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SIMULTANEOUS DETERMINATION OF NOREPINEPHRINE, DOPAMINE AND SEROTONIN IN RAT BRAIN REGIONS BY ION-PAIR LIQUID CHROMATOGRAPHY ON OCTYL SILANE COLUMNS AND AMPEROMETRIC DETECTION

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SUMMARY

A highly sensitive and specific ion-pair liquid chromatography—electrochemical detection method is described for the simultaneous determination of rat brain regional norepinephrine, dopamine and serotonin levels using octylsilane (C_8) columns. These amines were first isolated from tissue homogenates by adsorption on Amberlite CG-50 resin followed by separation on RP-8 columns with a mobile phase consisting of 0.05 *M* NaH_2PO_4 (pH 3.0) 0.02 m*M* EDTA, 1 m*M* heptanesulphonate—methanol (92:8, v/v) at 1.8 ml/min. Using 3,4-dihydroxybenzylamine as the internal standard, tissue recoveries ($\bar{X} \pm$ S.D.) for norepinephrine, dopamine and serotonin were 87.5 ± 2.6%, 61.8 ± 10.5% and 72.9 ± 7.5%, respectively. Assay sensitivities were sufficient for reliable quantitation of at least 200 pg of these compounds in a brain sample. The procedure is readily adaptable to determination of brain epinephrine and normetanephrine levels, as well. Finally, the reversed-phase system employed is highly flexible in that the same column and mobile phase conditions may be used for assay of biogenic amine metabolites.

INTRODUCTION

The utility of determining biogenic amines by reversed-phase liquid chromatography with electrochemical detection (LC—EC) has been clearly established Procedures have been described to measure brain norepinephrine (NE) and dopamine (DA) [1, 2] as well as a number of their respective metabolites [3, 4]. There are also a variety of methods for assaying brain serotonin (5-HT) and certain of its metabolites [5, 6]. While these separate procedures offer assays of either catecholamines or indoleamines, few methods permit the simultaneous determination of these compounds in biological tissues [7, 8].

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In all of the reports cited above reversed-phase octadecylsilane (ODS, C_{18}) columns were used for separation of biogenic amines or metabolites. The application of octylsilane (C_8) columns in LC—EC assay of these substances has not been previously described. We report here an LC—EC assay for the simultaneous measurement of NE, DA and 5-HT in rat brain regions, which employs reversed-phase separation on C_8 columns. The method described uses a convenient sample purification procedure and offers the following advantages compared to methods employing C_{18} columns: (1) shorter amine elution times without sacrifice in resolution, (2) higher sample through-puts, and (3) a single mobile phase suitable for separation of biogenic amines and metabolites, thus permitting greater versatility.

MATERIALS AND METHODS

Apparatus

Reversed-phase LC was performed on either a Spectra-Physics (Santa Clara, CA, U.S.A.) Model SP 8000 liquid chromatograph equipped with a Valco loop injector, or an Altex 110A liquid delivery system (Altex Scientific, Berkeley, CA, U.S.A.) mounted with a Rheodyne 7120 loop injector. Valve injector loops of 50 or 100 μl were employed. Stainless-steel columns (250 × 4.6 mm I.D.) packed with LiChrosorb RP-8 (10 μm; E. Merck, Darmstadt, G.F.R.) were used. Amperometric detection was performed with Bioanalytical Systems (BAS, West Lafayette, IN, U.S.A.) carbon paste electrodes or glassy carbon electrodes using either LC-2 or LC-4 controllers for signal amplification. The working electrode potential was maintained constant at +0.65 V vs. the Ag/AgCl reference electrode, unless otherwise stated.

The mobile phase consisted of phosphate buffer—methanol in a ratio of 92:8 (v/v). The phosphate buffer was 50 m*M* NaH_2PO_4 (adjusted to pH 3 at 22°C with H_3PO_4), 0.02 m*M* EDTA, and 1 m*M* sodium heptanesulphonate (HPS). This phosphate buffer was degassed under vacuum at 45°C prior to the addition of HPS and methanol and used within the same day. To remove any particulate matter, the mobile phase was passed through a 2-μm stainless-steel filter. A mobile phase flow-rate of 1.8 ml/min at room temperature (22 ± 1°C) was employed.

