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RAPID AUTOMATED ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method is described for the rapid automated analysis of: dopamine and norepinephrine; the major dopamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid; and the indoles tryptophan, serotonin, and 5-hydroxyindoleacetic acid in less than 0.5 mg of brain tissue. Samples are deproteinized, injected directly onto a reversed-phase high-performance liquid chromatography column, and quantitated using an electrochemical detector with a glassy carbon electrode. High sample stability permits the use of an automatic sample injector at ambient temperatures. Depending upon the column particle size, sample run times are less than 7—12 min. Thus, over 50 duplicate samples can readily be measured in a single day with very little operator attention. The chromatographic system used also resolves epinephrine, and the catecholamine metabolites: 3-methoxytyramine, normetanephrine, and 3-methoxy-4-hydroxyphenylglycol; and with very little modification this assay also could be used to measure these compounds.

INTRODUCTION

A wide variety of fluorometric and radioenzymatic assays for the monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT) and their metabolites exist [1—6]. These procedures are rapidly being replaced by methods which utilize high-performance liquid chromatography (HPLC) coupled with electrochemical detection [7—15]. However, most of these methods require some preliminary sample purification (e.g., ion-exchange chromatography or solvent extractions) and/or fairly long sample run times if both catecholamines (particularly NE) and indoles are to be resolved in the same system. Therefore, we have developed a rapid and highly sensitive assay for the measurement of the brain levels of the catecholamines DA and NE, the major DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), and homo-

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vanillic acid (HVA), and the indoles tryptophan (Tryp), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA). In addition, the HPLC system used resolves epinephrine (E), the NE metabolites 3-hydroxy-4-methoxy-phenylglycol (MHPG) and normetanephrine (NMN), and the DA metabolite 3-methoxytyramine (3-MT) from all of the other compounds measured. Since MHPG exists primarily as a sulfate ester in rat brain [16] and the levels of NMN and 3-MT greatly increase upon decapitation [17, 18] we have not yet used the following procedures to routinely quantitate the tissue levels of these compounds.

Depending upon the particle size (3 or 5 μm) and size of the HPLC column used, the run time per sample is only 7 to 12 min. Moreover, no sample purification other than deproteinization and centrifugation is required prior to the HPLC analysis. Furthermore, sample stability has been optimized so that the entire procedure, after deproteinization, can be fully automated by using an automatic sample injector. Thus, over fifty samples can be analyzed in duplicate in a single day with less than 1 h of operator attention once the samples have been deproteinized.

MATERIALS AND METHODS

Tissue preparation

Frozen samples are weighed and transferred to polypropylene centrifuge tubes which are kept on dry ice. To 39 vols. of a buffer consisting of 7 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH 4.0 using a saturated solution of citric acid) containing 1 m*M* disodium EDTA and 1 m*M* sodium octanesulfonic acid and 3 vols. of acetonitrile, one additional vol. of this buffer is added containing the internal standard, isoproterenol (ISO, 2 μg/ml). The solution containing ISO is stored at −20° C. Then, the samples are homogenized in 40 vols. (w/v) of this solution and centrifuged at 15,000 *g* for 20 min at 4° C. The supernatant is either injected immediately, kept at 4° C for up to 48 h, or stored at −20° C for longer periods. If samples are stored at −20° C, the centrifugation step is repeated.

Chromatography and detection

Aliquots (5—50 μl) of tissue samples or standards are injected onto a reversed-phase column using a WISP 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.). Routinely, a 5-μm, 15 cm × 4.6 mm Ultrasphere ODS® column (Rainin Instruments, Woburn, MA, U.S.A.) is used. However, as is indicated below, a 3-μm, 7.5 cm × 4.6 mm Ultrasphere ODS column can be used with small sample volumes. To protect the analytical column from sample contaminants, a 4.0 cm × 4.6 mm Ultrasphere ODS precolumn is placed between the analytical column and the sample injector. All connections are made using the shortest possible lengths of stainless-steel tubing (1.58 mm O.D., 0.25 mm I.D.), to minimize extra-column band-broadening.

