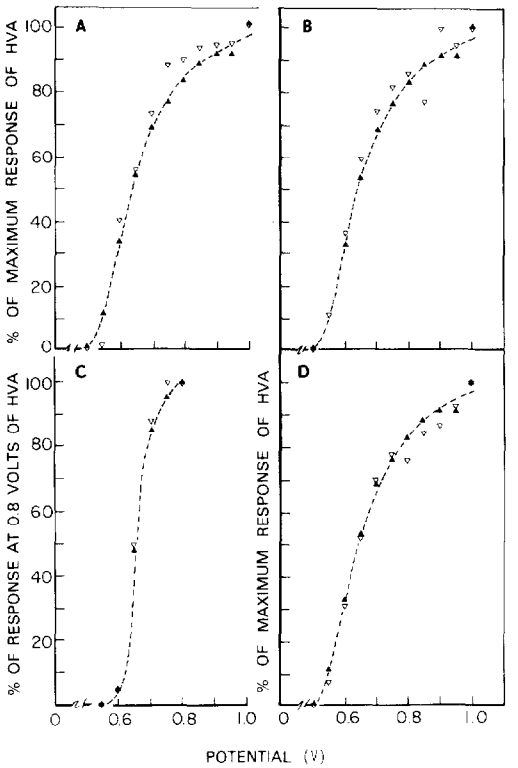


Fig. 4. (A) Voltammogram of homovanillic acid (HVA) in the standard (▲) and in rat caudate nucleus (△) expressed as a percentage of the maximum response of HVA; (B) voltammogram of HVA in the standard (▲) and in non-human primate lumbar cerebrospinal fluid (▽) expressed as a percentage of the maximum response of HVA; (C) voltammogram of HVA in the standard (▲) and in human plasma (▽) expressed as a percentage of the response of HVA at 0.800 V; (D) voltammogram of HVA in the standard (▲) and in rat urine (▽) expressed as a percentage of the maximum response of HVA.

Alterations in mobile phase affect retention times of neurotransmitters and metabolites

We have systematically varied mobile phase pH, methanol and ion-pairing reagent concentrations and have compared changes in retention times of the peaks of interest in the standard and the putative peaks in the biological samples. The results of these manipulations of the mobile phase are presented in Tables III and IV. The degree of protonation of the acid metabolites (DOPAC, 5-HIAA and HVA), and hence their retention times on a reversed-phase column, are altered by changes in the pH of the mobile phase. As seen in Table III, the retention time of HVA in both the standard and the biological sample increases as the mobile phase pH decreases.

Alterations in the concentration of the ion-pairing reagent, OSS, affect the retention of compounds with a functional amine group, e.g. the biogenic amines (NE, E, DA and 5-HT) [28]. As can be seen in Table IV, decreasing the concentration of OSS in the mobile phase by approx. 400% results in parallel decreases in the retention times of the biogenic amines in the standard and in the brain supernatant. Indeed, the reductions in retention times would have been even more dramatic if we had not also decreased the concentration

TABLE III

RETENTION TIME OF HOMOVANILLIC ACID UNDER DIFFERENT MOBILE PHASE pH VALUES AT 40°C

pH	Retention time (min)	
	Standard	Urine
4.0	12.43	12.39
3.85	13.41	13.43
3.75	14.28	14.31
3.50	15.26	15.21
3.45	15.61	15.70

TABLE IV

RETENTION TIME OF BIOGENIC AMINE NEUROTRANSMITTERS AND METABOLITES IN STANDARD AND SAMPLE UNDER TWO MOBILE PHASE CONDITIONS

Mobile phase 1: 100 m*M* NaH_2PO_4, 16% methanol, $2.3 \cdot 10^{-3}$ *M* octyl sodium sulfate, $1.0 \cdot 10^{-4}$ *M* Na_2EDTA, $2.5 \cdot 10^{-4}$ *M* triethylamine. Mobile phase 2: 100 m*M* NaH_2PO_4, 12% methanol, $6.5 \cdot 10^{-4}$ *M* octyl sodium sulfate, $1.0 \cdot 10^{-4}$ *M* disodium EDTA, $2.5 \cdot 10^{-4}$ *M* triethylamine.

Compound	Retention time (min)			
	Mobile phase 1		Mobile phase 2	
	Standard	Hypothalamus	Standard	Hypothalamus
DOPAC	5.65	5.65	6.54	6.57
NE	7.47	7.47	5.86	5.82
5-HIAA	8.14	8.14	10.47	10.38
E	9.10	9.09	7.27	7.21
DA	18.04	18.07	13.93	13.94
5-HT	41.85	42.43	33.80	33.76

of the organic modifier, methanol, in mobile phase 2 from 16 to 12% which resulted in a relative increase in retention times for all compounds. Because the shifts in retention time for these peaks in the standard and the biological sample are so similar, we have further indirect proof of the identity of the peaks.

Stability of neurotransmitters and metabolites following prolonged automated runs

Using the WISP 710B autosampler, we routinely made automated runs of 14–16 h. We have examined the stability of the neurotransmitters and metabolites by comparing concentrations of compounds of interest in brain supernatants determined after 10 h at room temperature with the same samples run with no delay before analysis. Fig. 5 demonstrates that there are no statistical differences between samples run with or without a 10-h hold at room temperature when the samples are homogenized in 0.2 *M* perchloric acid.

