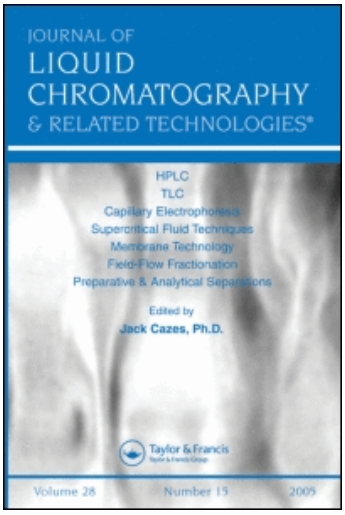

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Automated HPLC Analysis of Tissue Levels of Dopamine, Sertonin, and Several Prominent Amine Metabolites in Extracts from Various Brain Regions

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AUTOMATED HPLC ANALYSIS OF TISSUE LEVELS OF DOPAMINE, SERTONIN, AND SEVERAL PROMINENT AMINE METABOLITES IN EXTRACTS FROM VARIOUS BRAIN REGIONS

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ABSTRACT

Techniques have been developed to permit rapid and accurate automated analyses of rat brain regional extracts for dopamine, dihydroxyphenyl acetic acid, homovanillic acid, serotonin, and 5-hydroxyindole acetic acid. Separations on a C-18 reverse phase column packing were effected with the following mobile phases: one phase consisted of a solution of 1 mM EDTA pus 400 mM sodium acetate, at pH 4.0, mixed with 0.015 volume of methanol; the second phase consisted of 1 mM EDTA plus 500 mM sodium acetate, at pH 5.0. Chemical stability of the 5-hydroxyindole compounds was achieved by adding bisulfite to the perchloric acid homogenizing solution immediately prior to preparation of extracts. Time-related alterations in response of the electrochemical detector were monitored by repeated intermittent analysis of reference tissue extracts during an automated run; results obtained from analyses of the reference solutions permitted construction of detector response curves which were used to correct integrator area values to zero-time detector responsiveness.

INTRODUCTION

The development of this methodology was performed because of our interests in correlating animal behavior modification by toxic

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agents with modifications in tissue levels of, and/or turnover rates for, several biogenic amines in the brain. The aim of automating the analyses of neurotransmitter amines and their acid metabolites was to facilitate rapid acquisition of reliable quantitation results with a minimum of manpower. Several earlier HPLC techniques for analyses of the amines and their acid metabolites were complicated by the requirement that the compounds be partially purified by alumina adsorption (1) or by organic solvent extraction (2,3); more recently, perchloric acid extracts have been introduced into the chromatographic systems directly (4-7), thus, obviating the necessity of correcting for partial recoveries of the compounds of interest. However, automation necessitates the assurance that the compounds of interest remain chemically unchanged while awaiting analysis and that temporal variations in electrochemical (EC) detector response be accommodated; furthermore, the EC detector should permit good sensitivity for all compounds of interest. This report describes conditions which permit rapid and accurate automated analyses of rat brain regional extracts for dopamine (DA), dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA).

MATERIALS AND METHODS

Chemicals and Mobile Phases

DA, 5-HT, DOPAC, HVA, and pargyline were obtained from Sigma Chem Co., St. Louis, MO; 5-HIAA was obtained from Calbiochem-Behring Corp., San Diego, CA. Haloperidol was obtained from McNeil Lab., Inc., Ft. Washington, PA. HPLC grade water was obtained with the aid of a deionizer-charcoal filtration system from Hydro Services and Systems, Durham, NC.

Mobile phases were prepared by adjusting the pH of acetic acid solutions, containing appropriate quantities of EDTA, with sodium hydroxide; after dilution to final volumes and adequate stirring, the buffers were passed through 0.45 micron pore size filters (Millipore).

Equipment

A model 870 pump (Dupont Co., Wilmington, DE) was connected to a WISP (Waters Assoc., Inc., Milford, MA) automatic sample processor. The column system consisted of a Micro-Guard precolumn, containing a cartridge packed with BIO-SIL ODS-10, and a 4 x 150 mm analytical reverse phase column packed with BIO-SIL ODS-5S (BIO-RAD Labs, Inc., Richmond, CA). The column was operated at ambient temperature and a flow rate of 1 ml/min; optimal flow rates may change slightly during prolonged use of a column.