Slightly different mobile phases are used depending upon the particle size (3 or 5 μm) of the column used: 100 vols. of 0.1 *M* monobasic sodium phosphate containing 1 m*M* disodium EDTA and 1 m*M* sodium octanesulfonic acid are adjusted to pH 4.0 to 4.35 with a saturated citric acid solution, and

mixed with acetonitrile (8—11 vols.). Generally, satisfactory results were obtained at pH 4.0 using 10.5 vols. of acetonitrile with most of the analytical columns tested. These values for mobile phase composition are meant to serve as guidelines, since small changes in mobile phase composition were necessary for different columns and for columns that had been used for extended periods of time (see below for examples). Mobile phases are filtered through 0.2-μm nylon filters (Rainin Instruments), degassed under vacuum, and placed in a water bath at 35° C to keep them in a degassed state. All other components of the HPLC system are left at ambient temperature (22° C). Mobile phase flow-rate is maintained at 1 ml/min using a Beckman 112 pump (Beckman Instruments, Berkeley, CA, U.S.A.).

The effluent from the analytical column is passed through a TL-5 flow cell (glassy carbon electrode, 0.8 V; see below for details) of an LC-4A electrochemical detector (Bioanalytical Systems, W. Lafayette, IN, U.S.A.), and then to a waste receptacle or to a fraction collector, if desired. The electrical output of the detector is quantitated using a 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Sample values are calculated relative to the peak height of the internal standard, ISO. A calibration table, used for this purpose by the integrator, is generated prior to each sample run by making at least four 5-μl injections (3-μm column) or four 20-μl injections (5-μm column) of homogenization buffer containing 0.5 ng each of DA, NE, 5-HT, DOPAC and 5-HIAA and 1.0 ng each of ISO, Tryp, HVA, and 3-MT. These calibration solutions are stable for several months at —20° C. To avoid long start-up times (particularly for the detector), all equipment is left running when samples are not being assayed and the mobile phase flow-rate is reduced to 0.1 ml/min.

All tubing, the injector, and the solvent pump are passivated; and the columns are washed according to the instructions provided with the electrochemical detector.

RESULTS AND DISCUSSION

Sample preparation and stability

A major advantage of this assay is the ease of sample preparation which reduces assay time and the possibilities for technical errors. A second advantage is the relatively high stability of the compounds measured in the buffer used for tissue homogenization. Thus, even at room temperature (22° C), all compounds assayed are completely stable for at least 24 h. In comparison, samples homogenized in 0.2 *M* perchloric acid are unstable at room temperature. In particular, 5-HT and 5-HIAA are rapidly destroyed.

High sample stability permits the use of an automatic sample injector at ambient temperatures, and therefore eliminates the need for tedious manual injections. Furthermore, tissue samples and standards stored in the homogenization buffer are stable for at least 48 h at 4° C and are stable for at least one week at —20° C. In addition, fairly large variations of the pH of this buffer do not apper to affect sample stability. Thus, tissue samples homogenized in buffers prepared as described above and adjusted to pH values from 3.0 to 5.0 were stable for at least 16 h at room temperature. A buffer pH of 4.0 was chosen because this buffer is similar in composition to the assay

mobile phase. Therefore, the solvent front observed after sample injections is minimal.

If less than 20 vols. of buffer are used for homogenization, sample deproteinization is not complete and some loss (5—10%) of 5-HT and 5-HIAA is observed after about 12 h at room temperature. Additional protein can be removed from tissue samples homogenized in 20 vols. or less of buffer, by storing the partially deproteinized samples at −20°C overnight, followed by centrifugation. However, the sensitivity of this assay is more than sufficient to quantitate each of the compounds measured in an extract derived from less than 0.5 mg of tissue. The use of dilute tissue extracts also ensures the complete recovery (>95% from whole brain) of ISO and all of the compounds measured by this assay. This was verified by adding 50 ng of ISO and each assay standard (NE, DA, 5-HT, etc.) to 1-ml aliquots of whole rat brain samples which were homogenized as described above. Then, the samples were centrifuged and injected into the HPLC system. Peak heights from sample aliquots with and without added standards were compared to obtain a measure of sample recoveries.