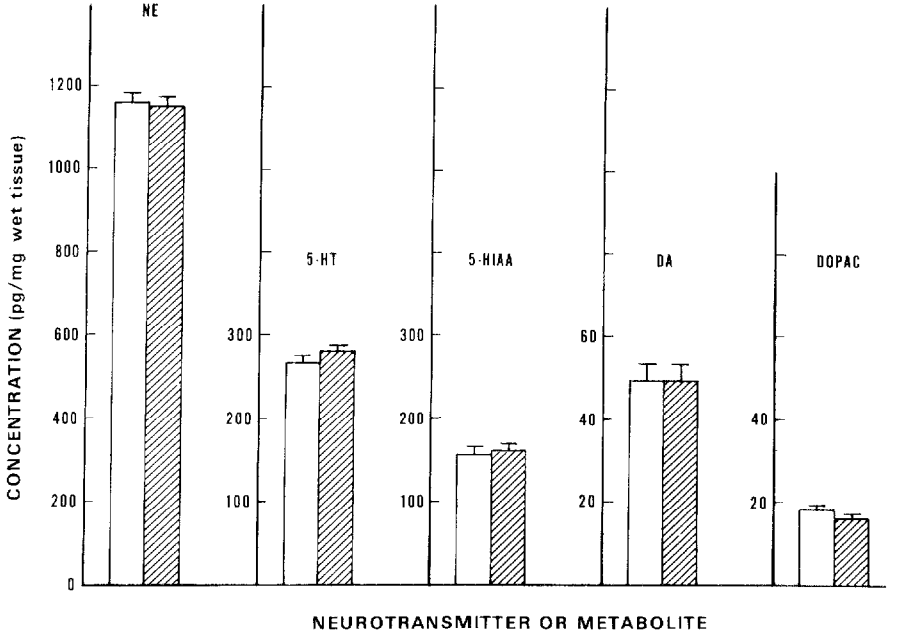


Fig. 5. Concentrations (mean ± standard error of the mean) of five neurotransmitters or metabolites determined in nine prepared rat brainstems analyzed immediately after thawing (□) and after a 10-h delay at room temperature (▨). In all cases, $n = 9$.

DISCUSSION

We have demonstrated for the biological samples brain, CSF, urine and plasma that the described HPLC method provides a rapid, sensitive, readily available means of simultaneously determining biogenic amines and their metabolites following a number of experimental perturbations.

The identity of the chromatographic peaks in the biological samples was verified by retention time, changes in retention time with alterations in mobile phase pH, ion-pairing reagent and methanol concentrations, voltammetry and the use of pharmacologic agents that modify endogenous metabolite concentrations. All of these techniques are in agreement on the identity of the putative compounds.

The autosampler and data automation system yielded retention times which were reproducible to within ± 0.01 min, and retention time is the first identifier of a peak from an endogenous compound.

The retention time of the peaks of interest in the samples changed by the same amount as the compounds in the standard when the methanol concentration and ion-pairing reagent concentration in the mobile phase were altered, suggesting that the putative peaks have been correctly identified. Similarly, the retention times of sample peaks thought to be acid metabolites of biogenic amines (DOPAC, HVA, 5-HIAA) were greatly altered by changes in mobile phase pH. The direction and magnitude of the changes in retention time were consistent with changes reported by Lammers et al. [28].

Throughout all of these changes in mobile phase composition the quantifica-

tion of each compound should remain the same. However, these proofs of chromatographic identity and purity are only indirect. It is conceivable that, as the retention time of the compound of interest is changed by altering the composition of the mobile phase, interfering compounds will either co-migrate with the compound of interest or the peak of interest may migrate and obscure additional small interfering peaks. The only method specific to electrochemical detection that will allow determination of the purity of a chromatographic peak is hydrodynamic voltammetry. This technique is based on the principle that each electrochemically active compound has a characteristic half-wave potential ($E_{1/2}$, the applied voltage that will yield a half-maximum response). If the voltage applied to the working electrode is varied across a wide range (e.g. + 1.00– 0.20 V in 0.05-V steps), a curve relating response to applied voltage will be generated. Therefore, if we are measuring the same compound in the standard as in the biological sample, as the applied voltage is changed, an identically shaped curve (voltammogram) should be generated. Fig. 4 confirms this and strongly suggests that there are no electrochemically active compounds that co-elute with the compounds of interest.

The chromatographic method we described permits quantification of neurotransmitters and metabolites down to the range of 50– 150 pg per injection. Indeed, for the majority of the samples analyzed, the problem we faced was determining the correct dilution factor(s) in order to maintain a linear detector response for all of the compounds of interest. We have determined, for the amplifier gain setting employed to produce the above chromatograms, that the majority of the neurotransmitters and metabolites were linear up to 3 ng.

The mobile phase we have developed permits baseline resolution and chromatographic separation of the compounds of interest. However, after several months of use the column undergoes changes that result in shifts in retention time of the compounds of interst — particularly the difficult chromatographic separation of HVA in urine (requiring modification of the mobile phase). Alterations in pH as small as 0.02 pH units will re-establish separation of HVA from neighboring interfering peaks and return the quantification of HVA in rat control urine to previously determined values.

Although catecholamines and their metabolites are subject to degradation by oxidative processes, we found little evidence of change in integrator counts during an automated series of runs when the standards and samples were placed in dilute (0.1—0.2 *M*) perchloric or (0.1 *M*) hydrochloric acid and maintained at room temperature. These findings allow us to make a series of runs of 12– 15 h or longer with confidence that any time/temperature-dependent changes in neurotransmitter/metabolite concentrations are statistically non-significant.

Thus, using the mobile phase and column described in the Experimental section, it is possible to quantify the catecholamines, their metabolites, 5-HT and its metabolite from brain in a single chromatographic run, and biogenic amine metabolites in CSF. Slight modifications in the pH of the mobile phase (from pH 4.25 to 4.0) permit the quantification of HVA and 5-HIAA in urine and a further reduction in pH to 3.85 allows for the quantification of HVA, 5-HT and 5-HIAA in plasma. Analysis of these disparate biological samples has applications in neuropharmacology, neurotoxicology and clinical medicine.

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