The EC detection system (Bioanalytical Systems, Inc., West Lafayette, IN) comprised a model TL-3 thin layer transducer with a carbon paste-wax electrode connected to an LC-4A amperometric detector control box. Stainless steel tubing was used for connections between components. The EC detector was operated at sensitivities of 1 to 50 namp/volt for automated analyses; the oxidation potential was 0.7 volts. Signals from the EC detector were processed by a model 3390A (Hewlett-Packard, Palo Alto, CA) reporting integrator. The reports from the integrator were recorded, on-line, by a MINC 11 (Digital Equipment Co., Maynard, MA) minicomputer; then, after suitable manipulation, results were printed out on an Omni 800 terminal (Texas Instruments Company).

Calibration Solutions

Solutions of the hydroxyindole compounds in 0.1 M $HClO_4$, containing 40 μM sodium bisulfite, (PCA-Bis-1) were stable for several hours at 22°C and could be stored for several weeks at -70°C. Stock solutions (1 mg/ml) of amines and acid metabolites were prepared in PCA-Bis-1, then aliquots of those solutions were mixed and diluted with PCA-Bis-1 to yield a working stock solution (WSS) which contained a mixture of the compounds which was 100- to 1000- fold more concentrated than the solution which was used to calibrate the integrator. Aliquots of the WSS were stored in the -70°C freezer; they were diluted, as necessary on a daily basis, with PCA-Bis-1 to permit integrator calibration.

Tissue Extracts

Brain dissections were performed according to the method described by Glowinski and Iverson (8). Tissue was frozen on dry ice then stored at -70°C until the day analyses were initiated.

In order to prevent decomposition of the hydroxyindole compounds while tissue extracts awaited analysis in the WISP, tissues were homogenized in 0.1 M $HClO_4$ which contained 2 mM sodium bisulfite (PCA-Bis-2). PCA-Bis-2 was prepared by adding crystalline sodium bisulfite to 0.1 M $HClO_4$ just prior to homogenization of tissues. In preparing the tissue extract, it was assumed that water comprises 70% of wet tissue, therefore, 19.3 µl of PCA-Bis-2 was added to the tissue for each mg wet weight; the contents of 1 mg of tissue were in 20 µl of the resulting extract. To express tissue levels on a per mg protein basis, the protein content of the PCA-insoluble sediment may be determined. After tissue was homogenized at 0°C, the sample was centrifuged at 25,000 X g for 20 min; then the supernatant was passed through a 0.2 micron pore size cellulose filter (Bioanalytical Systems, Inc., W. Lafayette, IN). Ten to 100 µl aliquots of calibration solution or of tissue extract were injected via the WISP in a typical analysis run; run time was 30 min.

RESULTS AND DISCUSSION

Chromatograms of Rat Brain Regional Extracts

Tissue extracts from various regions of the brain of a rat were prepared and subjected to HPLC analysis using two instrument assemblies, each of which contained identical reverse phase columns. The composition of the mobile phase in HPLC system 1 (Figure 1) differed from that of system 2 (Figure 2) with respect to the molarity and pH of the sodium acetate buffer; system 1 mobile phase also contained a small amount of methanol. There were obviously dramatic differences in relative elution order, as well as in absolute retention times, for each of the five compounds. Since estimates of the quantities of each of the five compounds in