Chemicals and reagents

Amberlite CG-50 resin (H^+, Type 1, 100—200 mesh; Sigma, St. Louis, MO, U.S.A.) was treated as previously described [9]. Formic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.) was glass-distilled to remove interfering electroactive substances. Methanol (LiChrosolv®; Merck), ethanol (Consolidated Alcohols, Toronto, Canada), NaH_2PO_4, H_3PO_4 (Aristar grade; BDH Chemicals, Toronto, Canada), EDTA (tetrasodium salt; Sigma) and HPS (Regis, Morton Grove, IL, U.S.A.) were used without further purification.

Stock solutions of the following amine and neutral standards were prepared in 0.01 *M* hydrochloric acid, whereas those of acidic standards were prepared in 0.1 *M* formic acid. The concentration of stock solutions was 100 μg/ml, calculated as free compounds for salt-complexed standards. NE bitartrate, DA hydrochloride, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenyl-

serine (DOPS), 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylglycol (MHPG) piperazine, normetanephrine (NMN) hydrochloride, metanephrine (MN) hydrochloride, 3-methoxytyramine (MT), 5-HT creatinine sulphate, 5-hydroxytryptophan (5-HTP) methyl ester hydrochloride mono hydrate, melatonin, L-tryptophan, 3,4-dihydroxymandelic acid (DOMA), 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) cyclohexyl ammonium salt were obtained from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). Epinephrine (EPI) bitartrate, 3,4-dihydroxybenzylamine (DHBA) hydrobromide, tyramine hydrochloride, N-acetyl-5-hydroxytryptamine (N-Ac-5-HT), bufotenine monooxalate, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid (VMA) and indole-3-acetic acid (IAA) were purchased from Sigma. (d,l)-α-Methylnorepinephrine (α-Me-NE) hydrochloride and 5-hydroxytryptophol (5 HTOL) were obtained from Regis. 5-Hydroxy-N-methyltryptamine monooxalate (N-Me-5-HT), epinine hydrochloride and 3,4-dihydroxy-hydrocinnamic acid (DHCA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Tryptamine hydrochloride (Sigma) was made up in ethanol (100 μg of free base per ml). Deionised water was used throughout. Standard solutions were diluted from their corresponding stock solutions in either freshly degassed water or the LC–EC mobile phase buffer, on the day of experiment.

Animal procedures

Male Wistar rats (High Oak Ranch, Ontario, Canada) weighing 200—500 g were acclimatized in a temperature (22°C) and light (lights on at 08.00—20.00 h) controlled environment for a week before use. Food and water were given ad libitum. Animals were sacrificed between 10.00 and 12.00 h by decapitation. The brains were rapidly removed from the crania and dissected into brain regions over an ice-bath using previously described lines of demarcation [10, 11]. The brain tissues were frozen on dry ice immediately on completion of the dissection and stored at —70°C (up to six months).

Assay of tissue samples

The determination of biogenic amines in rat brain regions was performed using the sample pre-purification technique previously developed in this laboratory [9, 12]. Briefly, individual brain regions (5—80 mg wet tissue weight) were homogenized in 1 ml of 80% (v/v) ethanol containing a pre-determined quantity of the internal standard, DHBA (4—25 ng). Homogenization was performed in an ice—water bath for 30 sec using a Biosonik Ultrasonic probe homogenizer (Bronwill Scientific, Rochester, NY, U.S.A.) at a setting of 5. Homogenates were centrifuged at 13,000 *g* (4°C) for 30 min and the clear supernatants were removed and diluted with 8 ml of degassed water. Supernatants were loaded into a reservoir (12-ml polypropylene disposable syringe) mounted on a 1-ml polypropylene disposable syringe filled with Amberlite CG-50 (bed height 13 mm) using a polypropylene two-way stopcock (Pharmaseal, Puerto Rico, U.S.A.). The diluted supernatants were passed through the Amberlite columns at a flow-rate of 0.3 ml/min regulated by a peristaltic pump. The columns were subsequently washed with 7 ml of degassed water and the amines were eluted with 0.6 ml of ethanol—2 *M* formic acid (1:1, v/v) into 3-ml Reacti-Vials

(Pierce) at a flow-rate of 0.2 ml/min. The eluates were evaporated to dryness under a stream of nitrogen and the residues stored at −70°C (1—7 days) for LC—EC assay. The residues were reconstituted in 200—1000 μl of the LC—EC mobile phase immediately prior to the introduction of the samples into the liquid chromatograph.