Chromatography

Shown in Figs. 1 and 2 are chromatograms of standards and tissue extracts obtained using a 5-μm Ultrasphere column. No additional peaks are observed beyond the peak corresponding to 5-HT. Similar chromatograms are obtained using 3-μm columns, except that the total sample run time is less than 7 min. Since the 3-μm column can accept only small sample volumes (about 5 μl), the 5-μm column is used routinely in most studies.

The capacity factors (k') of the compounds shown in Figs. 1 and 2 and several related compounds are listed in Table I for the 3- and 5-μm columns used. The monoamine precursors 3,4-dihydroxyphenylacetic acid (DOPA) and 5-hydroxytryptophan (5-HTP) are present in very small quantities in brain and are incompletely resolved from the solvent front (i.e., the initial large peak due

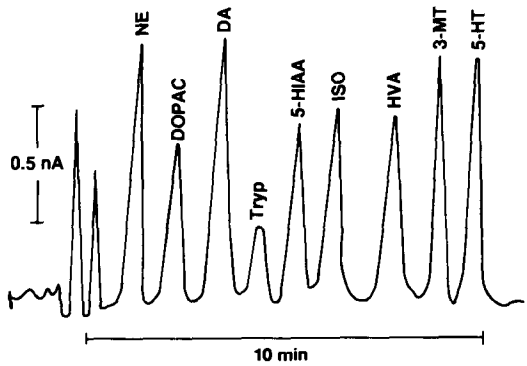

Fig. 1. Chromatogram of assay standards. A 20-μl aliquot of homogenization buffer (see Materials and methods) containing 0.5 ng each of DA, DOPAC, NE, 5-HT and 5-HIAA, and 1.0 ng each of Tryp, ISO, HVA and 3-MT was injected. Column: 5-μm Ultrasphere ODS; mobile phase: 100 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH 4.35 with citric acid) containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, mixed with 10 vols. of acetonitrile; flow-rate: 1 ml/min; detector: 0.8 V vs. Ag/AgCl reference electrode.

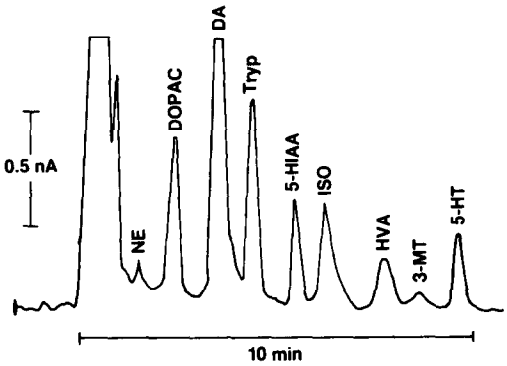

Fig. 2. Chromatogram of rat striatal tissue extract. Rats were decapitated and the striata were rapidly removed and frozen on dry ice. Then, frozen striata were homogenized in 40 vols. (w/v) of homogenization buffer (See Materials and methods) which contained 50 ng/ml of ISO, the internal standard. After centrifugation (15,000 *g* for 20 min at 4°C), a 20-μl aliquot of supernatant was injected and assayed using the conditions described in Fig. 1.

TABLE I

RETENTION (k') OF BIOGENIC AMINES, THEIR PRECURSORS, AND THEIR METABOLITES ON 3-μm (7.5 cm × 4.6 mm) AND 5-μm (15 cm × 4.6 mm) ULTRASPHERE ODS REVERSED-PHASE COLUMNS

Retention is expressed as k' values, which are the differences between the retention times of the compounds of interest and the retention time for an unretained compound (t_0) divided by t_0. Mobile phases: 3-μm column: 100 vols. of 0.1 *M* sodium phosphate buffer, pH 4.2, containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, and 9.5 vols. of acetonitrile; 5-μm column: 100 vols. of 0.1 *M* sodium phosphate buffer, pH 4.35, containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, and 10 vols. of acetonitrile. Buffer pH values were adjusted using a saturated solution of citric acid.