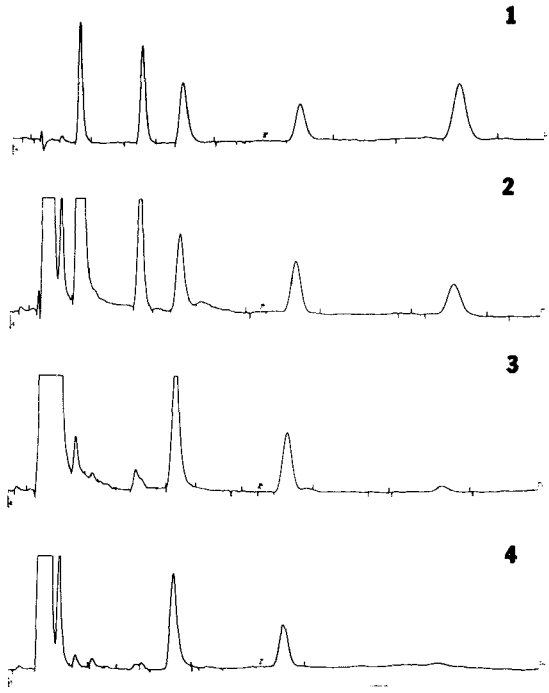

FIGURE 1

Panel #)Sample description, volume injected	Compounds in order eluted (retention time in min)				
	Dopamine (3.65)	DOPAC (7.16)	Serotonin (9.42)	5-HIAA (16.02)	HVA (25.01)
1) Standard mixture, 7 µl	0.70 ng	0.70 ng	0.70 ng	0.70 ng	1.40 ng
	Values below are: ng/mg wet tissue weight				
2) Caudate nucleus, 20 µl	9.32	1.07	0.49	0.54	0.92
3) Frontal cortex, 50 µl	0.08	0.08	0.31	0.22	0.05
4) Brain stem, 20 µl	0.06	0.00	0.56	0.41	0.00

System 1 HPLC of amines and acid metabolites. Mobile phase was 400 mM sodium acetate plus 1 mM EDTA at pH 4.0:methanol (100:1.5, v/v). Flow rate was 1 ml/min. Electrochemical detector oxidation potential was 0.7 volt and sensitivity was at 1 n amp/volt; full scale output to the reporting integrator was 10 m volts. Attenuation of the reporting integrator was set at 1. Run time was 30 min.

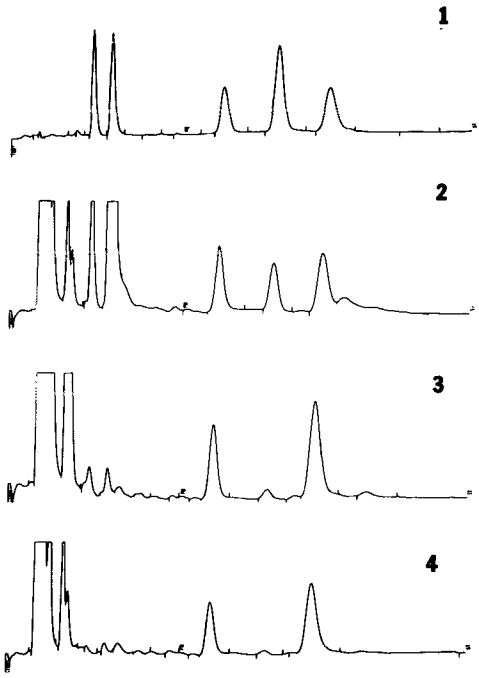

FIGURE 2

Panel #)Sample description, volume injected	Compounds in order eluted (retention time in min)				
	DOPAC (4.65)	Dopamine (5.74)	5-HIAA (12.30)	HVA (15.50)	Serotonin (18.50)
1) Standard mixture, 7 µl	0.70 ng	0.70 ng	0.70 ng	1.40 ng	0.70 ng
	Values below are: ng/mg wet tissue weight				
2) Caudate nucleus, 20 µl	1.10	10.04	0.52	0.74	0.45
3) Frontal cortex, 50 µl	0.10	0.10	0.22	0.06	0.29
4) Brain stem, 20 µl	0.07	0.11	0.39	0.05	0.54

System 2 HPLC of amines and acid metabolites. Mobile phase was 500 mM sodium acetate plus 1 mM EDTA at pH 5.0. Flow rate was 1.0 ml/min. Electrochemical detector oxidation potential was 0.7 volt and sensitivity was at 1 n amp/volt; full scale output to the reporting integrator was 10 m volts. Attenuation of the reporting integrator was set at 1. Run time was 30 min.

the tissue extracts were almost identical in each mobile phase, and since all norepinephrine metabolites appear in the solvent front or excluded volume, no detectable quantities of any unrecognized contaminant cochromatographed with any of the five compounds of interest.