Calibration curves were generated by processing solutions of authentic standards. Protein concentration of the tissue pellets was determined by the method of Lowry et al. [13].

RESULTS

Detection and separation of catechols and indoles by LC—EC

Using the instrumental and mobile phase conditions described here, all catechol and 5-hydroxyindole compounds investigated were readily oxidized at +0.65 V (Table I). Catechol compounds such as DOPA, DHPG, DOMA and DHBA yielded oxidative currents higher than or comparable to that of NE. All

TABLE I

COMPARISON OF RETENTION TIME AND ELECTROCHEMICAL RESPONSE OF CATECHOL AND INDOLE COMPOUNDS SEPARATED BY LC—EC

Compound*	Retention time (min)	Relative response**	
		+0.65 V	+0.8 V
DOPS	1.86	0.78	—
DOPA	2.10	1.18	—
DHPG	2.38	1.01	—
NE	3.28	1.00	1.00
VMA	3.40	—	0.66
DOMA	3.56	0.89	—
EPI	4.23	0.68	—
MHPG	4.33	—	1.35
α-Me-NE	4.68	0.66	—
DHBA	5.13	0.98	—
NMN	6.50	—	0.45
DA	7.91	0.64	—
5-HTP	8.95	0.65	—
DOPAC	9.45	0.53	—
MN	10.08	—	0.21
Epinine	11.00	0.28	—
5-HTOL	12.56	0.45	—
5-HIAA	15.75	0.37	—
DHCA	18.35	0.44	—
5-HT	20.26	0.24	—
N-Ac-5-HT	22.16	0.26	—
MT	23.63	—	0.16
HVA	28.08	—	0.18
N-Me-5-HT	36.83	0.15	—
Bufotenine	43.00	0.04	—

*For abbreviations, see text.

**The electrochemical response for all compounds (0.5 ng) has been normalized against NE, for which the peak response was 0.453 nA against a background of 5 pA.

other catechol compounds showed responses between 50% and 80% of that of NE, except epinine which gave a response of only 0.28% compared to NE. Of the hydroxyindole compounds, 5-HTP gave the highest response relative to the other hydroxyindoles tested. Among the N-substituted hydroxyindole compounds, bufotenine was the least sensitive (4% of the response of NE). O-Methylated catechols showed little (e.g. VMA, 0.045 nA; MHPG, 0.014 nA) or no response at +0.65 V but gave a substantial oxidative current at +0.8 V. At this latter voltage, these compounds yielded currents ranging from 135% (MHPG) to 16% (HVA) of that of NA. For 2 ng concentrations injected on column, tyramine, L-tryptophan, tryptamine, IAA and the methoxyindole, melatonin, did not give any response above a background current of 5 pA at either set voltage.

As demonstrated by the data in Table I, and the reconstructed pictorial chromatogram in Fig. 1, the reversed-phase LC separation system described would not adequately resolve a variety of indole and catechol compounds, should they co-exist in a sample. For example, compounds such as DOPA and DHPG, detected at +0.65 V, eluted quickly and were poorly separated. Substantial overlapping occurred between the peaks for NE and DOMA, whereas there was very little overlapping of NE, EPI and DHBA peaks. Clear separation of DA and DOPAC was not achieved while DOPAC and 5-HTP co-eluted isographically. The chromatographic peaks of hydroxyindoles like 5-HT and N-Ac-5-HT also showed a high degree of overlap. At a higher working electrode potential of +0.8 V, the 3-methoxycatechol compounds were also detectable and still further overlapping of chromatographic peaks was revealed. Complete overlap of peaks was evident for compounds such as VMA with NE and DOPAC, MHPG with EPI, MN with DOPAC, and HVA with N-Me-5-HT. Com-