Compound	Retention (k')	
	3-μm Column	5-μm Column
DOMA	0.34	0.35
VMA	0.38	0.36
DOPA	0.39	0.42
DHPG	0.43	0.46
5-HTP	0.47	0.52
NE	0.51	0.59
MHPG	0.63	0.67
E	0.78	0.85
DOPAC	1.18	1.71
NMN	1.33	1.97
DA	1.52	2.14
Tryp	2.17	3.09
5-HIAA	2.80	3.43
HVA	3.38	4.95
3-MT	3.61	5.39
5-HT	4.06	5.79

to the injection of poorly retained tissue constituents and solvent). Tyrosine, the amino acid precursor of DOPA, is not detected at the electrode potentials used in this assay. The catecholamine metabolites 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxymandelic acid (DOMA), and vanillylmandelic acid (VMA) are also poorly resolved from the solvent front, and therefore do not interfere in this assay. Decreasing the pH value and/or acetonitrile concentration of the mobile phase might permit the resolution of these compounds (see below).

Although the k' values differ between the 3-μm and 5-μm columns, the relative positions of the compounds listed in Table I are almost identical ($r > 0.99$). However, minor differences in mobile phase composition have been found to be necessary to permit satisfactory compound resolution with these two types of columns. Likewise, depending upon the particle size and previous history of use of the HPLC column, minor variations in the composition of the mobile phase are necessary to adequately resolve all of the tissue components measured with minimal retention times. Therefore, the following data on mobile phase composition are presented both as a description of and as guide to the development of a suitable chromatographic system.

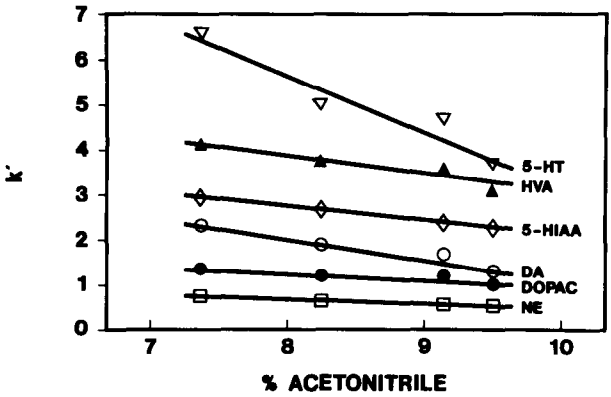

Fig. 3. Effect of acetonitrile concentration on retention (k'). Column: 3-μm Ultrasphere ODS; mobile phases: 100 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH 4.2 with citric acid) containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, mixed with 8—10.5 vols. of acetonitrile; flow-rate: 1 ml/min.

The concentrations of acetonitrile and octanesulfonate ion, and buffer pH markedly affect the resolution and retention times of the compounds measured. In Fig. 3 the effects of acetonitrile concentration on the capacity factors for the amines DA, NE and 5-HT, and the acidic metabolites DOPAC, HVA, and 5-HIAA are shown. The capacity factors for all compounds decrease as the concentration of acetonitrile is increased. Increasing the mobile phase acetonitrile concentration decreases the k' values of the amines roughly in proportion to their elution order (i.e., the greatest effect is observed for 5-HT).