Correction for Alterations in Responsiveness of the EC Detector

In order to optimize responsiveness of the EC detector to DOPAC and HVA the working electrode should contain carbon paste with wax binder. Responsiveness of the working electrode has been found to vary to some extent during the course of a 24 hour automated run; thus, it has been necessary to employ a correction technique to facilitate accurate analyses. Our approach to this problem has been to repeatedly analyze a reference solution (RS) of the compounds at convenient intervals before, during, and after the automated run; the resulting information has been utilized to construct a series of correction curves as shown in Figure 3. The RS may be either an extract of an appropriate region of the brain or an extract of whole brain which had been fortified with

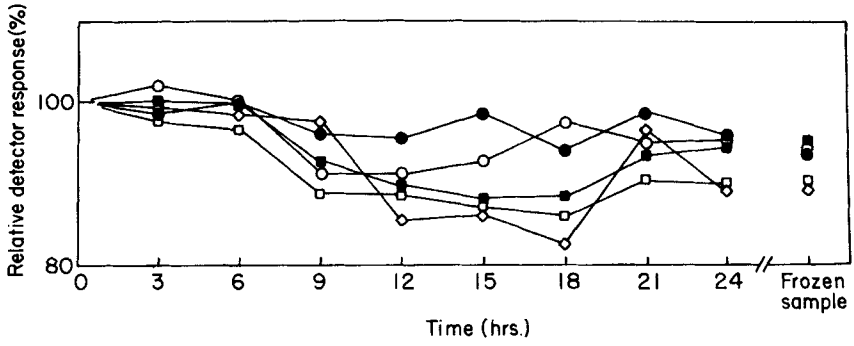

FIGURE 3

Relative responsiveness of the EC detector to components in an extract of caudate nucleus during a typical 24 hr run using HPLC system 2. The compounds are dopamine - ■ , DOPAC - □ , HVA - ◇ , serotonin - ● , and 5-HIAA - ○ .

the WSS (Materials and Methods). Tissue extracts, used as RSs, were obtained from untreated rats and were prepared on the same day that samples of unknowns were prepared. The RSs were interspersed with samples of unknowns as follows: A sample of RS was analyzed at the beginning of an automated run; subsequently, every fifth or sixth sample was a RS; finally a sample of RS which had been stored in the freezer overnight was thawed and immediately analyzed. As indicated in Figure 3, detector responsiveness to the compounds in the RS which had remained in the WISP for 24 hours were within 2% of those in the sample which had been frozen for 24 hours; this indicated that the compounds in the extract had undergone no decomposition while standing in the WISP awaiting analysis.

Correction of integrator values for each compound was performed by dividing the determined value by the fraction of zero-time, detector responsiveness appropriate for the actual time during which the sample underwent analysis. Determination of the fraction of zero-time, detector responsiveness was accomplished by performing a simple linear interpolation using the values for the fractions of zero-time, detector responsiveness obtained for that compound in the two most recently analyzed RSs. For instance, detector responsiveness to DA in the RSs at 6 and 9 hours was 1.00 and 0.94, respectively, (Figure 3); thus, integrator area values for DA in unknowns analyzed at 6.5, 7, 7.5, 8, and 8.5 hours were divided by fractions ranging from slightly less than 1.00 to slightly more than 0.94. This correction technique yielded replication values within 5% over a 24 hour interval; routinely, within group, standard error values for each of the five compounds range from 3 to 6% in extracts from control animals.

For reasons which are discussed later, internal standards were not used in this automated analysis procedure. Tyramine, at a concentration of 5 nmols, may be used as an internal standard in system 2 (Figure 2), however, this compound is not very useful in the automated analyses since corrections in detector responsive-

ness also have to be applied to it. Tyramine elutes, with baseline separation, between DA and 5-HIAA in system 2. We have not found a compound to serve as an internal standard in system 1 (Figure 1).

Applications of the Technique

A convenient aspect of our ability to simultaneously estimate tissue levels of the neurotransmitter amines and their major acid metabolites is that this method permits one to obtain insight into the nature of the perturbation(s) effected by xenobiotic exposure when proper strategy is employed. For instance, administration of the moamine oxidase inhibitor- pargyline- leads to enhancement of tissue levels of dopamine and serotonin concomittant with decreases in levels of the major acid metabolites of these amines in the caudate nucleus (Table 1).