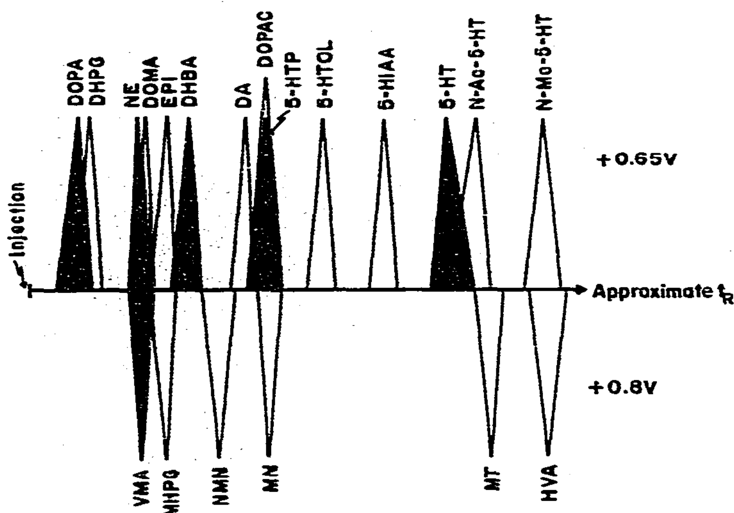

Fig. 1. Reconstructed schematic illustration of the separation of various biogenic amines and their metabolites (for abbreviations, see text) on RP-8 columns. Substances in the upper frame were detected at 0.65 V, whereas in the lower frame 0.8 V was applied to the working electrode for detection.

plete separation was not observed between MNM and DA, and between MT and N-Ac-5-HT.

Simultaneous determination of catecholamines and indoleamines in tissue

The inability of the reversed-phase LC system described here to resolve completely all catechol and indole compounds and possibly many other electroactive compounds in a biological matrix indicates the necessity for adequate preliminary sample purification prior to LC—EC analysis. Using sample purification by adsorption on a weak cation-exchange resin [9], biogenic amines were first separated from their corresponding acidic or neutral metabolites. A representative chromatogram of a processed rat hypothalamic extract is shown in Fig. 2. After pre-purification of tissue samples on Amberlite columns, NE, DA and 5-HT were well separated from each other and the reversed-phase chromatogram was clean and free of interfering substances.

Table II shows the overall yields of the method for NE, EPI, DA and 5-HT estimated by simultaneously processing 1 ng each of the four biogenic amines through the entire procedure. Yields of 60% or greater were obtained for NE, EPI and 5-HT, whereas for DA the yield was only 42%. For pooled-brain extracts augmented with the same concentration of standards and processed, yields ranged from 50 to 72% (Table II). The coefficients of variation (C.V.) for determination of these standards were about 10% of their respective mean values. An exception was DA, for which the C.V. was greater for standards added to tissue extracts than when processed in solution only.

Using DHBA as an internal standard to control for procedural losses, the recoveries for NE, EPI and DA were virtually the same as when they were

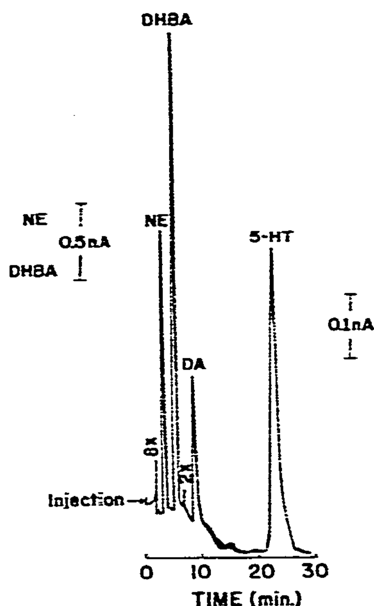

Fig. 2. Chromatogram demonstrating the LC—EC separation and detection of NE, DA and 5-HT from a portion of a single rat hypothalamus. DHBA was used as an internal standard. X represents the magnitude of attenuation. See text for abbreviations and details of the chromatographic conditions.

TABLE II

YIELDS, RECOVERIES AND INTRA-ASSAY VARIABILITY OF SELECTED BIOGENIC AMINES DETERMINED BY LC—EC

	Compounds*	Standards (1 ng) alone**		Standards (1 ng) added to a pooled-brain extract**	
		X̄ ± S.D. (%)	C.V. (%)	X̄ ± S.D. (%)	C.V. (%)
Yields	NE	61.3 ± 4.9	8.0	69.7 ± 5.3	7.6
	EPI	67.5 ± 6.2	9.2	72.6 ± 4.6	6.3
	DA	42.4 ± 4.5	10.6	50.1 ± 10.6	21.2
	5-HT	67.9 ± 6.9	10.2	57.6 ± 5.4	9.4
Recoveries***	NE	82.4 ± 1.6	1.9	87.5 ± 2.6	3.0
	EPI	90.8 ± 4.1	4.5	91.3 ± 3.0	3.3
	DA	57.6 ± 5.1	8.9	61.8 ± 10.5	17.0
	5-HT	91.4 ± 8.0	8.8	72.9 ± 7.5	10.3

*For abbreviations, see text.
**$n = 6$.
***DHBA (1 ng) was used as the internal standard.