The retention times of the amines are also increased in the presence of anionic ion-pairing reagents such as sodium octanesulfonate. In agreement with others, preliminary investigations indicated that the k' values for the amines increase as the carbon chain and/or concentration of the ion-pairing

reagent are increased [7, 10, 12]. A concentration of 0.75 m*M* sodium octanesulfonate is sufficient for the resolution of all of the amines of interest. During the development of this assay, this concentration of sodium octanesulfonate was used. Since then, it was found that increasing the sulfonate concentration to 1 m*M* resulted in a better resolution of NE from the solvent front. This change, after minor adjustments in mobile phase composition, did not significantly affect the k' values of the other compounds of interest. Thus, increasing the sodium octanesulfonate to 1 m*M* concentration necessitated a 1—2% increase in the concentration of acetonitrile, so that the retention times of compounds with high k' values were not substantially increased. Likewise, minor corrections in mobile phase pH (0.1—0.3 pH units) were also necessary. These were accomplished as described in the following paragraph. In the absence of octanesulfonate ions, NE remains in the solvent front. It is important to note that considerable time (12—16 h) is necessary for equilibration of a column with a mobile phase containing an ion-pairing reagent, otherwise retention times may increase and peak heights may vary between sample runs [12]. The presence of octanesulfonate ions does not affect the retention times of the acidic compounds measured (DOPAC, HVA, 5-HIAA) or MHPG (a neutral compound).

Mobile phase pH greatly affects the k' values for the acidic compounds and Tryp (data not shown, see ref. 15), but not the retention of the amines (Fig. 4). The k' values for MHPG (a neutral compound) at different mobile phase pH values paralleled, but were slightly greater than those of NE. As mentioned previously, some differences in the characteristics of various columns may occur. Therefore, the construction of curves such as are shown in Figs. 3 and 4 may be necessary to ascertain what slight variations in the mobile phases described in Materials and methods may be necessary for the complete resolution of all compounds of interest. The data in Figs. 3 and 4 also indicate that once the composition of a mobile phase for use with a particular column is established, very careful control over the mobile phase acetonitrile concentration and buffer pH is necessary, otherwise large fluctuations in k' values will

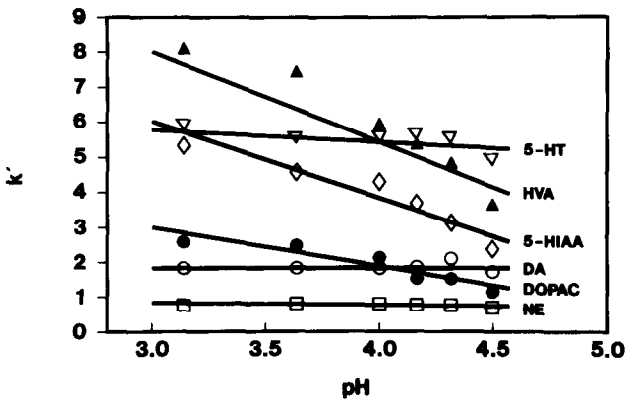

Fig. 4. Effect of pH on retention (k'). Column: 5-μm Ultrasphere ODS; mobile phases: 100 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH values of 3.25 to 4.50 with citric acid) containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, mixed with 10 vols. of acetonitrile; flow-rate: 1 ml/min.

occur. Similarly, the concentration of octanesulfonate ions must be held constant within narrow limits.

Using the mobile phases and the sample preparation described above, analytical columns have been in use for several thousand sample injections. The use of a guard column greatly extends analytical column life. Moreover, guard columns have been in use for well over 3000 injections. Column back pressure is generally about 120 bars. Once column back pressure increases by over 30 bars, often all that is necessary to place the system back into service is to clean or replace the guard column frit which is closest to the sample injector. It is important that no voids in the column packing develop when the guard column frit is cleaned or replaced, since pressure changes may occur following sample injections and these pressure changes may results in spurious peaks on the sample chromatogram. Slowly raising and lowering mobile phase flow-rates is essential for maintaining the column packings. If column voids do occur, column performance can sometimes be returned to normal by filling the voids with a packing material similar to that already present in the column.