TABLE 1

Effects of pargyline treatment on levels of amines and acid metabolites in rat caudate nucleus

Time (minutes) after pargyline[a]	DA	DOPAC	HVA	5-HT	5-HIAA
	Nanogram compound/ mg tissue (mean ± s.e.m.)				
0 saline (control)	9.43 ± 0.46	0.96 ± 0.04	0.74 ± 0.04	0.60 ± 0.01	0.54 ± 0.02
30	11.23[b] ± 0.22	0.16[c] ± 0.05	< 0.02	0.82[c] ± 0.03	0.41[c] ± 0.01
60	11.87[c] ± 0.66	< 0.02	< 0.02	0.99[c] ± 0.04	0.33[c] ± 0.03

[a] A solution of pargyline in saline was injected intraperitoneally to provide a dosage of 75 mg per kg body weight. Control rats received saline.

[b] $P<0.05$ vs control group. [c] $P<0.01$ vs control group, Fishers LSD test (9). ($n = 4$ or 5 rats per group.)

Turnover rates for amines, estimated from rates of disappearance of acid metabolites (10), indicate that the rate of turnover of dopamine is more than 5-fold greater than that of 5-HT in the caudate nucleus (Table 1).

Estimates of 5-HT turnover in several brain regions (Table 2) yielded results similar to those reported by Neckers and Meek (10). For instance, 270 ng 5-HT/ g tissue/hr in the caudate nucleus (Table 2) is reasonably close to the 2.3 ng/mg protein/hr reported by Neckers and Meek (10) when one assumes that protein accounts for about 10% of the wet tissue weight.

A comparison of tissue levels, molar ratios, and turnover rates for 5-HT and 5-HIAA in several brain regions (Table 2) shows that tissue levels and molar ratios are rather similar, whereas, turnover in the hypothalamus and brain stem appears to be much greater than in the caudate nucleus. The absence of a strict

TABLE 2

Tissue levels, molar ratios, and turnover rates for 5-HT and 5-HIAA

Brain region	Tissue levels (pg/ mg tissue)		Molar ratio [5-HIAA] / [5-HT]	Turnover rate[a] (ng 5-HT/ g tissue/ hour)
	5-HT	5-HIAA		
	mean ± s.e.m. (n = 5)			
Hypothalamus	799 ± 61	499 ± 23	0.62	586
Brain stem	508 ± 32	380 ± 30	0.75	680
Caudate nucleus	537 ± 42	471 ± 38	0.88	270
Hippocampus	486 ± 46	392 ± 16	0.81	

[a] The turnover rate was estimated from the rate of disappearance of 5-HIAA following administration of pargyline (10).

correlation between tissue levels, molar ratios, and turnover rates for 5-HT in these various regions of the rat brain may be indicative of different rates of regional elimination of 5-HIAA.

Haloperidol, a dopamine antagonist, is known to elicit marked elevation in dopamine turnover as a consequence of its binding to the dopamine receptor (11). As indicated in Figure 4, haloperidol effects several fold elevations of levels of DOPAC and HVA; it also effects a slight elevation of 5-HIAA and slight depressions of 5-HT and DA in the caudate nucleus.

In addition to methodological verification efforts such as those in Figure 4 and Tables 1 and 2, the automated analysis methods have also been applied to studies involving animal expo-

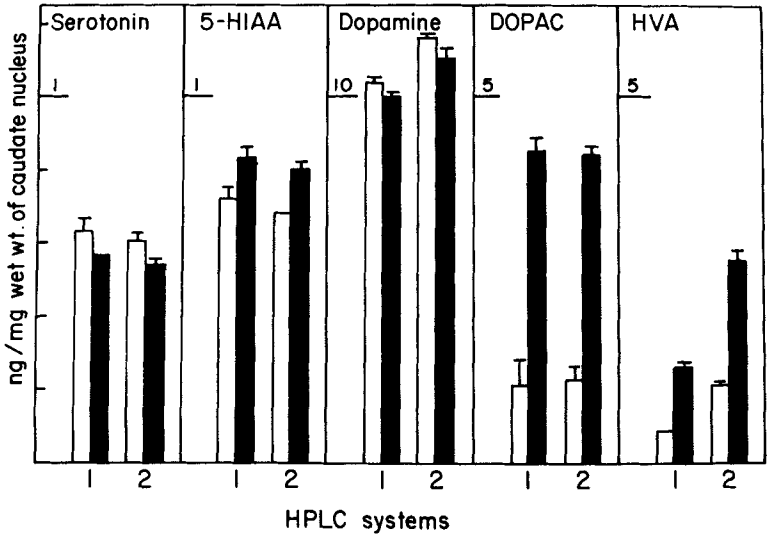

FIGURE 4

Levels of amines and their major acid metabolites in the caudate nucleus one hour after intraperitoneal administration of haloperidol (1 mg/kg body wt). Dark bars represent treated groups. Means and s.e.m. values are for extracts from four control and four treated rats analyzed on the same day using HPLC systems 1 and 2, as described in legends to Figures 1 and 2. In several instances s.e.m. values were too small to be represented.