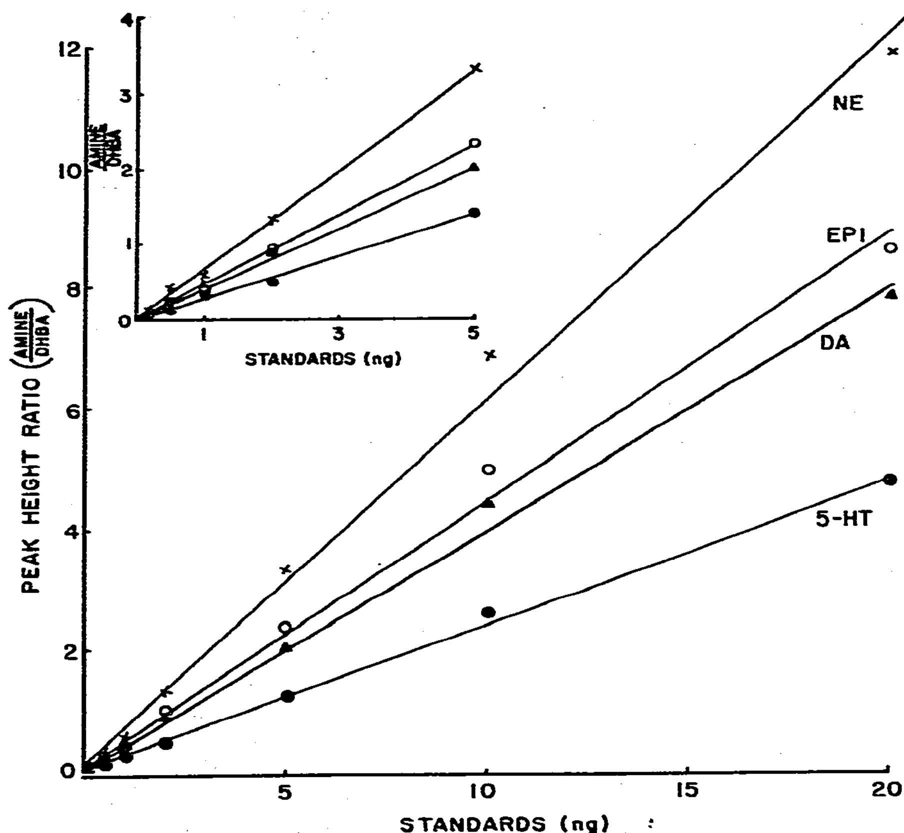

Fig. 3. Calibration curves of the peak height ratio of various amine standards versus amine/DHBA concentration. See text for abbreviations.

processed in the absence of tissue. This was not the case for 5-HT, which showed a decrease in recovery to 73% in the presence of tissue. As shown in Fig. 3, linear calibration curves were obtained for processed standards of NE, EPI, DA and 5-HT, ranging from 0.2 to 20 ng. Divergence from linearity was observed at 50 ng, however (data not shown). The present method permitted working assay sensitivities of at least 200 pg for NE, DA and 5-HT in the same sample. Recoveries and intra-assay reproducibility ($n = 5$) at this low concentration of standards were as follows: NE, 78.8 ± 6.6% ($\overline{X}$ ± S.D.); DA, 66.2 ± 7.4%; and 5-HT, 92.1 ± 4.7%.

Table III shows typical values for NE, DA and 5-HT determined simultaneously in several rat brain regions. All three compounds were readily quantitated in the septum and hypothalamus. However, the small amount (< 30 mg

TABLE III

RAT BRAIN AREA BIOGENIC AMINE* LEVELS DETERMINED BY LC—EC

Results (ng/mg protein) are expressed as the mean ± S.E.M. for the number of animals indicated in parentheses.