Electrochemical detection

The high sensitivity of this assay is largely due to the great sensitivity of electrochemical detection. Thus, 20—50 pg of DA, NE, 5-HT, DOPAC, and 5-HIAA and 50—150 pg of MNM, MHPG, 3-MT, HVA and Tryp can be easily detected in a 20-μl sample. The sensitivity of electrochemical detection depends partially on the background current (electrical noise) which is a function of the voltage applied to the electrode. Beyond 0.85 V, the background current increases tremendously (Fig. 5). Therefore, with this type of electrode and mobile phase, higher potentials are unfeasible. Lower background currents and somewhat higher sensitivity are possible using carbon paste electrodes. However, carbon paste electrodes are not very durable and require frequent repacking; whereas glassy carbon electrodes can be used continuously for several months with this system without resurfacing. In addition to electrode characteristics, the presence of EDTA in the mobile phase is useful in reducing background currents. In the absence of EDTA, background currents may double.

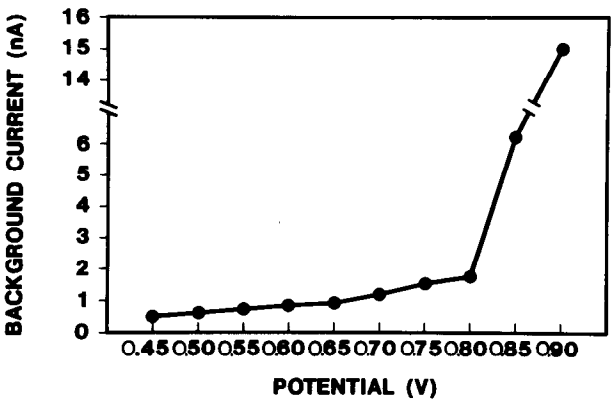

Fig. 5. Effect of electrode potential on background currents. Assay conditions were as described in Fig. 1. Background currents were measured once the currents had stabilized at each potential. Different electrodes give somewhat different responses.