ures to several xenobiotics of environmental interest, including: acrylamide (12), chlordecone (Kepone^{R}) (13,14) and triethyl lead (15). It was interesting that elevation of brain regional levels of 5-HIAA was effected by each of these compounds (12-15). The elevation in 5-HIAA levels in response to Kepone administration was investigated more extensively and was found to reflect enhancement in the rate of turnover of 5-HT (14). As an aside, enhancement of tissue levels of 5-HIAA and of turnover of 5-HT have been reported by others (16,17) to occur as a consequence of animal exposure to the toxic levels of the pesticide DDT.

Validity of the Interpolation-Correction Technique

Since it is a common pratice to utilize internal standards when HPLC analyses are performed manually, we conducted the following test to attempt to ascertain the relative merits of the employment of internal standards versus the simple linear interpolation technique, described in this report. We recently adapted an amino acid analysis technique developed by D. Klapper (18) to permit automated analysis of free amino acids in brain tissue extracts. Results from adaptation of that method indicated that repeated analyses of tissue extracts leads to slight deviations in the ideal results which should be expected from utilization of internal standards. In our amino acid HPLC analysis technique, leucine is used as an internal standard, separation is effect by ion exchange chromatography, the eluting amino acids are allowed to mix, then react, with a reagent which converts the amino acids to isoindoles which are quantitated with the aid of a fluorescence detector. Results obtained from a number of amino acid analyses have indicated the following: (1) the sampling precision of the WISP is essentially as was claimed in the manufacturer's specifications (i.e., less than 1% variation in runs which ranged in duration from 13 to 30 hours); (2) time-dependent variations in fluorescence detector response of as much as 10% occurred when reference solutions of amino acids were chromographed on the strong cation exchange resin, however, variations were not constant for each of the amino acids in the

analyses; (3) the interpolation-correction technique described in this report yielded more accurate and precise estimations of the amino acids than did the internal standard technique. To rephrase the results, an internal standard for which detector response went uncorrected would not permit appropriate correction for estimates of other amino acids in that sample; utilization of the interpolation-correction technique created a situation such that inclusion of the internal standard represented an unnecessarily redundant operation. While the time dependent variations in EC detector output voltage (Figure 3) do not exactly mimic those of the fluorescence detector used in the amino acid HPLC analysis method, the simple linear interpolation correction technique has permitted acquisition of precise, accurate estimates of amines and acid metabolites, as well as of amino acids, without the aid of an internal standard.

Other Methodological Factors

The mobile phases described in this report are concentrated buffers and, as such, they may facilitate cochromatography of the acids and amines by ion-pairing with the amines. Utilization of concentrated buffers can create problems when used with pumps which do not have proper piston-seal design. The seals of the pump described in the Materials section have not leaked in over six months of continuous usage with the mobile phases described in this report.

Column performance is influenced by the quantity of extract utilized in the analyses, however, after 400 to 600 analyses the precolumn cartridge is usually replaced. If necessary, the analytical columns may be regenerated by suitable washing procedures involving utilization of water and acetonitrile.

The carbon paste-wax electrode undergoes surface changes which eventually lead to diminutions in detector responsiveness; the diminutions are more pronounced for DOPAC and HVA than for the other compounds.

The sensitivity of this method is such that the contents of 1 to 2 mg of tissue are adequate for satisfactory results. Preparation of tissue extracts is optimally performed as described in the Methods section, however, if the instrument system is inoperable for a day or two, tissue extracts may be stored in the refrigerator and then analyzed. Should this happen the extracts may be further diluted with 0.5 to 1 volume of PCA-Bis-2, then, a proportionately larger size aliquot may be injected on column. It is assumed that the bisulfite facilitates maintenance of tissue thiol compounds (glutathione, cysteine, etc.) in the reduced state.

The method described in this report has been used for over a year for overnight automated analyses for brain regional levels of the various amines and their acid metabolites.

ACKNOWLEDGEMENT

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