Brain regions	NE		DA		5-HT	
Septum	7.32 ± 0.53	(11)	13.83 ± 2.17	(11)	8.59 ± 0.81	(11)
Hypothalamus	21.99 ± 0.29	(6)	4.68 ± 0.20	(6)	12.79 ± 1.05	(6)
Hippocampus	2.24 ± 0.17	(6)	n.d.**		2.37 ± 0.14	(6)
Cerebral cortex	1.67 ± 0.10	(6)	n.d.		2.56 ± 0.17	(6)

*For abbreviations, see text.
**n.d. = not determined.

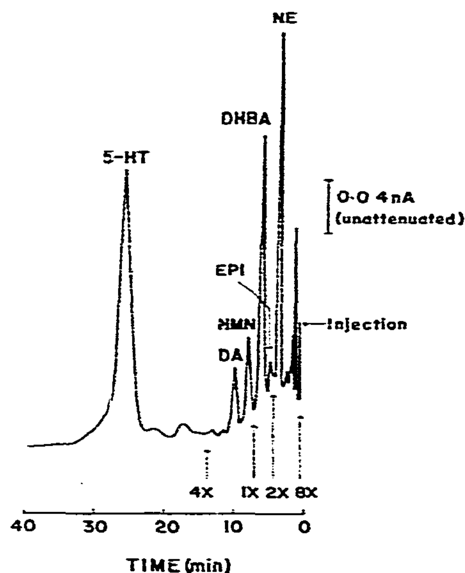

Fig. 4. Chromatogram demonstrating the LC—EC separation and detection of biogenic amines (for abbreviations, see text) in rat hippocampus. Mobile phase: 0.05 M NaH_2PO_4 (pH 3), 0.02 mM EDTA, 1 mM octanesulphonate—methanol (95:5, v/v). Flow-rate: 3 ml/min. Column temperature: 35°C. X = magnitude of attenuation.

wet weight) of cerebral cortical and hippocampal tissues used in the present study did not permit accurate measurement of DA in these latter regions. Using the same sample purification procedure, the present method was readily adaptable to determination of brain NMN, NE, DA and 5-HT, simultaneously (Fig. 4). This required slight modification of the LC conditions, such as substitution of the pairing-ion HPS with octanesulphonate, along with higher column temperature (35°C) and flow-rates (3 ml/min), to offset the prolongation of elution times for the biogenic amines when octanesulphonate was used.

DISCUSSION

The simple tissue purification procedure combined with the LC—EC conditions described here offers an accurate and sensitive method to determine NE, DA and 5-HT simultaneously in small rat brain regions. Reversed-phase separation of this group of compounds on RP-8 columns was superior to that achieved on the extensively used C_{18} columns [1, 14]. The latter columns do not permit short retention times for 5-HT ($t_R > 60$ min) under the conditions required for adequate separation of catecholamines (Warsh et al., unpublished observation). Furthermore, when C_{18} columns (μBondapak C_{18}; Waters Assoc., Milford, MA, U.S.A.) were used with 0.2 M acetic acid (pH 3.0)—methanol (9:1, v/v) as the mobile phase at 1 ml/min, NE, EPI, DHBA and DA were inadequately resolved. These observations contrast with the findings of Freed and Asmus [15] that NE, DHBA and DA were well separated when simple organic acids such as nitric or acetic acid were employed as the mobile phase using the same type and source of column.

In the LC—EC assay reported here the same mobile phase is suitable for determination of catechol- and indoleamines, as well as their respective acid metabolites. Under acidic conditions (pH 3) ion-pairing of biogenic amines with HPS is unaltered, as the amines are predominantly in dissociated form at this pH. On the other hand, the acid metabolites of catechol- and indoleamines are weak acids and have pK_a values above 3. Ionization of the carboxylic moiety of these compounds is suppressed when the pH of the mobile phase is kept below their pK_a values. When the pH of the mobile phase was decreased from 4.8 to 3, we found only a slight loss (about 10%) in detector response for NE and EPI, whereas the response for DA was not changed (see also ref. 16). Moreover, a mobile phase pH of 3 or 4.8 did not influence retention times for NE, EPI, DHBA and 5-HT [17].