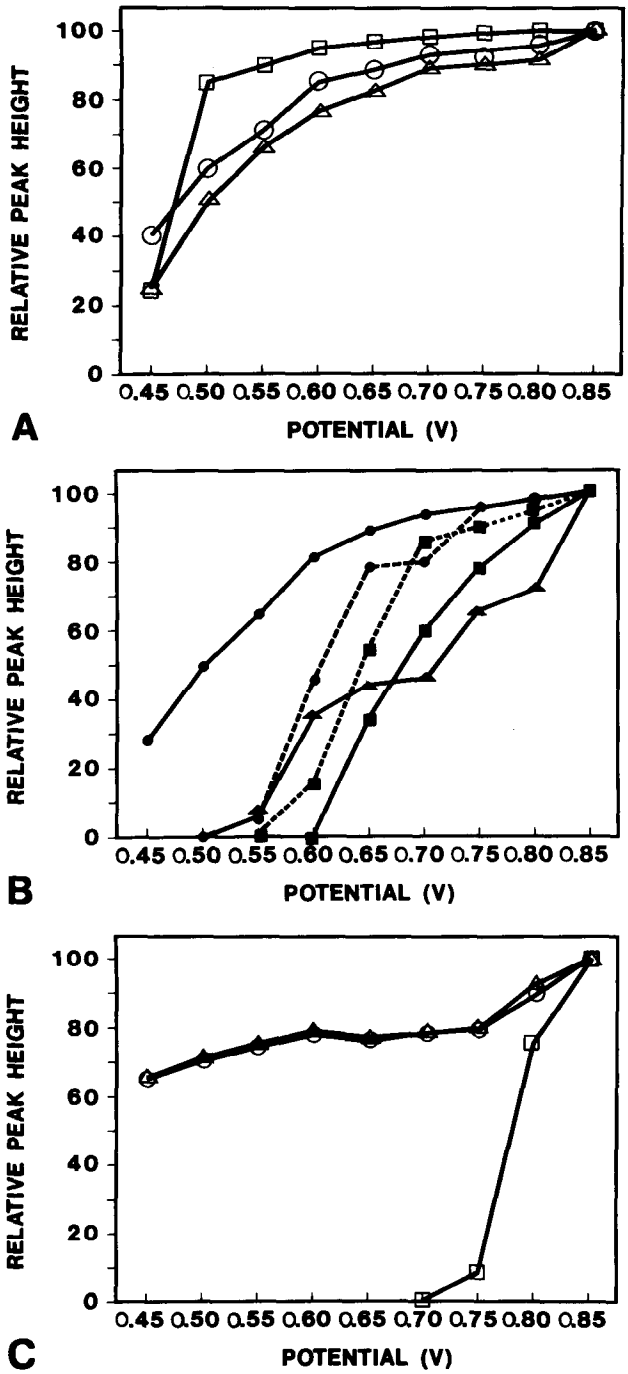

Fig. 6. Relative peak heights of: (A) the catecholamines DA (○) and NE (□), and the catecholamine-derivative ISO (△); (B) the catecholamine metabolites DOPAC (●—●), HVA, (▲—▲), MHPG (■—■), 3-MT (●- -●), and NMN (■- -■); and (C) the indoles Tryp (□), 5-HT (△), and 5-HIAA (○) as a function of electrode potential. The peak height observed at 0.85 V (vs. Ag/AgCl reference electrode) for each compound was set equal to 100. Assay conditions were as described in Fig. 1.

The magnitude of oxidation currents and, therefore, detector responses vary as a function of oxidation potential in a manner which is characteristic of the specific compounds measured by this system. The relative oxidation currents for some of the compounds which are resolved (detector response at 0.85 V for each compound set equal to 100) at various oxidation potentials are shown in Fig. 6. For the purpose of comparison, the peak heights of each compound in Fig. 6 relative to the internal standard ISO (set equal to 1) are given in Fig. 7. Because the detector response for each compound is near maximal and background currents are low (Fig. 5) and stable (i.e., almost no baseline drift over 24 h) at 0.8 V, this potential is generally used in the assay.

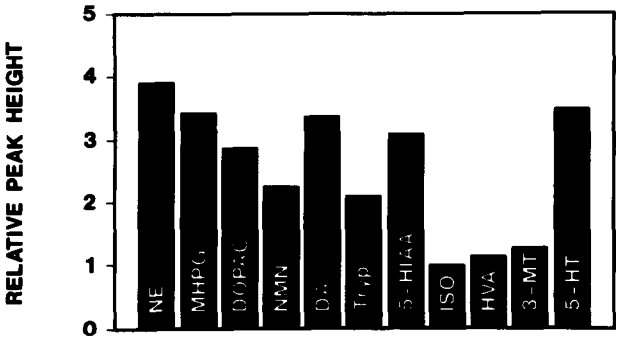

Fig. 7. Relative peak heights of equimolar amounts of the compounds in Fig. 6. Compounds are shown in the order of elution from the HPLC column and all peak height values are relative to ISO (set equal to 1). Chromatographic conditions were as described in Fig. 1. Detector: 0.85 V vs. Ag/AgCl reference electrode. Small differences in peak heights are observed depending upon the exact chromatographic conditions used.

The use of an internal standard, such as ISO, greatly contributes to the high reliability of this assay (see below). ISO does not overlap with any peaks produced by tissue constituents and is completely (> 95%) recovered from brain tissue homogenates. Since tissue values are calculated relative to the ISO peak, the effect of variations in detector sensitivity or injection volume are minimized. Thus, the coefficient of variation of samples injected over a 24-h period was less than 5% for each compound measured. The use of an internal standard also eliminates the need for repeated injections of reference samples and computer assisted manipulations of integrator data, such as are required by a recently published automated assay for some of the compounds measured by this assay [19].

The detection of NE with this assay poses a special problem. Unless the advantage of relatively short sample run times is compromised, the NE peak is near the tissue solvent front. The Hewlett-Packard integrator used in quantitating sample values can employ a tangent skimming procedure to calculate sample values from peaks which occur on the tail of the solvent front peak. However, the size and slope of the solvent front peak can vary with time, making this type of procedure unreliable. Fortuitously, the substances responsible for the solvent front of tissue samples are relatively unstable even at 4° C and the size of the solvent front greatly decreases with time. Since NE

and the other compounds measured by this assay are stable in the dark at room temperature (22° C) for at least 24 h, all that is generally necessary to reliably quantitate these compounds is to let tissue extracts sit in the dark for about 6–8 h at room temperature prior to analysis. This delay time may be programmed into the automatic sample injector.

The size of the solvent front from tissue samples may also be reduced substantially by decreasing the electrode potential of the electrochemical detector. For example, the size of the solvent front of tissue samples is much smaller at 0.65 V than at 0.80 V. Since the detector response is low for HVA and nonexistent for Tryp at 0.65 V (Fig. 6), the use of dual electrodes set at 0.65 V (or lower) and 0.8 V might be useful when low levels of NE and these compounds are to be measured in the same samples.

Verification of compound identity in tissue extracts

The identity of chromatographic peaks resulting from the analysis of brain extracts is demonstrated in several ways. First, there is a complete correspondence between the retention times of the tissue components and those of standards (Figs. 1 and 2). Second, varying the acetonitrile concentration and pH value of the mobile phase markedly alters the retention times for standards (Figs. 3 and 4) and tissue components in a similar manner. Third, the ratio of the peak heights for a compound at various oxidation potentials is relatively specific for that compound (Fig. 6), and the ratio of peak heights of standard and tissue samples measured at 0.8 V and 0.6 V are the same. For example, HVA is about twice as electrochemically active at 0.8 V as it is at 0.6 V, whereas Tryp is inactive at 0.6 V. An exact correspondence between the peak height ratios for standards and tissue extracts would be unlikely if a peak from the tissue extract is due to the oxidation of one or more compounds which differ from the standard. Fourth, the levels of the compounds measured vary in a predictable manner after various drug treatments. For example, reserpine, which disrupts biogenic amine storage, depletes tissue DA, NE, and 5-HT (Table II). Conversely, pargyline, a monoamine oxidase inhibitor, elevates tissue amine levels and decreases the levels of their deaminated metabolites: DOPAC, HVA and 5-HIAA (Table II). Likewise, although we have not routinely used this assay to measure 3-MT levels in brain tissues, the chromatographic peak corresponding to 3-MT increased when 3-MT catabolism was inhibited by

TABLE II

DA, 3-MT, DOPAC, HVA, NE, Tryp, 5-HT AND 5-HIAA CONCENTRATIONS IN RAT STRIATUM AFTER PARGYLINE AND RESERPINE

Rats were decapitated either 1 h or 20 h after receiving intraperitoneal injections of either pargyline or reserpine, respectively. Each value is the mean of six to eight duplicate determinations. The analytical conditions were as described in Figs. 1 and 2. All values for the drug-treated rats, except for Tryp and for 3-MT in the reserpine-treated rats, are significantly different from control values ($p < 0.05$, Newman–Keuls test). Values are expressed as ng/g ± S.E.M.

	DA	3-MT	DOPAC	HVA	NE	Tryp	5-HT	5-HIAA
Control	8701 ± 375	320 ± 27	1497 ± 7	1073 ± 54	108 ± 10	4621 ± 227	516 ± 27	738 ± 15
Reserpine (5 mg/kg)	6666 ± 307	327 ± 25	658 ± 40	738 ± 66	65 ± 8	4098 ± 222	470 ± 27	812 ± 22
Pargyline (50 mg/kg)	10,645 ± 1005	849 ± 136	271 ± 45	464 ± 62	136 ± 6	4506 ± 382	857 ± 61	484 ± 63

pargyline (Table II). The 3-MT levels in Table II are similar to those reported by others for striata taken from untreated decapitated rats [18, 20].

In summary, by a variety of criteria, this assay appears to adequately resolve and measure the compounds it is intended to quantitate. Because no preliminary sample purification, other than deproteinization, is needed, the possibilities for technical errors are reduced. Moreover, the high stability of the compounds measured in the homogenization buffer permits the use of an automatic sample injector at room temperature. Therefore, many samples can be reliably analyzed with very little operator attention.

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