Inclusion of EDTA was essential when phosphate buffer was used in the mobile phase since omission of this metal chelator resulted in high detector background currents (> 20 times) with an unsteady spiking baseline [17]. When 0.1 mM EDTA was used, as described by Moyer and Jiang [14], detector responses for biogenic amines, using either carbon paste or glassy carbon electrodes, deteriorated very rapidly. Inclusion of 0.02 mM EDTA was necessary in the present mobile phase system to suppress the baseline noise adequately without affecting the detector life. Alternatively, citrate buffer could be used to stabilize the baseline [9]. However, corrosion of stainless-steel materials of the LC—EC apparatus [18] or permanent deterioration of ODS sorbents [15] may occur with citrate buffers. We found that 1 mM HPS and 8% methanol in

phosphate buffer (v/v) provided the most satisfactory separations of NE, EPI and DHBA yet permitted 5-HT elution within 20 min. Under the present chromatographic conditions, the retention and resolution of these compounds was constant for at least 200 analyses on the same column. Varying the volumes of sample injected (20—100 μl) also did not alter resolution. Deterioration of peak shape occurred only after about 500 analyses. Column rejuvenation to the same efficiency was readily accomplished by washing sequentially with 200 ml each of methanol, methanol—chloroform (1:1, v/v) and methanol.

The high sensitivity and specificity of the present method were achieved partly through the adequate pre-purification of the tissue samples by the Amberlite column procedure. Neutral and acidic biogenic amine metabolites were not retained on the Amberlite columns, thus these compounds did not interfere with the subsequent LC—EC analysis of NE, DA and 5-HT. Although methoxyphenylethylamines such as NMN, MN and MT are also absorbed on Amberlite columns, these compounds were readily resolved on the RP-8 reversed-phase system used here (see Fig. 1) and therefore would not affect the determination of NE, DA and 5-HT. Furthermore, these methoxyphenylethylamines were electrochemically inactive at an electrode potential of +0.65 V. However, as shown here, the method can be readily adapted for determination of methoxyphenylethylamines such as NMN.

Recently, some biogenic amines and their metabolites have been assayed in unprocessed biological samples by direct injection into the LC—EC system. For example, tryptophan, 5-HIAA, IAA and indolepropionic acid have been determined in untreated rat cerebrospinal fluid [19]. Similarly, combinations of 5-HT and 5-HIAA [20] and 5-HTP, 5-HT and 5-HIAA [21] have been assayed in supernatants of rat brain homogenates. However, these procedures are best applied to the analysis of less complex biological matrices such as cerebrospinal fluid, or to determine indole compounds which are relatively strongly retained on octyl or ODS columns. These methods are not the most satisfactory procedures for determination of catecholamines that have short retention times on C_8 and C_{18} columns. In direct-injection analysis the elution of catecholamines occurs superimposed on the frontal elution peak of unretained compounds, thereby compromising the accuracy of this technique.

The present method is of greatest utility for the simultaneous determination of NE, DA and 5-HT. Procedures employing pre-purification with alumina or boric acid gel [14] permit determination of catechols only, while methods employing butanol—heptane extraction for biogenic amines are subject to very low recoveries [22]. The recoveries for NE, EPI, DA and 5-HT found here were generally high. The lower recoveries (about 65%) and higher C.V. for DA suggest that the chemical characteristics of DHBA are inadequate to control for procedural losses of DA. However, when epinine was used as internal standard [9,12], interassay (n = 5) C.V. values for NE, DA and 5-HT standards were 27%, 25% and 13%, respectively, whereas with DHBA as internal standard the corresponding C.V. values were 8.6%, 9.8% and 8.7%, respectively.

The rat brain regional concentrations of NE, DA and 5-HT determined by the present method agree closely with previously reported values determined by alternative LC—EC assays [1, 9, 23] and radioenzymatic methods [24, 25]. The application of the present reversed-phase system for EPI determination

should be undertaken with some caution, however, since this compound elutes very closely to NE and DHBA in this system. In the presence of large NE/EPI sample ratios accurate determination of EPI may be compromised.

In summary, the present method employing octylsilane reversed-phase columns offers a sensitive and specific means for the simultaneous determination of NE, DA and 5-HT in small brain regions. Furthermore, through slight modifications of the mobile phase composition, concurrent measurement of NMN is also possible.

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